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(54) Title: SOLID-PHASE LIPOSOME IMMUNOASSAY SYSTEM

(57) Abstract

An assay system for determination of an analyte. The system includes liposomes having surface-bound, analyte-related binding molecules and entrapped reporter molecules, and a cellulose acetate surface reagent having surface molecules adapted to bind the liposomes to the reagent in proportion to the amount of analyte present. The reagent is prepared, according to the invention, by treating a cellulose acetate substrate with a base, to form a hydrophilic, hydroxyl-group shell on the substrate surface, reacting the modified substrate with a bifunctional reagent in an organic solvent, and coupling the surface molecules to the activated substrate in an aqueous solution.

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SOLID-PHASE LIPOSOME IMMUNOASSAY SYSTEM

1. Field of Use

The present invention relates to a solid-phase liposome immunoassay system, and to an improved reagent for use in the system.

2. References

The following references are referred to herein by corresponding number.

1. Kay, G., et al, Nature, 216:514 (1967).
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3. Background of the Invention

Various types of solid-phase immunoassay systems for determination of chemical and biochemical analytes are known. One system employs a solid support having bound surface molecules, and a reporter/ligand bimolecular conjugate which binds to the solid support in proportion to the concentration of analyte present in the assay system. The conjugate may compete with the analyte for binding to the support, in a competitive-inhibition type assay, or may bind to the support through a bifunctional analyte, in a sandwich-type assay. After the initial conjugate binding reaction in

the presence of analyte, the solid (support) and liquid phases are separated, and the reporter activity associated with one or both phases, typically the solid phase, is measured to determine the presence and/or amount of analyte.

The bimolecular conjugate immunoassay system has limited sensitivity, inasmuch as each analyte-binding event is "reported" by one reporter molecule only, e.g., a single enzyme molecule. Another limitation is that the conjugate must be formed from a relatively pure ligand preparation. Otherwise, a significant amount of the reporter/ligand conjugate formed will not show analyte-specific binding to the support, and relatively high levels of non-specific binding and/or interference with specific binding may occur.

The limitations of the bimolecular conjugate system just described are overcome, in part, in a solid-phase system employing a liposome assay reagent in place of the reporter/ligand conjugate. The liposomes are prepared to include a surface array of analyte-related binding molecules (analogous to the ligand moiety of the reporter/ligand conjugate), to produce binding to a solid support in proportion to the amount of analyte present, either analyte-mediated sandwich binding or direct binding to the support in competition with the analyte. The reporter molecules, typically enzyme molecules, are entrapped in the liposomes at relatively high concentration, either in liposome-encapsulated form, or bound to the surface of the liposomes. A solid-phase immunoassay system employing a liposome reagent having encapsulated reporter enzyme has been disclosed in U.K. patent application GB 215360. Co-owned patent application for

"Lipid-Vesicle Surface Assay Reagent and Method", Serial No. 452,798, filed 23 December 1982, describes a liposome immunoassay containing surface-bound reporter enzymes.

Experiments conducted in support of the latter application indicate that each liposome can bind to the support through a relatively small number of surface binding events, e.g., individual antigen/antibody binding events. Accordingly, this system has the potential for very high sensitivity due to the high ratio of reporter molecules to analyte-related binding events.

Despite the potential advantages of a liposome reagent in a solid-phase immunoassay system, the sensitivity has been limited heretofore by relatively high levels of non-specific liposome binding to the solid surface. Studies conducted in support of the present invention indicate that a variety of solid-support materials, including glass beads, nylon beads, and polystyrene beads are quite sticky to liposomes, even when the liposomes were prepared in a variety of ways calculated to reduce non-specific binding.

4. Summary of the Invention

It is therefore an object of the invention to provide a solid-phase liposome immunoassay system which substantially overcomes the sensitivity limitation of earlier-proposed solid-phase immunoassay systems.

A more specific object of the invention is to provide in such a system, a novel surface reagent which gives specific-to-nonspecific liposome binding ratios which are several times higher than those achievable using prior art surface reagents.

Another object of the invention is to provide methods for making such a surface reagent, and for using the same in a liposome immunoassay.

The invention includes, in one aspect, a surface reagent for use in a solid-phase immunoassay based on immunospecific liposome attachment to the reagent. The reagent comprises a cellulose acetate substrate whose surface has been treated to replace surface acetate groups with hydroxyl groups, and an array of surface molecules attached to the substrate through the hydroxyl groups. In a preferred embodiment of the invention, the reagent is base-hydrolyzed under conditions which result in a hydrophilic surface shell of hydroxyl groups that protects the material from dissolution by organic solvents capable of dissolving cellulose acetate, and the surface molecules are attached to the substrate through bifunctional linking groups which are attached to the support in the presence of an organic solvent.

The immunoassay system of the invention includes the surface reagent and liposomes containing surface-bound, analyte-related binding molecules and entrapped reporter molecules. The liposomes are adapted to bind to the surface reagent by direct binding to the reagent surface molecules, in a competitive-inhibition assay, or by binding to a bifunctional analyte in a sandwich-type assay. Reporter groups in the liposome reagent are preferably surface-bound and/or encapsulated enzymes

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows a reaction scheme for producing a solid surface reagent having surface-bound ligand molecules coupled to the reagent substrate by means of a cyanuric chloride-activating agent; and

Figure 2 shows a reaction scheme for producing a solid surface reagent having surface molecules attached to the reagent substrate through a carbonyl diimidazole coupling agent.

Detailed Description of the Invention

I. Preparing the Solid Surface Reagent

The reagent of the invention is prepared from a cellulose acetate solid-support substrate. According to one finding of the invention, this type of substrate gives significantly lower levels of non-specific liposome binding than a number of other substrate materials which are available. To illustrate, the binding of two different types of liposomes to each of four different types of bead substrates was examined. One liposome type (#1 in Table 1 below) was prepared to contain surface-bound anti-hCG antibodies and ß-galactosidase. A second liposome type (#2) was prepared to include encapsulated alkaline phosphatase and surface-bound anti-hCG antibody, as described in Example VI. The liposomes were incubated with three beads of each bead type in 50 mM phosphate buffer, pH 7.4, for 30 minutes at room temperature, then washed four times with incubation buffer. Each bead was assayed separately for associated enzyme activity, according to procedures discussed below, and the mean value obtained for three beads for each bead and liposome type, expressed in OD unit/bead, was

determined. The results, seen in Table 1 below, show a severalfold improvement in non-specific binding levels obtainable with the cellulose acetate substrate.

