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(54) Title: COMPLEX FOR IN-VIVO TARGET LOCALIZATION

(57) Abstract

To localize targets within the body, antibodies are linked via polysaccharide or polymer spacer arms to rapidly cleared, Tc-99m labeled submicron-sized, albumin microspheres. The polysaccharide or polymer spacer arms are attached to the surface of the microspheres to provide hydrophilic, sterically free binding sites with a high loading capacity for targeting or effector molecules. These labelled albumin targeting microspheres may be used to detect a variety of sites of clinical interest using non-invasive external imaging devices and may be employed to carry therapeutic agents to these sites.

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COMPLEX FOR IN-VIVO TARGET LOCALIZATION

This application is a continuation-in-part of U.S. Serial No. 525,258, filed May 17, 1990, the contents of which are hereby incorporated by reference into the subject application.

Background of the Invention

Field of the Invention

This invention relates to macro-molecular complexes which are used to locate specific targets within the body of an animal or human. More particularly, this invention relates to a microsphere core coupled with a spacer arm, which may additionally be coupled with a specific targeting molecule. The specific targeting molecule may be an antibody or fragment of an antibody which is directed to the desired target. By labeling the macro-molecular complex, e.g. with a radioisotope, the location of the target can be detected by locating the macro-molecular complex.

Description of the Prior Art

To detect a site of interest or disease within an organism, a radioactive tracer is often employed that localizes at that site through mechanical or biochemical means. The localization or uptake of the tracer is then defined by external imaging devices such as a radionuclide gamma camera. The ability to externally detect the site of interest is dependent on a variety of factors such as amount localized, specific activity of the isotope, the attenuation of the isotope radiation and the detector efficiency. The overall detectability of a site is best summarized by the concept of a ratio of the target signal to background noise.

Mono- or polyclonal antibodies (e.g. immunoglobin G or IgG) and their fragments are being applied as carriers of radioactive isotopes to increase the localization specificity of the tracer molecule. Unfortunately there is a large amount of non-specific background in radiolabeled antibody images which decreases an observer's ability to detect sites of specific localization. To enhance target signal relative to background noise, investigators have delayed imaging from hours to days, used background subtraction and image filtering algorithms, modified the size of the antibody molecule, varied antibody dose level, altered routes of administration, added side groups to the antibody, administered secondary antibodies, and have attempted to label the antibody after localization. The problem of non-specific background is most evident with short lived tracers such as Tc-99m or when there is an acute need for clinical information. Tc-99m labelled anti-fibrin, for

example, has not been efficacious in detecting pulmonary embolism.

One successful method to clear background is widely applied in liver/spleen scanning and gastro-intestinal blood loss studies. Colloidal suspensions of Tc-99m albumin microspheres are rapidly cleared by the reticulo-endothelial system (RES) leaving a small fraction of the injected dose within the vascular pool. This rapid clearance, with only a small fraction of the injected dose remaining in the vascular pool, results in a reduction of background noise. These rapidly cleared albumin microspheres can be targeted to a site of interest by attaching antibodies to them. Monoclonal antibodies can be attached to the microspheres by means of absorption, direct coupling and indirect coupling via an intermediate (spacer) molecule.

With absorption, IgG molecules bind firmly to hydrophobic surfaces by van der Waals-London forces. However, competitive displacement of adsorbed proteins (IgG) has been shown to occur. This imposes limitations on the usefulness of this technique for in vivo targeting.

Antibody can be adsorbed non-covalently onto the surface of the microspheres by means of a ligand which interacts specifically with the intact or modified antibody. For such purposes, avidin-biotin and protein A have been used in coupling IgG to albumin microspheres. The latter approach has limited usefulness in-vivo as certain subclasses of IgG bound via protein A can activate the complement system.

Direct binding to microspheres may occur if functional groups capable of covalently bonding with proteins, e.g., aldehyde groups, are available on the

surface of the microspheres. Proteins and other molecules, for example, can be covalently bound to latex spheres under a variety of mild conditions using water soluble carbodiimides, cyanogen bromide and glutaraldehyde. Indeed, polystyrene latex particles have been used as immunochemical markers for scanning electron microscopy. In vivo applications of such a reagent, however, are limited because they are not biodegradable and their hydrophobic surfaces adhere non-specifically to many tissues and molecules. Microspheres polymerized from natural materials such as albumin and gelatin contain surface amino and carboxyl groups that can be used to attach antibody molecules. Coupling sites may also be introduced, i.e., free aldehyde groups can be generated, by cross-linking albumin with glutaraldehyde. Unfortunately, direct linkage of antibody to microsphere surfaces may compromise the active site or block it through stearic effects.

Thus, a need exists for an efficient method of coupling antibodies to microspheres, in which the resulting macro-molecular complexes provide suitable target signal to background noise ratios.

The resulting macro-molecular complexes must also be efficiently labelled for detection. Albumin microspheres are labelled with Tc-99m in preparations of colloids for liver and lung scanning. The most common method of labelling uses stannous chloride, but a variety of techniques have been employed. The labelling of microspheres with technetium is highly dependent on the microsphere composition and is made difficult by the tendency of technetium and tin to form into colloidal suspensions at body pH. This confounds the labelling of the microspheres.