Table 1

Solid surface	Absorbance (OD/bead) Liposome #1	Liposome #2
glass	>2.0	>2.0
nylon	1.286±.035	0.428±.037
polystyrene	0.105±.002	0.118±.001
cellulose acetate	0.027±.003	0.044±.018

The cellulose acetate substrate used in preparing the surface reagent may be beads, strips, rods, or other substrate forms which are readily separated from the liquid phase of an assay mixture. Alternatively, the assay vessel itself, such as a tube, may be formed of cellulose acetate. As a first step in preparing the reagent, the substrate is treated with a strong base to replace surface acetate groups with hydroxyl groups. In some applications, the surface molecules may be coupled to the surface-hydroxyl groups through chemical coupling reagents which can be attached to the substrate hydroxyl groups in an aqueous reaction medium. As an example, the surface-treated substrate can be reacted with an alkalating agent, such as iodoacetic acid, to attach carboxyl groups to the substrate surface in an aqueous reaction. Amine-containing surface molecules could then be coupled to the acid groups through amide linkages by reaction in the presence of a water-soluble carbodiimide, according to known methods. In such applications, where only

aqueous solvents are used in coupling the surface molecules to the substrate, it is unnecessary to treat the substrate to resist solvent attack.

For a variety of reasons, including greater coupling efficiency and to avoid the problem of molecules which are to be attached to the surface becoming cross-linked in solution, it is generally preferred to couple the molecules to the surface by first activating the substrate surface with a bifunctional linking agent, then reacting the molecules with the activated, washed substrate. The activation reaction used to couple the bifunctional agent to the substrate is typically carried out in an organic solvent or solvent mixture, to minimize hydrolysis of the activated reactive group in the bifunctional agent, and, in many cases, to increase compound solubility.

Heretofore, cellulose acetate has not been a suitable substrate for surface activation reactions which need to be carried out in organic solvents, because the polymer material is soluble in most solvents, such as acetone, dioxane, dimethyl sulfoxide, which are used in surface activation reactions. This limitation has been overcome, according to the present invention, by treating the cellulose acetate substrate under conditions which produce extensive base hydrolysis, to form a hydrophilic shell of hydroxyl groups on the substrate surface. The hydrophilic shell confers substantial resistance against dissolution in organic solvents such as dioxane and acetone, during the course of such activation reactions.

The hydrolysis conditions used in forming the solvent-resistant shell may be determined empirically, for example, by carrying out the base hydrolysis until the substrate material acquires the requisite solvent

resistance. The hydrolytic treatment described in Example I, in which 1/8 inch cellulose acetate beads were suspended in 3 N NaoH for 36 hours, is generally suitable. The surface-treated substrate may be dried or stored in solution.

The substrate-activation reaction just described is carried out by suspending the surface-treated substrate from above in a solution of the bifunctional agent in a suitable organic solvent. After a reaction period typically between a few seconds up to 1-2 hours, depending on the concentration and reactivity of the agent, the substrate is washed several times to remove bifunctional agent and is dried. The dried substrate may be resuspended in a suitable solvent, such as benzene, to remove trace amounts of non-covalently attached bifunctional agent. Example II below describes the activation of modified cellulose acetate beads with cyanuric chloride and Example V, activation of the beads with carbonyl diimidazole.

The washed, activated substrate is reacted in aqueous medium with a selected concentration of molecules which will form the surface molecules in the surface reagent. The surface molecules may be either (1) analyte or analyte-like molecules capable of competing with the analyte for binding to anti-analyte molecules carried on liposomes, or (2) anti-analyte molecules capable of binding a bifunctional analyte to the support, preferably through only one of two or more analyte epitopic sites. As used herein, the terms "analyte" and "anti-analyte" refer to the opposite members of a binding pair composed of a target molecule having one or more specific epitopic features, and a target-binding molecule which recognizes at least one such feature to bind the target molecule specifically

and with high affinity. The analyte/anti-analyte binding pairs which are suitable for use in the invention are antigen-antibody, immunoglobulin-protein A, carbohydrate-lectin, biotin-avidin, hormone-hormone receptor protein, and complementary nucleotide strand pairs, where the analyte may be either member of the pair, and the anti-analyte, the opposite member. More generally, the anti-analyte may include any portion of an anti-analyte molecule which is capable of participating with the analyte in specific, high-affinity binding, for example, in an antibody-antigen pair, an anti-analyte antibody may include either analyte-binding $F(ab')_2$ or Fab' fragments.

The concentration of surface molecules in the coupling reaction is selected to produce a desired surface concentration of coupled molecules on the support, under the reaction conditions employed. For coupling proteins to cyanuric chloride-activated substrate, the coupling reaction is usually carried out at a pH of about 7.5 at 2-8°C for one hour, at a protein concentration between about 0.5 and 5 mg/ml. Similar conditions are used for coupling to diimidazole-activated substrate, except that longer reaction times may be employed. After terminating the coupling reaction, the substrate is washed and may be incubated with an amine, such as ethanolamine, to tie up any unreactive activating groups. The substrate can be stored in standard physiological buffer, and is typically incubated overnight with 1% serum albumin before use.

Figure 1 illustrates the reactions used in coupling a protein ($P-NH_2$) to a modified cellulose acetate surface with cyanuric chloride. The reaction,

which follows the scheme described in reference 1, involving initial covalent attachment of cyanuric chloride to the support substrate (S-OH) through an ether linkage, and subsequent reaction of the dichlorotriazine with a protein amine group to couple the protein to the triazine ring. The initial activation reaction is carried out in acetone, and the protein-coupling reaction in an aqueous buffer.

A similar series of reactions, illustrated in Figure 2, are used in coupling a protein to a base-modified cellulose acetate surface by means of a carbonyl diimidazole linking agent. As seen, the diimidazole reacts with a surface hydroxyl group to form an imadazolyl carbamate which can then react with the amine group of a protein to attach the protein to the substrate. The general reaction scheme is described in reference 2. The coupling of hCG and anti-hCG antibody to a modified cellulose acetate substrate activated with cyanuric chloride is described in Examples II and III below, and the coupling of hCG to the modified substrate activated with carbonyl diimidazole, in Example IV.

The activated substrate may also be reacted with an "intermediate" coupling compound which has one functional group, such as an amine group, adapted to react with the surface activating agent and another group such as an amine, carboxyl, or sulfhydryl group, to which the surface molecules can be conveniently coupled, according to well-known coupling methods. The intermediate coupling compounds may provide a spacer arm, e.g., a 3-20 atom carbon or carbon-nitrogen chain with the desired reactive end group. Having the surface molecules attached to the support through a spacer arm may reduce steric binding constraints near the substrate

surface and thereby provide enhanced immunospecific binding at the substrate surface.

II. Preparation of Liposomes for the Immunoassay Systems

The liposomes in the immunoassay system of the invention are composed of lipid vesicles having surface-bound, analyte-related binding molecules and reporter molecules which are entrapped in the vesicles, either in encapsulated or surface-bound form.

Lipid vesicles are prepared from lipid mixtures which preferably include phospholipids at a mole ratio between about 40 and 90%, and sterols, at a mole ratio between about 10 and 50 mole percent. The lipid mixture may include one or more negatively charged lipids, such as phosphatidyl glycerol, typically at a mole percent between about 5 and 20%, to impart a negative surface charge. Experiments conducted in support of the present invention indicate that liposomes having a slight negative surface charge produce less flocculation on storage and also reduced levels of non-specific binding to the cellulose acetate support. The vesicles also preferably include one or more lipid components having reactive or activated polar head groups which can be used in coupling analyte-related binding molecules to the vesicle surfaces. Such lipids include those having reactive polar groups such as amine, carboxyl, hydroxyl, or sulfhydryl groups, or activated groups capable of reacting directly with hydroxyl, carboxyl, amine, or sulfhydryl groups on the molecules to be attached to vesicles. Methods for coupling proteins to lipid vesicles containing either thiol-reactive lipids, oxidized amine reactive lipids, or carboxyl groups will be discussed further below. The reactive or activated lipids are included in the lipid vesicle components at a

mole ratio preferably between about 0.1 and 10%. The lipid components used in preparing the liposomes described in Examples VI and VII include 40% cholesterol, 48% phosphatidylcholine (PC), 6% phosphatidylglycerol (PG), and 6% (MPB-PE), a thiol-reactive phophatidyl ethanolamine (PE) described in references 3 and 4.

The lipid vesicles may be formed by any of a variety of methods, such as those detailed in reference 5. One preferred method, referred to as a reverse-phase evaporation method, involves initial formation of an emulsion of aqueous particles in an organic solvent containing the vesicle lipids. Removal of the organic solvent by evaporation leads to a reverse-phase emulsion that can be converted to reverse-evaporation vesicles (REVs) by agitation in an aqueous medium. Within a fairly broad range of lipid/aqueous solvent ratio, the REV method produces predominantly uni- and oligolamellar vesicles having relatively large sizes, i.e., greater than about 1 micron. Vesicles of this type may be readily converted to a population of substantially homogeneous sizes by extrusion through a unipore polycarbonate membrane having selected pore sizes, e.g., between 0.1 and 1 micron. Examples VI and VII below describes the preparation of REVs and subsequent vesicle sizing by extrusion through 0.4 micron unipore membranes.

Large multilamellar vesicles can be formed readily by hydrating a film of lipid vesicle components in an aqueous medium, and these vesicles can also be made homogeneous in size by extrusion through a unipore membrane filter. This liposome formation technique is described in reference 6.

With both methods, the vesicles may be prepared under conditions which lead to encapsulation of reporter molecules, for use in preparing liposomes having surface-bound binding molecules and encapsulated reporter. Various types of reporters, such as fluophores, chromophores, spin-labeled molecules, and enzymes may be used. Of these, enzymes generally provide greater assay sensitivity due to high enzyme turnover numbers in converting a substrate to a detectable product. Where an enzyme reporter is used, the enzyme preferably is one which: (1) is capable of producing an easily measured colorometric or fluorometric effect in the presence of a suitable substrate; (2) is available in pure or nearly pure form; (3) can be encapsulated in lipid vesicles without significant loss of activity; and (4) is stable in encapsulated form on storage in solution, or is resistant to freezing and lyophilization in encapsulated form.

The two lipid vesicle preparation methods mentioned above are both suitable for preparing liposomes having encapsulated enzyme reporter molecules, as are a variety of other liposome preparation methods. In the REV method, the initial water and oil emulsion is formed using an aqueous enzyme solution, leading to lipid vesicles which may encapsulate up to about 50% of the enzyme solution used in forming the emulsion. A high encapsulation efficiency is a significant advantage of the REV method, but enzyme inactivation by organic solvents may limit use of this method for some enzymes. Co-owned patent application for "Encapsulated Enzyme Liposome Reagent", Serial No. 699,860, filed 8 February 1985, describes a novel method for stabilizing glucose-6-phosphate dehydrogenase against inactivation

by organic solvents in the REV procedure and against inactivation on storage in liposome-encapsulated form.

As indicated above, the reporter may be entrapped either in encapsulated form or surface-bound form or both. Methods for forming liposomes having surface-bound reporter molecules, such as enzyme molecules, in addition to surface-bound binding molecules, such as antibodies, are described in the above-noted patent application for "Lipid-Surface Vesicle Reagent". Protein-to-liposome coupling methods described in that application, and methods described below for attaching analyte-related binding molecules to liposome surfaces are applicable. One enzyme which has been used advantageously in forming a "co-conjugate" liposome having surface-bound enzyme and binding molecules is bacterial-derived ß-galactosidase. Among the advantages of this enzyme are: (1) the enzyme is available in purified form with high specific activity; (2) the enzyme contains free thiol groups which can be used in coupling the enzyme to thiol-reactive lipids in liposomes, without affecting the enzyme activity; and (3) both fluorogenic and chromogenic substrates are available. Example VII below describes the preparation of co-conjugate lipisomes having surface-bound ß-galactosidase.

The liposomes are further prepared to include surface-bound molecules adapted to produce lipid binding to the solid-support reagent in proportion to the amount of analyte present in an immunoassay reaction mixture. The analyte-related binding molecules are typically anti-analyte molecules which form one member of an analyte/anti-analyte binding pair, as defined above. In a competitive inhibition assay, the analyte compete with analyte or analyte-like molecules carried on the surface

reagent for binding to anti-analyte carried on the liposomes. Where the free analyte is an antigen, the liposome-bound anti-analyte is typically an anti-antigen antibody or antibody fragment. In the sandwich type assay, the anti-analyte is one which typically has binding specificity for one binding moiety (epitopic site) of the analyte, with the anti-analyte carried on the reagent preferably having binding specificity for a second, distinct binding moiety. For example, where the analyte is a bifunctional antigen having A and B epitopic sites, the reagent molecules may include anti-A site antibodies, and the liposome binding molecules, anti-B site antibodies.