Thus, a need also exists for an efficient method of labelling the macro-molecular complexes.

Summary of the Invention

To this end, the subject invention provides for the production of submicron sized, stable hydrophilic albumin microspheres without the use of oil emulsion media or organic polymer solutions. The albumin microsphere complexes are designed for intravascular targeting and incorporate long hydrophilic spacer molecules. The microsphere complex is novel in its application to external imaging because it can be efficiently coupled with glycoproteins (antibodies) and labelled with large quantities of Tc-99m to provide it with a highly detectable signal at a target site. Furthermore, the complex can be cleared from the circulation with a rapid but controllable rate to allow a low background noise. This original combination is effective in producing a high signal to background noise ratio that is important in external detection, and which is very difficult to achieve with previous antibody mediated tracer localization technology. Highly directed coupling of targeting/effector molecules to the microsphere complex can be obtained, as well as the efficient labelling of the complexes.

In one embodiment, the invention provides a macro-molecular complex constructed from a unique formulation of a microsphere central core together with a long spacer arm that is chemically prepared to attach to specific targeting or effector molecules. This construction provides a favorable signal to noise ratio due to the contrast between the high targeted sphere activity and the low, RES cleared, background. The

macro-molecular complex described in this disclosure will hereafter be referred to as the complex.

In one embodiment, the complex comprises a supporting central core, a hydrophilic spacer arm greater than 20 Angstroms in length connected to the central core, and a specific targeting molecule that is attached to the spacer arm. The central core is typically heat denatured albumin and the targeting molecule is typically an antibody or antibody fragment. The spacer arm is typically a polysaccharide or polymer, preferably a polysaccharide such as dextran or a polymer such as polyethylene glycol. The complex resulting from the preferred embodiment is essentially a huge multi-valent antibody with hundreds of thousands of targeting molecules and a high tracer activity. The central core restricts the antibody-tracer complex to the intravascular space where non-specific activity can be cleared. However, the size of the complex does not restrict its access to the endothelium that is altered by vascular disease (clots, plaque), or which contains neovascular antigens or the molecular signals controlling cell traffic in regional inflammation.

Effector molecules, such as drugs, can also be attached to the complex for direction to the target of interest.

The complex is produced by forming the central core from a material that is appropriate for in-vivo use, by linking the core to a spacer arm appropriate for in-vivo use that is sufficiently long to provide high cross section for targeting molecule attachment, and by connecting a specific targeting molecule to this arm. A symmetric hydrazide may be used for coupling the spacer arm to both the central core and the specific targeting molecule. The complex may be

labelled with a radio-isotope, such as Tc-99m, to allow its in-vivo localization.

The complex is injected into the blood to allow external identification (through the use of an imaging device or detector) of sites that are located by the specific targeting molecules attached to the complex. The complex provides a means for achieving high target signal to background noise ratio and hence improved target detection. This results from a combination of high complex tracer loading and low background noise due to controlled clearance of non-targeted complex from the blood. Alternatively, the complex may be used to clear the blood of targets, such as antibodies, drugs, viruses, bacteria, or cells, that become attached to the specific targeting molecules.

Brief Description of the Drawings

These and other objects, advantages and features of the present invention will be more fully understood from the following detailed description of certain embodiments thereof when considered in conjunction with the accompanying drawings in which:

FIG. 1 is a diagrammatic representation of a macro-molecular complex which is one embodiment of the subject invention;

FIG. 2 is a schematic of the process of one embodiment of the subject invention in which albumin microspheres as linked to whole immunoglobin molecules. The surface of the spheres is first derivatized using succinic anhydride to provide a linkage site for the dextran spacer arm and to keep the particulates from cross-linking during subsequent modification.

Succinic dihydrazide is used to couple the dextran to the sphere and the antibody; and

FIG. 3 is a schematic of the process of one embodiment of the subject invention in which dialdehyde stabilized albumin microspheres are linked to an antibody fragment (Fab'). The spacer, diamino dextran, is coupled to the aldehyde groups on the microsphere via reduction of Schiff base bonds. The opposite end of the spacer is converted to an activated sulfhydryl using S-acetyl mercaptosuccinic anhydride and 5,5'-dithiobis (2-nitrobenzoic acid). The final disulfide bond is formed with sulfhydryls on the antibody fragment far from its active site.

Detailed Description of the Invention

The subject invention provides a macro-molecular complex which can be used for localizing a target, for example within a human or animal. In one embodiment, the macro-molecular complex comprises a bio-compatible in-vivo circulatable central core, and a bio-compatible hydrophilic spacer arm greater than 20 Angstroms in length coupled to the central core.

Preferably, the central core comprises albumin and provides a solid foundation for covalent attachment of antibodies. The core may be formed in any suitable way, but the preferred method for forming the core (microspheres) from albumin is in the absence of oil emulsion media, organic polymer solutions, or wetting agents. The preferred method includes a flash heat annealing step which stabilizes the microspheres to achieve the better foundation for covalent attachment of antibodies. The central core may be further stabilized by chemical crosslinking.

Other circulatable biocompatible materials may be used to form the central core, in addition to albumin, such as iron oxides. Iron oxide cores impart the properties of magnetic responsiveness to the central core.

The hydrophilic spacer arm preferably comprises a hydrophilic molecule greater than 20 Angstroms in length. The spacer arm has two or more attachment points, typically on its extremes, each of which is capable of attaching, for example, to a sphere or to an antibody. In the preferred embodiment, attachment of the spacer arm at each of its ends, as opposed to along the length of the spacer arm, helps to maintain the structural integrity of the spacer arm molecule. The preferred molecules which comprise the spacer arm are polysaccharides and polymers which are greater than 20 Angstroms in length and which are hydrophilic. These preferred molecules are also characterized by being linear or having long linear segments, having native or introducible attachment sites, and being of low immunogenecity. Examples of suitable molecules include dextrans, modified starches, chitosans, heparin, chondroitin sulfates, polyethylene glycols, polyvinyl alcohol, and polyacrylic acids, or the derivatives of any of these molecules. Preferred suitable molecules include dextran, diamino-dextran, polyethylene glycol, and diamino polyethylene glycol. The characteristics of each of these preferred molecules which enable them to be used as spacer arms is that they are hydrophilic, biocompatible, linear and have ends which allow attachment of the spacer arm at each end. Hydrophilic, biocompatible, branched molecules can also be used as spacer arms.

In order to target the macro-molecular complex within a subject, one embodiment of the macro-molecular complex further comprises a specific targeting molecule coupled to the spacer arm. Typically, this targeting molecule is an antibody or a fragment of an antibody. The antibody is directed to a specific antigenic substance within the subject. When the macro-molecular complex is introduced into the subject, the antibody comes in contact with, and binds to, its specific antigenic substance. The macro-molecular complex will thereby localize and target the specific substance within the subject.

In order to then detect the target, the macro-molecular complex is labeled with a detectable marker. Preferably, this marker is a radio-isotope such as technetium (Tc-99m). Any suitable means for labelling and detecting the presence of the macro-molecular complex can be used in addition to technetium 99m. For example, other radio-isotopes commonly used in medicine and well known to those skilled in the art include iodine-123, iodine-125, iodine-131, gallium-67, indium-111, or indium-113m, or mixtures thereof. The marker could also be a paramagnetic ion, contrast agent, or magnetic material such as those commonly used in medicine. These could include gadolinium and chelated metal ions of chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), ytterbium (III), or iron oxides, or mixtures thereof.

The labeled macro-molecular complex can be used to detect a target by contacting the complex with the target under conditions such that the complex binds to the target. The bound complex is then detected, thereby detecting the target.

Alternatively, the macro-molecular complex, with or without a label, can be used to remove a target from a subject. This is accomplished by contacting the macro-molecular complex with the target under conditions such that the complex binds to the target. The macro-molecular complex is then removed, thereby removing the target which is bound thereto. This is useful when the targeting molecule of the macro-molecular complex is, for example, an antibody directed to a toxin, a drug, a virus, or a bacteria. By contacting the macro-molecular complex with the toxin, drug, virus or bacteria, the complex binds thereto and then the substance can be removed from the subject by removing the macro-molecular complex. Typically, this removal is effected via the subject's reticulo-endothelial system.

In a further embodiment of the subject invention, an effector molecule is also bound to the complex. The effector molecule may be any molecule which is to be directed to the target location. For example, if the target is a fibrin clot, an effector molecule such as a thrombolytic agent may also be attached to the macro-molecular complex. A targeting molecule such as anti-fibrin antibody is used to localize the complex to the fibrin clot, and the thrombolytic agent, such as streptokinase, is used as the effector molecule. Thus, when the targeting molecule locates the fibrin clot and binds thereto, the thrombolytic agent is also delivered to the site of the fibrin clot. The thrombolytic agent will have an "effect" at the targeted site, for example, dissolving the clot. Effector molecules which can be bound to the complex of the subject invention include any drugs or therapeutic agents, with thrombolytic agents being merely one example.

Macro-molecular complexes of the subject invention may be provided in a liquid form in combination with a physiologically acceptable carrier. Such a composition comprising the macro-molecular complex and the physiologically acceptable carrier may be prepared in a lyophilized form. When the complex is ready to be used, the composition is reconstituted by adding a physiologically acceptable carrier back into the lyophilized complex.

The spacer arm of the subject invention, which comprises a chemical compound, may also be provided in composition form. The composition comprises the spacer arm and a suitable carrier, and may also be provided in lyophilized form.