Several methods for coupling anti-analyte molecules, such as antibodies, covalently to the reactive or activated polar head groups of the components in lipid vesicles are known. One efficient coupling method suitable for coupling thiol-containing molecules to lipid vesicles through thioether or disulfide bonds has been mentioned above with respect to references 3 and 4. This method is particularly suited to attaching antibody fragments, after reduction, to lipid vesicles. A second efficient method, described in reference 7, involves oxidation of lipid vesicle glycolipids followed by coupling of proteins or other amines to the lipids through a Schiff-base intermediate. A third efficient method, which has been described in co-owned patent application for "Carboxylated Lipid Coupling Reagent", Serial No. 692,679, filed 18 January 1985, involves coupling of proteins to a carboxyl group carried at the end of a 3-18 atom spacer arm attached to the vesicle through a hydrophobic anchor. The first two methods mentioned above are capable of producing protein-to-vesicle

coupling ratios of up to 200-300 µg protein per µmol lipid; the third method, ratios of up to about 600 µg protein/µmole lipid. Example VI below describes the coupling of anti-hCG antibody to lipid vesicles containing encapsulated alkaline phosphatase. In Example VII, which describes the preparation of the co-conjugate liposome containing surface-bound anti-hCG antibodies and ß-galactosidase, the antibody and enzyme are coupled to the enzyme in a single coupling reaction containing thiol-reactive liposomes and antibody and enzyme, at a selected concentration of each.

3. Solid-Phase Liposome Immunoassay

This section describes the use of the above solid-phase immunoassay system in two general types of solid-phase immunoassays. In the first type, referred to above as a competitive inhibition assay, the analyte competes with analyte and analyte-like surface molecules on the solid-phase reagent for binding to anti-analyte molecules on the liposomes. The extent of the liposome binding to the solid support is therefore inversely proportional to the amount of analyte present in the reaction mixture. The initial binding reaction is carried out in a reaction medium whose pH and ionic strength are compatible with analyte/anti-analyte binding, typically in a buffered medium, pH 5-8. The concentration of liposome vesicles in the assay medium is preferably adjusted such that the extent of the liposome binding to the support is inversely proportional to the amount of analyte present in the mixture within the concentration range of analyte to be tested. Typical liposome vesicle concentrations are in the range between about 10^{-12} and 10^{-7} mole lipid per ml total assay. The binding reaction is typically

carried out at room temperature for a period of between about 5 and 60 minutes.

In the usual assay procedure, the assay is calibrated using serially diluted analyte solutions, such as diluted serum control samples containing known concentrations of analyte, to establish a standard curve of reagent-associated enzyme activity versus analyte concentration over a selected analyte range. The test sample itself may also be assayed at each of a series of different concentrations to give at least some sample points which are within the standard curve range.

Following the binding reaction step, the solid support is washed, e.g., with the binding-reaction buffer, to remove non-specifically bound liposomes. After the wash step, the solid support bearing the lipsomes is placed in an enzyme-assay medium containing enzyme substrate and, where the encapsulated enzyme liposome reagent is being used, a detergent such as Tween-20 effective in solubilizing the liposomes and releasing the encapsulated enzyme for reaction. The level of enzyme in the reaction mixture is measured conventionally, either spectrophotometrically or in a qualitative test by visible detection of color change. Example VIII below illustrates a competitive-inhibition assay for determination of hCG using a liposome reagent containing surface-bound anti-hCG antibodies and ß-galactosidase. The measured enzyme activity, expressed as OD/bead, ranged from about 1.6 in the absence of analyte to 0.112 at the highest concentration of analyte added. Example IX describes a similar type of competitive-inhibition assay using a liposome reagent having surface-bound anti-hCG antibodies and encapsulated alkaline phosphatase. Here the OD/bead

levels ranged from 1.2 in the absence of added analyte to a low of 0.033 at high analyte concentration.

A second general type of immunoassay involves sandwich-type binding of liposomes to the solid-phase reagent through analyte bridging. Typically, the analyte is a bivalent antigen having two or more epitopic binding sites which combine simultaneously to anti-analyte molecules carried on the support and to liposome-bound anti-analyte binding molecules. This assay type gives greater liposome binding to the solid support in the presence of greater amounts of analyte.

In the usual procedure, the assay involves an initial reaction step which is carried out under conditions like those used in the competitive-inhibition assay described above. In particular, the concentration of liposomes in the reaction mixture is preferably selected to give proportionally more binding to the solid support with increasing amounts of added analyte, over the desired analyte concentration range.

The assay is usually carried out by preparing a standard curve from known analyte controls, in a linear range of analyte concentrations, and assaying for the analyte at one of a number of serial dilutions.

After the initial incubation step, the solid and liquid phases are separated, the solid support washed, and the amount of enzyme bound to the support assayed conventionally. Example X below describes a sandwich-type assay for determination of hCG using liposomes containing either surface-bound polyclonal anti-hCG or monoclonal anti-hCG antibodies. The test can accurately measure hCG to a sensitivity level of about 15 mIU/ml, and shows a concentration dependence in the range from about 15 to 250 mIU/ml. An OD range of more than 2 OD units/reagent bead over the analyte range

tested, and a maximum signal-to-noise ratio of about 30 were attained.

From the foregoing, it can be appreciated how various objects and features of the present invention are met. The modified cellulose acetate substrate of the invention is readily prepared for attachment of surface molecules through surface hydroxyl groups, and the hydrophilic surface shell formed in accordance with the invention allows surface molecule coupling by means of and initial surface activation reaction carried out in an organic solvent.

According to another important feature of the invention, non-specific liposome binding to the modified-substrate reagent is severalfold less than to other types of solid phase reagent which have been used heretofore. As a result, the signal-to-noise ratio and hence assay sensitivity attainable in a solid-phase liposome immunoassay, is enhanced severalfold. Also, the wider range of detectable reporter signal achievable with the reduced non-specific (background) binding levels markedly enhances the accuracy and reliability of solid-phase liposome immunoassay tests.

The immunoassay system thus overcomes a serious limitation in prior art liposome immunoassay systems, without compromising the inherent advantages of such a system, particularly high signal levels, due to the large number of reporter molecules which report each analyte/anti-analyte binding event on the reagent surface.

The following examples illustrate various aspects of the invention, but are in no way intended to limit the scope thereof.

Example I

Preparing Modified Cellulose Acetate Beads

Cellulose acetate beads (1/8 in. diameter) were obtained from Precision Plastic Ball Co. (Chicago, IL). The beads were suspended in 3 N NaOH for 36 hours, and then washed extensively with water until the pH of the bead suspension dropped to between about 6 and 7. The beads were dried in a Buchner funnel under vacuum.