The subject invention further provides a method of producing a stabilized albumin bio-compatible in-vivo circulatable central core (microsphere) for use in macro-molecular complexes. The method involves: (a) dissolving the albumin in an aqueous solution; (b) raising the pH of the resulting solution to about pH 9-11; (c) heating the then-resulting solution having a pH of about 9-11 at about 70-90°C for about 15-25 minutes; (d) cooling the heated solution to about 20-30°; (e) lowering the pH of the cooled solution to about 6-8; (f) adjusting the pH of the resulting solution having a pH of about 6-8 to about 5.8-6.6 so as to produce the albumin microspheres; (g) heating the then-resulting solution having a pH of about 5.8-6.6 to 70-120°C until the albumin microspheres are stabilized; and (h) recovering the resulting stabilized microspheres.

The heat annealing step (g) which involves heating the solution containing the albumin microspheres and having a pH of about 5.8-6.6 to 70-120°C, results in the stabilized microspheres. Any microspheres in an

aqueous solution can be stabilized using this unique step which comprises flash heat annealing.

The central core may be further stabilized by chemical crosslinking with a suitable dialdehyde, such as glutaraldehyde or acrolein, or with a carbodiimide such as 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide.

The subject invention also provides an improved method of labeling, rapidly with very high efficiency, the macro-molecular complex of the subject invention. The method of labeling utilizes a tin reduction procedure. Prior art labeling of microspheres used in lung scintiscanning for example, typically utilizes a tin reduction method. These methods generally comprise dissolving $SnCl_2$ at low pH to form a solution and exposing the microspheres to the dissolved $SnCl_2$. Subsequently the microsphere/$SnCl_2$ composition, usually in a lyophilized form, is exposed to a solution of Tc-99m to effect the labelling of the microspheres. The subject invention provides an improvement of this method, thereby achieving a high labeling efficiency without exposing the complex to potentially damaging low pH levels.

This efficiency is obtained by combining the $SnCl_2$ dissolved in a low pH solution with a transfer ligand, preferably sodium saccharate, and then neutralizing the pH of this combined solution prior to exposing the microspheres to the solution. This results in the binding of $SnCl_2$ to the microspheres. Unbound $SnCl_2$ and transfer ligand are then removed prior to exposing the resulting microspheres to Tc-99m. The interactions between the microsphere, the $SnCl_2$ and the transfer ligand result in a labeling efficiency typically in excess of 99.5%. The use of a transfer ligand thus

avoids exposure of the complex to low pH levels, provides high efficiency of the labeling, and is also a rapid method of labeling.

Production of the macro-molecular complexes of the subject invention is accomplished by forming the bio-compatible in-vivo circulatable central core, and coupling the spacer arm to the central core. Where a specific targeting molecule is also included in the complex, this specific targeting molecule is coupled to the spacer arm.

Stabilized albumin bio-compatible in-vivo circulatable central cores for use in macro-molecular complexes are provided by the method described above. Stabilized microspheres which are produced by the method, involving the unique heat annealing step, are efficiently labeled by the method discussed above which uses a transfer ligand.

The following examples are illustrative of the preferred embodiments of the subject invention, but are not intended to limit the scope of the invention to the specific examples.

Preparation of Central Core

The central core 1 (see Figure 1) is typically 1,000 - 10,000 Angstroms (Å) in diameter but may vary in size depending on the preparative procedure. The submicron or micron sized central core is synthesized to withstand the chemical manipulations necessary to attach targeting or effector molecules, such as monoclonal antibodies 3 (see Figure 1). In the prior art, albumin colloids used for liver scanning are dissolved by high or low pH, ultrasonication or reductions in stabilizing quantities of free albumin. The central cores of the subject invention provide a

better foundation for covalent attachment of antibodies because the microspheres are formed from albumin in the absence of oil emulsion media, organic polymer solutions or wetting agents and are heat annealed. This formation, especially with the heat annealing step, provides hydrophilic, relatively uniform, stable spheres that are easily labelled with Tc-99m 4 (see Figure 1).

Microspheres of the subject invention are produced using simple equipment. Mild heat and elevated pH serve to uncoil the protein (albumin) such that subsequent cooling and reduced alkalinity cause refolding into globular clumps. The pH of the solution during the refolding process largely determines the final size of the spheres. Spheres between 10 and 20 nm (nanometer) are formed with neutral pH solutions while larger spheres result from mildly acidic solutions (pH >5.0). In the preferred embodiment, at about pH 6.2, the solution is moderately opalescent and particulates are barely visible microscopically (45x). At this point the solution is flash heated over a 30 - 60 second interval in an 85°C water bath. A similar procedure described by Taplin [J Nucl Med 1964, 5:259-275] does not include a flash reheating step. Heating the microspheres strongly stabilizes them, for without this step the particulates are poorly resistant to high (>11) or low (<2) pH, ultrasonication and chemical surface modification.

In the preferred embodiment, a 1% solution of essentially fatty acid-free albumin (Sigma A-6289) is prepared by dissolving 2 grams of albumin in doubly distilled deionized water. Tracer quantities of desalted (PD-10 Sephadex column, Pharmacia) I-125 Human serum albumin (Mallinckrodt Diagnostic Products) are

added and the solution is raised to pH 10 with 1 M NaOH. The mixture is heated at 80°C for 20 minutes and cooled on ice to room temperature. After the solution pH is neutralized with 0.1 M HCl, 0.05 M HCl is added drop wise with continuous stirring. As the solution approaches pH 6.2, one half ml aliquots are swirled in small glass test tubes in an 85°C water bath and are examined on a hemocytometer. When particles of .5-1.0 micron are evident microscopically (45x), the solution is passed through 1 meter of intravenous tubing (Baxter 2C5545s) that is immersed in the water bath (30 second transit time). The particulates are centrifuged at 100 rcf for 5 minutes to remove any large aggregates. Protein mass incorporated into microspheres is determined by measurements of activity recovered in samples spun at 4300 rcf. Average size is measured from electron micrographs. Typical yields vary between 40 and 70% protein recovery in microspheres ranging from 0.1 to 1 micron in diameter. The microsphere produced by this method will be referred to hereafter as the foundation microsphere.

Production of Polysaccharide or Polymer Derivatized Microspheres

As it is desired to give the antibody a high degree of rotational freedom and reduced stearic hindrance, a polysaccharide (e.g. dextran) or polymer (e.g. polyethylene glycol) spacer arm 5 (see Figure 1) is used to link antibodies to the microspheres. In this embodiment, dextran or polyethylene glycol (PEG) provides an uncharged, hydrophilic, relatively inert, linear arm for which mild procedures can be used in antibody coupling. The polysaccharide or PEG linked to the surface of the albumin core allows a very high

complex targeting molecule loading capacity which far exceeds that previously reported. The polysaccharide or PEG spacer arms provide a very high cross-section for molecular attachment that shorter spacer arms or direct microsphere coupling is not capable of providing. Based on a dextran loading of 200 ug dextran per milligram of microspheres and 2 billion microspheres of 1 micron diameter per milligram, there is a potential for over 6 million attachment sites per microsphere.

The polysaccharide or PEG coat provides a substrate for attaching groups that determine the rate of particulate clearance by the reticuloendothelial system. It is possible to vary the rate of particulate clearance by changing type or the amount of spacer molecule on the sphere surface or by adding to the polysaccharide/polymer coat other substances that modify clearance. To study the effect of different surface coats on the blood residence time of microspheres, experiments were carried out in rabbits using native microspheres, commercially available albumin colloid and microspheres coated with both dextran and polyethylene glycol spacer arms. Images obtained with all particulate preparations show clearance by the reticuloendothelial system. The native spheres, albumin colloid and dihydrazide covered spheres demonstrate a rapid and comparable clearance with a disappearance half time of between 2.0 and 4 minutes. The spheres with dextran or polyethylene glycol spacer arms show a longer clearance half-life. The optimum rate of clearance will depend on the kinetics of complex localization at the target site. Although it is desirable to clear background prior to imaging, very rapid clearance may result in an

antibody-antigen interaction time that is too short to provide a detectable signal at the target site.

The preparation of the polysaccharide or polymer derived microsphere complex can be effected by several methods.

Method 1. Derivatization by sequential reactions involving the microsphere core and a polysaccharide.

Dextran is coupled after treatment of the foundation microspheres with succinic anhydride and succinic dihydrazide. Treatment with succinic anhydride provides a uniform chemical interface of carboxyl end groups, and a highly electronegative surface that maintains microsphere separation during linkage to dihydrazide. Dihydrazide is coupled to the microsphere via a water soluble carbodiimide which also serves to further stabilize the microsphere core. Once attached, the dihydrazide end groups convert the spheres to an electropositive state which again prevents particulate cross-linking.

In this method, the polysaccharide molecule is oxidized to provide the aldehyde groups that will subsequently undergo Schiff base formation with microsphere hydrazide groups. Sodium periodate which attacks vicinal hydroxyls within the glucose rings of dextran, for example, generates these aldehyde attachment points.

Suitable conditions for dextran attachment are determined from experiments wherein the dextran concentration, reaction time and pH are varied. Four and ten millimolar dextran solutions are combined with hydrazide derived spheres after the dextran is oxidized

with equimolar quantities of sodium periodate. The reactions are carried out for 90 minutes in 0.05 M NaOAc, pH 4.0 at room temperature. Dextran loading is found to be initially rapid but tends to level off after twenty minutes. The nearly proportional relationship between dextran concentration and microsphere loading suggested by these results is further evaluated at various reaction hydrogen ion concentrations. These preparations are reacted for 1 hour, again using dextran that is oxidized with equimolar amounts of periodate. Dextran loading appears nearly proportional to dextran concentration at pH 6, but tends to plateau as the solution pH drops to 4.0. This pH dependence is also evident with increasing dextran oxidation (i.e., as molar ratio of periodate to dextran increases from 1 to 4).

To couple antibody to the dextran spacer arm, a succinic dihydrazide bridge is attached to the glucitol at the reducing end of the dextran molecule. The borohydride used to "stabilize" the microsphere-to-dextran Schiff base bond also reduces the terminal glucose ring on dextran to glucitol allowing it to be rapidly oxidized by sodium periodate.