Example II

Activation of Cellulose Acetate Substrate with Cyanuric Chloride

Mouse immunoglobulin G (IgG), was obtained from Cappel Lab (West Chester, PA). The IgG was radiolabeled with ^{125}I according to a chloramine T iodination method. The specific activity of the radiolabeled protein was about 6 μCi/mg.

Hydrolyzed cellulose beads (2-5 g) from Example I were immersed in 1 N NaOH for ten minutes. The excess base was removed by filtration, and the wet beads were added to 20 ml of an acetone solution containing 20% cyanuric chloride (w/v). Distilled water (25 ml) was immediately added with stirring. After a reaction period of 12-15 sec. at room temperature, 25 ml glacial acetic acid was added to stop the reaction. The mixture was filtered and washed three times with a cold acetone:water mixture (1:1, v/v). The beads were dried in a Buchner funnel under suction, and the dry beads were resuspended in benzene to remove trace amounts of non-specifically attached cyanuric chloride.

The extent of protein coupling to cyanuric chloride activated and non-activated beads was determined separately for three substrate batches. Three activated or washed, non-activated beads were reacted

with mouse IgG (0.5 mg/ml) in 0.25 ml of 50 mM phosphate buffer, pH 7.4, at 2.5-8°C, 1 hr. After removing the protein solution, any reactive cyanuric chloride groups on the surface of the beads were blocked by addition of 1 M ethanolamine, pH 9.0, for 1 hour with moderate shaking. After washing several times with the reaction buffer, the ^{125}I radioactivity associated with each batch was determined. The results are shown in Table 2 below, along with the calculated protein coupling ratios for activated (A) to non-activated (NA) material. The total radioactivity associated with the activated beads corresponds to about 1 µg protein/bead (1/8").

Table 2

Batch #	Activated	Non-Activated	A/NA
1	17534±2140	2722±515	6.5
2	17383±1480	2429±82	7.2
3	17218±674	2249±109	7.7

Example III

Coupling hCG to Activated Substrate

Human chorionic gonadotropin (hCG) was obtained from Calbiochem-Behring (San Diego, CA).

A 5 ml solution of hCG (1 mg/ml) in 50 mM phosphate buffer, pH 7.4, was reacted with the freshly activated cellulose beads (2-5 g) from Example II. The reaction was carried out at 2-8°C for 1 hour with moderate shaking. After removing the hCG solution, reactive groups remaining on the surface of the bead were blocked with incubation with 1 M ethanolamine, pH 9.0, for one hour with moderate shaking. The hCG beads were stored in a phosphate-buffered saline containing 1%

bovine serum albumin (BSA), 0.2% NaN_3, pH 7.4, at 4°C overnight before use.

Liposomes having surface-bound anti-hCG in either encapsulated alkaline phosphatase (preparation #1) or surface-bound ß-galactosidase (preparation #2) were prepared as described in Examples VI and VII, respectively. Levels of specific binding to the solid surface support were measured by incubating preparation #1 (approximately 50 nmole phospholipid) or #2 (approximately 10 nmole phospholipid) in 50 mM phosphate buffer, pH 7.4, with 3 beads for each liposome type. Non-specific binding levels were determined similarly, using the two liposome preparations which were first preincubated with excess hCG for 30 minutes to block anti-hCG antibody binding sites on the liposomes. After incubating the beads with the respective liposome preparations, the liquid material in each sample was removed by aspiration and the beads were washed four times with the incubation buffer.

Beads having bound preparation #1 liposomes were assayed by addition to each bead of 0.5 ml assay solution containing 0.5% Tween-20 (to solubilize the bound liposomes) and 1.9 mM of the substrate p-nitrophenyl phosphate in 1.0 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8. The reaction was followed at 405 nm. Beads having bound preparation #2 liposomes were assayed by addition to each bead of 0.5 ml of assay solution containing 2.7 mM of the substrate orthonitrophenyl-3-D-galactopyranosides (Calbiochem-Behring, San Diego, CA) (ONGP) in phosphate buffer, pH 7.8. The reaction was followed at 405 nm.

The data in Table 3 below are mean values of the 3 beads for each incubation sample. As seen, the signal-to-noise ratio (specific to non-specific liposome

binding ratio) was at least about 40 for both liposome types.

Table 3

Liposome Preparation	Specific	Non-Specific	S/NS
#1	2.173±0.045	0.055±0.004	40
#2	3.113±0.1	0.058±0.007	50

Example IV

Coupling of Anti-HCG Antibody to Activated Substrate

Mouse monoclonal anti-hCG antibody was obtained from Bioclone Australia Pty (Marrickville, Australia).

A 1 mg/ml solution of the antibody (5 ml) in 50 mM phosphate buffer, pH 7.4, was added to freshly activated cellulose beads (2-5 g) from Example II. The coupling reaction was carried out at 2-8°C for 1 hour with moderate shaking, after which the anti-hCG solution was removed. Any reactive groups remaining on the surface of the beads were blocked for incubation with ethanolamine, as in Example III. The antibody-coupled beads were washed and stored overnight with 1% BSA, as in Example III. For control purposes, a bead reagent having surface-bound mouse immunoglobulin was similarly prepared.

The specificity of binding of hCG to the antibody-coated beads is seen from the data in Table 4 below, which shows, in row 1, binding of I^{125}-labeled hCG to the beads, and, in row 2, the binding level where 1 mg non-radioactive hCG was added to the incubation

medium to compete ^{125}I-hCG for binding to the support. The third row in the table shows levels of ^{125}I-hCG binding to the beads having surface-bound immunoglobulin.

Table 4

Bead Coated with	Non-radioactive hCG	Radioactivity (cmp/beads)
Anti-hCG	-	10396±1412
Anti-hCG	+	1234±71
Mouse IgG	-	218±42

The data show that non-labeled hCG effectively competes with ^{125}I-hCG for binding to the anti-hCG beads, and that the level of non-specific ^{125}I-hCG binding to the beads is only about 2% of the specific binding level.

Example V

Coupling hCG to Beads Activated with Carbonyl Diimidazole

Previously hydrolyzed cellulose beads (2-5 g) from Example I were reacted with 5 ml dry acetone containing 0.12 g carbonyl diimidazole. After a reaction period of 15 minutes, the mixture was filtered and the beads were washed 3 times with cold acetone and then dried in a Buchner funnel under suction. The dried beads were resuspended in benzene to remove trace amounts of non-specifically attached diimidazole, and again dried.