Prior oxidation of the dextran does not destroy this potential linkage site. Coupling a dihydrazide molecule to the reducing end of the dextran chain provides an average spacer arm of approximately 125 Å, i.e. half of the native dextran chain length.

Procedure: The foundation microsphere solution is maintained at pH 7.5 with 2 M NaOH while 10 grams of finely powdered succinic anhydride (Sigma S-7676) is added in small quantities. To remove reaction products, the particulates are titrated to pH 4.0, and

washed 4 times by 4300 rcf centrifugation and resuspension in distilled water using low wattage ultrasonication (3M Ultrasonic bath). The succinylated microspheres are suspended in 0.5 M succinic acid dihydrazide (SDH, Alfa Products 16200) and are reacted at pH 5.0 with 0.1 M of EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, Sigma E-7750) for 2 hours at room temperature. The hydrazide derivatized microspheres are washed 4 times and stored at 4°C. Dextran (MW 9400, Sigma D-9260) is prepared in a 20 mM solution and added to an equal volume of 20 mM sodium meta-periodate (Sigma S-1878). This is reacted in the dark at room temperature, pH 4.0, for 30 minutes. The reactants are dialyzed to remove residual periodate and iodate byproducts before combining with the hydrazide microspheres. Dextran is coupled to microspheres via Schiff base bonds formed during a one hour reaction at pH 4.0. These bonds are stabilized with 50 mM sodium borohydride (Sigma S-9125, pH 9.0, in borate buffer) for 1 hour at room temperature. Dextran loading per mg microspheres is determined by the phenol-sulfuric acid colorimetric reaction. Dextran coupled microspheres are suspended in equal volumes of 20 mM native dextran and sodium periodate buffered to pH 4.0 with 0.05 M sodium acetate (NaOAc). The particulates are immediately washed 4 times in distilled water and resuspended for 2 hours in 100 mM SDH, pH 6.0. The Schiff bases formed between the dextran and SDH are reduced with 50 mM sodium borohydride for 1 hour at pH 9-10. The product is washed 4 times and stored at 4°C.

Method 2. Microsphere derivatization by coupling dextran-succinic dihydrazide construct.

Dextran is first reduced to produce terminal glucitols with sodium borohydride and then oxidized to create a terminal aldehyde and several other reactive aldehydes at random sites along the polysaccharide backbone. The iodate reaction products are removed by dialysis and succinic dihydrazide is reacted with the dextran chain. Schiff bases formed between SDH and dextran aldehyde functions are reduced with sodium borohydride and the reaction product is exhaustively dialyzed. The dextran succinic dihydrazide spacer arm is coupled to succinylated foundation microspheres using a water soluble carbodiimide.

Procedure: The foundation microsphere solution is maintained at pH 7.5 with 2 M NaOH while 10 grams of finely powdered succinic anhydride (Sigma S-7676) is added in small quantities. To remove reaction products, the particulates are titrated to pH 4.0, and washed 4 times by 4300 rcf centrifugation and resuspension in distilled water using low wattage ultrasonication (3M Ultrasonic bath).

Ten grams of dextran and 3 ml of 1 M $NaHCO_3$ buffer are diluted to 30 ml with distilled, deionized water and 480 mg of NaBH4 are added. The pH is maintained at 9.5 with 1 M NaOH over a 3 hour reaction period, following which the pH is reduced to 4.5 using 2 M HCl. A three molar ratio of NaIO4 (680 mg) was added and reacted for 30 minutes at room temperature in the dark. The pH was maintained at 4.5 during the reaction and the reactants are dialyzed to remove iodate byproducts.

The dialysate was combined with 25 grams of succinic dihydrazide that had been recrystallized in the cold after solution in 20 cc of water and 4300 rcf centrifugation to remove solid impurities. The reactants were adjusted to pH 5.5 with .1 M NaOH and the Schiff base coupling reaction was allowed to proceed overnight at room temperature. The pH of the solution was then raised to 9.5 with 2 M NaOH and 240 mg of $NaBH_4$ was added to stabilize the SDH - dextran bonds. The resulting product is exhaustively dialyzed against distilled water using 2,4,6-trinitrobenzenesulfonic acid [Fields, Meth. Enzymol, 25,464 (1972)] to check for free SDH and determine the molar ratio of SDH to dextran. Typically, 4-6 moles of SDH are found per mole of dextran.

To couple the SDH derivatized dextran to succinylated microspheres, a final concentration of 40 mM EDC is achieved in a solution containing 5 mg/ml microspheres and 50 mg/ml of SDH-dextran. The reaction is carried out at pH 5 for two hours at room temperature. Typical dextran loading determined by the phenol-sulfuric acid test and microsphere I-125 counts is 300-350 ug dextran per mg of microspheres.

Method 3. Microsphere derivatization using hydrazinodextran.

Sodium borohydride reduced dextrans are reacted with p-toluenesulfonyl chloride. The product, mainly 6-O-p-tolylsulfonyldextran is converted into the 6-hydrazino-6-deoxydextran by refluxing with hydrazine and the product is coupled to the carboxylated microspheres. As dextran is mainly a 1-6 glycan, the nitrogen functions are mainly located at the original

terminal primary hydroxyls; for instance C1 of the reducing end glucitol and C6 of the non-reducing end glucopyranoside. Periodate oxidized IgG may be then directly coupled to the terminal hydrazines via reductive amination with sodium cyanoborohydride. This approach reduces the number of coupling steps considerably. The hydrazinodextrans are prepared in bulk and characterized in respect to molecular weight and substitution. The hydrazinodextran is also well defined as to the sites of sphere or effector molecule interaction. The production process favors sites at either end of the dextran chain, keeping the glucose rings intact. These spacer molecules are much less prone to the breakage that relates to the presence of open ring forms found in Methods 1 and 2 resulting from periodate treatment.

Procedure: Succinylated foundation microspheres are prepared as indicated in Methods 1 and 2. Dextran (10,000 MW) 32.4 g is dissolved in 200 ml of 95% pyridine and water is removed at 60°C by rotary evaporation. To the swollen dextran in 2 hours is added 85 grams of p-toluenesulfonyl chloride and the mixture kept for 24 hours at 25°C. After addition of 100 grams of ice and 100 ml of methanol, the mixture is agitated in a blender and the powder is washed with 80% methanol and dried. Twenty-five grams of this material is refluxed for 7 days with 35 ml of anhydrous hydrazine under nitrogen. Excess hydrazine is removed in vacuo and the residue dissolved in 5% acetic acid, dialyzed against water, and lyophilized. This material is analyzed for sugar content and hydrazinosugars by ion exchange chromatography. To calibrate against known compounds the hydrazinodextran is reduced with excess

Raney-nickel (1g/g/10ml water of hydrazinodextran) for 24 hours. After heating to 100°C, the catalyst is removed by filtration and the product dialyzed and lyophilized. Hydrolysis and analysis for constituents gives a high ratio of glucose: 6-amino-6-deoxyglucose : 1-amino-1-deoxy-D-glucitol (D-glucamine) [50:1].

Coupling of the hydrazinodextran to the succinylated microsphere is accomplished using EDC as described under method 2 above.

Method 4. Microsphere derivatization using diaminodextran.

Other embodiments of the complex may be created using a modified foundation microsphere or variations on the preparation of the dextran spacer arm. For example, the foundation microsphere can be both prepared for coupling to the spacer molecule and stabilized as well, using a variety of dialdehydes. In this example, glutaraldehyde is used, but acrolein produces similar results. Furthermore, modification of the procedure defined under method 3 may be used to prepare other spacer arms with attachment points at their ends. The procedure for preparation of diaminodextran and diamino polyethylene glycol are described in this method and in method 5 respectively.

Procedure: The foundation microsphere solution is maintained at pH 6.8-7 with .1 M NaOH. A sufficient volume of 25% glutaraldehyde solution is added to achieve 100mM concentration. The reaction is allowed to proceed for 1 hour at room temperature and the spheres are washed 3 times by 16,000 rcf centrifugation

and resuspension in distilled water using low wattage ultrasonication (3M Ultrasonic bath).

Dextran (10,000 MW) 32.4 g is dissolved in 200 ml of 95% pyridine and water is removed at 60°C by rotary evaporation. To the swollen dextran in 2 hours is added 85 grams of p-toluenesulfonyl chloride and the mixture kept for 24 hours at 25°C. After addition of 100 grams of ice and 100 ml of methanol, the mixture is agitated in a blender and the powder is washed with 80% methanol and dried. Twenty-five grams of this material is shaken with 75 ml of concentrated aqueous ammonia and the resulting homogeneous solution reacted for 16 hours at 20°C. The syrupy material resulting from rotary evaporation was diluted with 50 ml of 5% acetic acid and applied to a 5 by 100 cm Sephadex G25 column. The column fractions containing the product are pooled and lyophilized. Typical yields are 50-65% of starting dextran weight.

Coupling of the diamino-dextran to the glutaraldehyde derivatized microsphere is accomplished at pH 6.5 in 0.1M phosphate buffer over 16 hours at 20°C using a 2:1 dextran/microsphere w/w solution. The reactants are then combined with borate buffer (0.1M final concentration) and adjusted to pH 9.0 to reduce the Schiff base bonds with 100mM $NaBH_4$. The reduction is allowed to proceed for 1 hour prior to washing (4 times) and storage at 4°C.

Method 5. Microsphere derivatization using diamino-polyethylene glycol.

Polymeric spacer arms with the appropriate properties of hydrophilicity and biologic compatibility may also serve the role of the spacer molecule in the

microsphere complex. Polyethylene glycol is an inert, hydrophilic linear polymer that has very low toxicity. It has hydroxyl groups at either end of the molecule that may be modified to serve as attachment points. Diamino derivatives of PEG have been used as examples of polymeric spacer molecules in embodiments of the complex.