The freshly activated beads were reacted with 5 ml of hCG (1 mg/ml) in 50 mM phosphate buffer, pH 7.4,

under substantially the same reaction conditions as those described in Example III, except that the reaction was carried out overnight. After removing the hCG solution, the beads were incubated with ethanolamine, to tie up free amine groups, and the beads washed and stored according to the method described in Example III.

Liposomes containing encapsulated alkaline phosphatase and surface-bound anti-hCG antibodies were prepared as in Example VI. The binding of the liposomes to beads having surface-bound hCG coupled to the substrate surface either through cyanuric chloride (Example II) or carbonyl diimidazole was compared. A suspension of the liposomes in 20 mM phosphate buffer, 150 mM NaCl, pH 7.4, was serially diluted with the same buffer and added to each of 4 assay tubes, for each surface substrate, in the total amount of liposomes shown at the left in Table 5 below. After incubation for 1 hour at room temperature, the liposome suspensions were removed by aspiration, and the beads washed 4 times with the incubation buffer. Surface-bound enzyme was assayed as in Example III and is expressed in Table 5 as mean values for 3 separately measured beads. Immunospecific binding of liposomes to the support was proportional with liposome concentration and substantially the same for both bead types.

Table 5

Amount of Liposome (nmole pL)	hCG-Coupled Beads Absorbance, 405 nm/bead (cyanuric chloride)	(diimidazole)
1	2.34	2.4
0.5	1.80	1.5
0.25	1.04	0.916
0.125	0.589	0.544

Example VI

Preparing Liposomes Having Surface-Bound Anti-hCG Antibody and Encapsulated Alkaline Phosphatase

The sulfhydryl-reactive phospholipid derivative MPB-PE was prepared as substantially as described in reference 3. Alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Lipid vesicles were prepared by a reverse evaporation phase method as described in reference 6. Briefly, a lipid mixture containing phosphatidylcholine (8 µmol), cholesterol (6.7 µmol), phosphatidyl-glycerol (1 µmol), and MPB-PE (1 µmol) was dissolved in 1.0 ml diethylether. An enzyme solution (8.5 mg/ml) in 40 mM phosphate buffer, 0.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6.0 was added (350 µl) and two phases emulsified by sonication for 1 min. Ether was removed under reduced pressure at room temperature and resulting dispersion was extruded through 0.4 micron unipore polycarbonate membranes (Bio-Rad Laboratories, Richmond, CA).

Mouse monoclonal antibodies specific against the beta-subunit of hCG were obtained from Bioclone Australia Pty (Marrickville, Australia). The antibodies were thiolated by reacting with 20 µM succinimidyl 4-(p-maleimidophenyl)butyrate (Pierce Chemical, Rockford, IL) at a reagent-to-protein molar ratio of 8:1. The thiolated antibodies were reduced with 20 mM dithiothreitol at pH 5.0. Fresh reduced antibodies were reacted with above prepared vesicles (0.25 mg/µmole PL) by adjusting the pH of the mixture to 6.5-6.8 with 1 N NaOH. The reaction was carried out for 14 hr at 4°C under constant stirring. The vesicles were separated from the unreacted proteins by ultracentrifugation at 50,000 rpm for 2 hr using a metrizamide gradient.

Example VII

Preparing Liposomes Containing Surface-bound Anti-HCG Antibody and ß-Galactosidase

ß-galactosidase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Lipid vesicles were prepared by a reverse phase evaporation method as described in Example VI. The enzyme solution used in preparing the liposomes in Example VI was replaced by a buffer containing 150 mM NaCl, and 1mM EDTA, pH 6.0. The buffer (300 µl) was added to a lipid mixture dissolved in 1 ml diethylether and the two phases emulsified by sonication for one minute at 25°C in a bath sonicator. Ether was removed under reduced pressure at room temperature and the resulting dispersion was extruded successively through 0.4 micron unipore polycarbonate membranes (Bio-Rad Laboratories, Richmond, CA). Freshly reduced antibody (0.1 mg) and ß-galactosidase (0.375 mg) was reacted with 1 µmol vesicle lipid in 1 ml reaction buffer described in Example VI. After stirring under a stream of argon for 14 hours at room temperature, the vesicles were separated from the unreacted proteins by ultracentrifugation at 50,000 rpm for 2 hr using a metrizamide gradient.

Example VIII

Competitive Inhibition Assay

A surface reagent having surface-bound hCG molecules was prepared as in Example III. A suspension of liposomes having surface-bound ß-galactosidase and anti-hCG was prepared as in Example VII.

A 0.05 ml test sample containing hCG, at one of the concentrations indicated at the left in Table 6 below, was added to one of six assay tubes containing 3

nmoles liposomes to a final liquid volume of 0.25 ml in 20 mM phosphate buffer, 150 mM NaCl, pH 7.4. Each assay mixture was pre-incubated at 25°C for 30 minutes. Three surface reagent beads were then added to each assay tube and incubated at 25°C for 1 hr. The reagent beads were washed 2 times with the assay buffer.

The washed reagent from each sample was placed in 0.5 ml of assay solution containing phosphate buffer, pH 7.8, and 2.7 mM ONGP (Example III). The conversion of the substrate was followed at 405 nm, and enzyme activity expressed in terms of OD per bead. The results obtained are shown in the second column in Table 6 below. Each data point is the mean value for three separately measured beads.

Table 6

[hCG] (IU/ml)	Absorbance, 405 nm (O.D./bead)
0	1.592±0.068
0.128	1.616±0.088
0.640	1.531±0.046
3.2	0.608±0.076
16	0.281±0.029
80	0.112±0.030

The assay was sensitive to changes in hCG concentration in the range between about 0.64 and 80 IU/ml.

Example IX

Competitive Inhibition Assay for HCG

A competitive inhibition assay for determination of hCG was carried out substantially as described in Example VIII, but using liposomes from Example VI having surface-bound anti-hCG and encapsulated alkaline phosphatase. The assays were carried out in a 20 mM phosphate buffer, 150 mM NaCl, pH 7.4, the analyte being initially introduced to one of the concentrations shown at the left in Table 7 below in an initial analyte volume of 0.05 ml. To each of the ten samples was added 0.3 nmole of liposome reagent in 0.2 ml. Each mixture was preincubated at 25°C for 30 min, 3 surface-reagent beads were added. The reaction mixtures were incubated at 25°C for 1 hr.

After removing the reaction fluid by aspiration, the beads were washed four times with reaction buffer and surface-bound enzyme was assayed as in Example III. Each data value shown in Table 7 is the mean value for three separately determined beads. The results show a substantially linear relationship between the log of the analyte concentration and the enzyme activity measured, in the analyte range between about 16 and 3,200 IU/ml.

Table 7

[hCG] (mIU/ml)	Absorbance, 405 nm (O.D./bead)
0	1.202±0.052
8	1.043±0.065
16	1.039±0.065
32	0.843±0.017
64	0.504±0.059
128	0.297±0.013
640	0.081±0.008
3200	0.033±0.002

Example X

Sandwich-type Assay for Determination of hCG

Mouse monoclonal antibodies specific against either the α or ß subunits of hCG were obtained from Bioclone Australia Pty (Marrickville, Australia). Rabbit polyclonal anti-hCG antibody was obtained from New England Immunology (Cambridge, MA). A solid support reagent composed of cellulose acetate beads having surface-bound antibody against the α subunit was prepared as in Example III, reacting cyanuric chloride-activated substrate with a solution of 1 mg/ml antibody.

Liposomes having encapsulated alkaline phosphatase enzyme and either surface-bound monoclonal antibodies against the ß-subunit of hCG or polyclonal anti-hCG antibodies were prepared substantially as in Example VI.

Two samples containing each of the increasing concentrations of hCG, in a sample volume of 0.05 ml were prepared. To each sample was added 0.2 ml of the

monoclonal- or polyclonal-antibody liposomes to a final liposome concentration of about 0.3 nmole PL/ml, and 3 beads of the surface reagent. Each sample was incubated at 25°C for 1 hr in a final assay mixture containing 50 mM phosphate buffer, pH 7.4. The sample beads were washed 4 times with the assay buffer and each bead suspended in 0.5 ml of the enzyme assay medium employed in Example III. The enzyme activities for each hCG concentration, expressed as mean OD/bead, are shown in the middle column in Table 7 below for the liposome reagent prepared with surface-bound polyclonal antibody and, at the right in Table 7, for the liposome reagent prepared with surface-bound monoclonal antibody.

With both types of liposomes used, increased analyte led to increased liposome binding over the hCG concentration range between about 15 and 250 mIU/ml.

Table 8

Concentration of hCG (mIU/ml)	Absorbance (OD/bead) Polyclonal Antibody	Monoclonal Antibody
0	0.068±0.004	0.024±0.002
15.6	0.368±0.023	0.140±0.008
31.2	0.762±0.009	0.316±0.020
62.5	1.495±0.065	0.718±0.050
125	2.283±0.025	1.438±0.046
250	2.630±0.010	2.280±0.020
375	2.650±0.017	2.570±0.017

While preferred embodiments for making and using the invention have been described, it will be apparent that various changes and modifications can be made without

departing from the invention. In particular, it will be appreciated from the foregoing that many different analyte/anti-analyte pairs of the type mentioned above will function in substantially the same way, to produce substantially the same results as those exemplified herein, in a solid-phase liposome immunoassay system for determination of a variety of analytes, either by competitive-inhibition or sandwich-type liposome binding to a solid support (see, for example, the above-mentioned co-owned patent application for "Lipid-Vesicle Surface Assay Reagent and Method").

IT IS CLAIMED:

1. A cellulose acetate substrate whose surface acetate groups have been replaced by hydroxyl groups to form a hydrophilic surface shell which protects the substrate from dissolution in organic solvents capable of dissolving cellulose acetate.

2. A cellulose acetate substrate having surface-bound amine-reactive groups.

3. The substrate of claim 2, having a hydrophilic shell of surface OH groups adapted to protect the substrate from dissolution in organic solvents capable of dissolving cellulose acetate.

4. The substrate of claim 2, wherein the reactive groups include hydroxyl-coupled cyanuric chloride or carbonylimidazole groups.

5. A surface reagent for use in a liposome immunoassay based on immunospecific attachment of liposomes to the reagent comprising

a cellulose acetate substrate whose surface has been treated to replace surface acetate groups with hydroxyl groups, and

an array of surface molecules attached to the substrate through bifunctional linking agents linking the substrate hydroxyl groups to reactive groups in the surface molecules.

6. The reagent of claim 5, wherein the bifunctional linking agents includes cyanuric chloride or carbonyl diimidazole.

7. The reagent of claim 5, wherein the substrate has been treated to form a hydrophilic surface shell adapted to protect the substrate from dissolution by solvents capable of dissolving cellulose acetate.

8. The reagent of claim 5, wherein the surface molecules include hCG molecules.

9. The reagent of claim 5, wherein the surface molecules include antibody or antibody fragment molecules.

10. An assay system for determination of an analyte, comprising

liposomes having surface-bound analyte-related binding molecules and entrapped reporter molecules, and

a surface reagent composed of a cellulose acetate substrate whose surface has been treated to replace surface acetate groups with hydroxyl groups, and, attached to the substrate surface through such hydroxyl groups, surface molecules adapted to bind the liposomes to the reagent in proportion to the amount of analyte present.

11. The system of claim 10, wherein the reagent surface molecules are adapted to bind immunospecifically with analyte molecules, and the liposome binding molecules are adapted to bind to the analyte molecules, with such bound to the reagent.

12. The system of claim 11, wherein the analyte is hCG and the surface and analyte-related binding molecules are one and another antibody or

antibody fragments capable of binding immunospecifically to the α and ß hCG subunits, respectively.

13. The system of claim 10, wherein the analyte is adapted to compete with analyte or analyte-like surface molecules on the reagent for binding to anti-analyte binding molecules carried on the liposomes.

14. The system of claim 13, wherein the analyte is hCG, the ligand molecules on the reagent are hCG, and the analyte-related liposome binding molecules are anti-hCG antibody or antibody fragments.

15. The system of claim 10, wherein the liposome-entrapped reporter molecules are encapsulated within the liposomes.

16. The reagent of claim 10, wherein the entrapped reporter molecules are attached to the surface of the liposomes to form a co-array of binding and reporter molecules carried on the liposomes surfaces.

17. A method of attaching ligand molecules to a cellulose acetate substrate comprising

treating the substrate with a strong base to replace surface acetate groups with hydroxyl groups, and

coupling the ligand molecules to the substrate through such hydroxyl groups.

18. The method of claim 17, wherein said treating is carried out under conditions which produce a hydrophilic shell of hydroxyl groups capable of

protecting the substrate from dissolution in solvents which are capable of dissolving cellulose acetate.

19. The method of claim 18, wherein said coupling includes reacting the treated substrate with a bifunctional linking agent in an organic solvent.

20. The method of claim 23, wherein the linking agent is cyanuric chloride or carbonyl diimidazole.

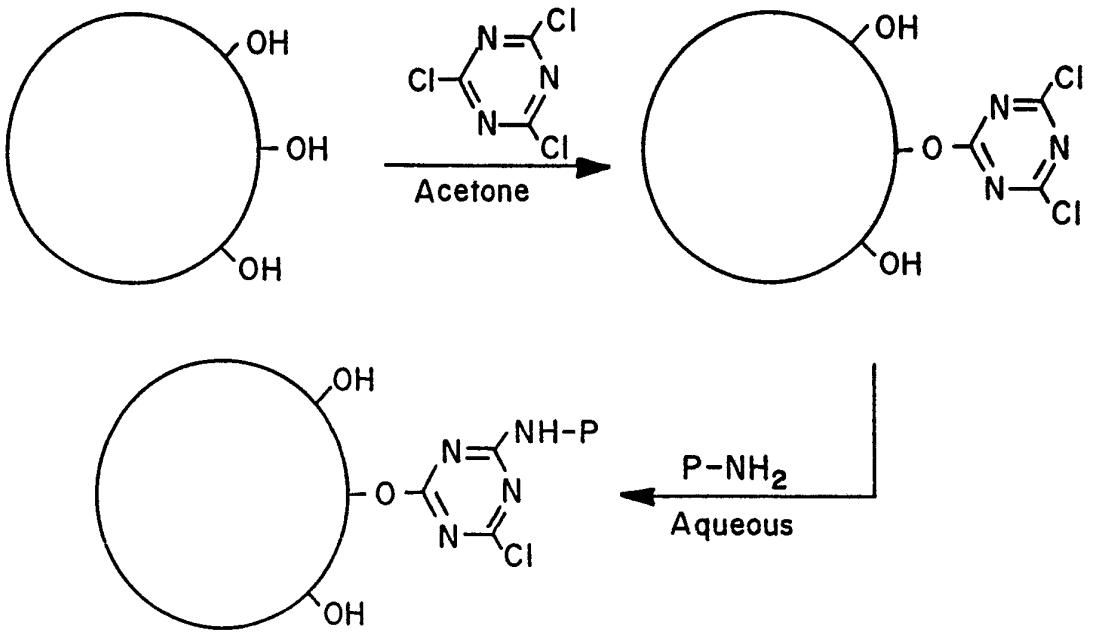

OH
OH
OH
Cl
Cl
N
N
N
Cl
Acetone
O
Cl
N
N
N
Cl
NH-P
P-NH2
Aqueous

FIG. 1

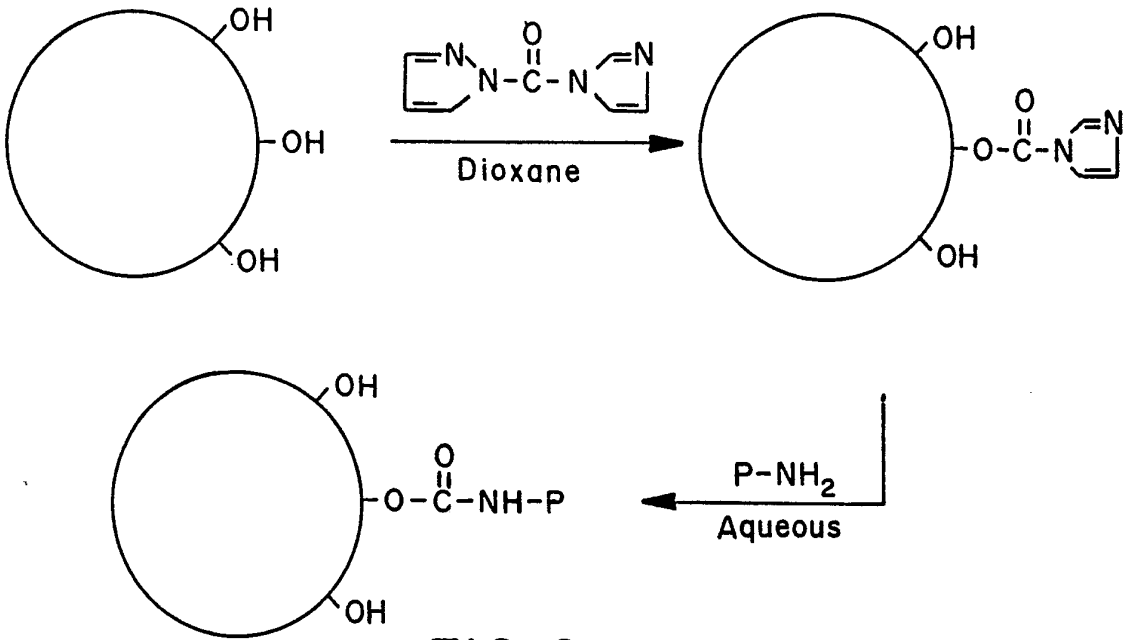

OH
OH
OH
O
N
N
N
N
C
Dioxane
O-C-N
O-C-NH-P
P-NH2
Aqueous

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02233

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.(4); G01N 33/531, 546, 548; B32B 23/00; C08L 1/00
U.S. CL : 435/179, 436/528, 530; 427/2, 324.

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/179 427/2, 324 436/528,530,818,829 530/814

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CAS ON LINE SEARCH BI/AB
(ACETYL CELLULOSE OR CELLULOSE ACETATE OR 9004-45-7) AND (liposom/ or lipid vessicle?) AND (IMMUNO? OR ANTIBOD? OR ANTIGEN?)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US,A, 3,824,150 (LILLY ET AL) 16 July 1974 see column 2, line 41; column 3, lines 9-24; Examples 1,4	1-7,9, 17-20
X	US,A, 4,012,571 (DEAN ET AL) 15 March 1977 see column 2, lines 31-49	2,4
X	US,A, 4,033,822 (GREGOR) 05 July 1977 see Examples I-III	1-7,9, 17-20
X	US,A, 4,090,022 (TSAO ET AL) 16 May 1978 see column 3, line 18- column 4, line 42; column 6, lines 45-55.	1-7,9, 17-20
Y	US,A, 4,195,128 (HILDEBRAND ET AL) 25 March 1980 see column 1, lines 9-52.	2,4-6,20

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

"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

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
05 Dec. 1986	02 JAN 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	David A. Saunders David A. Saunders

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	US,A, 4,357,311 (SCHUTT) 02 November 1982 see column 2, line 40- column 3, line 41; Example 1.	1-7,9, 17-20
Y	US,A, 4,483,929 (SZOKA) 20 November 1984 see column 4, line 8; column 10, line 22- column 11, line 7.	5-11,13-15
Y	US,A, 4,514,505 (CANFIELD) 30 April 1985, see column 3, lines 50-58	12