Procedure: The foundation microsphere solution is prepared as in method 4. Diamino-polyethylene glycol is synthesized as follows: 45.5 grams of polyethylene glycol (MW 1450) is dissolved in 200 ml of toluene and brought to a boil. Twenty-five ml of pyridine is added and heated until about 10 ml of solvent has evaporated, thereby removing water from the reactants. Five grams of tosylchloride is added and the solution stirred for 16 hours at 45°C. The tosylated PEG is crystallized in 20 ml of methanol. Seventy-five ml of concentrated aqueous ammonia is added to the waxy crystals and the materials are shaken to achieve a homogeneous solution which is reacted at 20°C for 16 hours. The syrupy material obtained after rotary evaporation is diluted to 250 cc with distilled water and applied to a 4 by 25 cm column bed of Dowex 50X2(H). The column is then washed with 500 ml of distilled water and the bound product is eluted with 100 ml of concentrated ammonia in 400 ml of distilled water followed by another 250 ml of distilled water. The eluates are allowed to crystallize following rotary evaporation, redissolved in 30 cc of distilled water and passed over a 5.0 X 80 cm Sephadex G25 column. Ninhydrin positive fractions of the column eluate are pooled and rotary evaporated. The residue is dissolved in 50-100 ml tertiary butanol

and lyophilized. Typical yields range from 15 to 20% of starting PEG weight.

Coupling of the diamino-PEG to glutaraldehyde derivatized microspheres is accomplished at pH 6.5 in 0.1M phosphate buffer over 16 hours at 20°C using a 1:1 diamino-PEG/microsphere w/w solution. The reactants are then combined with borate buffer (0.1M final concentration) and adjusted to pH 9.0 to reduce the Schiff base bonds with 100mM $NaBH_4$. The reduction is allowed to proceed for 1 hour prior to washing (4 times) and storage at 4°C.

Preparation of Antibody and Effector Molecules and their Coupling Procedures

Although target localization rates are a function of many physiologic and antibody factors, antibody uptake at intravascular sites should be significantly faster than at extravascular sites. For example, an extracorporeal perfusion study of a fibrin specific antibody demonstrated 70 percent of maximal antibody localization within 10 minutes of systemic circulation. Despite the fact that individual antibody molecules may dissociate from a target, the complex is less likely to uncouple due to its multivalency. It can be expected that the strength of the binding should grow rapidly after initial target interaction as the number of antibody-antigen combinations increases at the target surface.

There are several methods that may be applied to link the microsphere to targeting molecules, depending on the nature of the targeting molecule. For example, whole immunoglobin molecules or other targeting molecules may be coupled to the complex as described in methods 6 and 7 below. Antibody fragments (Fab') may

be linked to the complex as illustrated in method 8 below.

Method 6: Coupling whole IgG molecules to the complex via antibody carbohydrate moiety.

This method for antibody-complex coupling is based on the reactivity of aldehyde groups generated at the C_H2 region sugar moiety of the IgG molecule. In contrast to immunoglobulin amino acid residues (usually used to couple other molecules to IgG), the carbohydrate prosthetic groups furnish linkage sites that do not usually interfere with antibody activity. Because of their solubility, hydrophilicity, and bulk, oligosaccharides are found on the exterior of the protein and are thus accessible. Favorable conditions for treatment of IgG with periodate and subsequent condensation of the oxidized product with amino compounds have been established. Sodium meta-periodate is used to oxidize the IgG carbohydrate cis-vicinal hydroxyl groups. The resulting aldehyde functions react at pH 4-6 with hydrazide or amide groups on the complex spacer arms to form Schiff bases. The conditions described in the embodiments preserved more than 90% of antibody activity.

Procedure: Anti-glucose oxidase (AJG-0122 Accurate Chemical and Scientific Corporation) and anti-sulfanilic acid are used in the antibody studies. Tracer quantities of antibody labeled with I-131 (IODO-GEN, Pierce 28600) are employed to follow preparative steps and antibody coupling yields. Prior to each use, the labeled antibody is desalted through Sephadex (PD-10 column). Antibody is exchanged into 0.1 M sodium

acetate buffer, pH 4.0, and combined with 20 mM sodium periodate for 30 minutes in the dark at room temperature. The oxidized antibody is separated from periodate and reaction byproducts by desalting through a PD-10 column equilibrated with 0.1 M sodium acetate, pH 4.0. Antibody bioreactivity after oxidation is checked by ELISA assay. The oxidized antibody is coupled to microspheres in 0.1 M sodium acetate buffer, pH 5.5, overnight, and then stabilized with 50 mM borohydride for 1 hour. Preservation of antibody function is checked by precipitation of washed anti-sulfanilic acid coupled complex with native microspheres derivatized with the diazonium salt of sulfanilic acid.

Method 7: Coupling effector or targeting molecules to the complex via cross linking agent or heterobifunctional reagent.

This method for coupling effector and/or targeting molecules to the complex employs a bridging molecule that is coupled to the amino group at the end of the complex spacer arm. The bridging molecule need only be free of potential for internally coupling to the complex itself. For example, a heterobifunctional such as m-Maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) or N-gamma-Maleimidobutyryloxy-succinimide ester (GMBS) bound to the sphere via its succinimide ester then may couple to sulfhydryl groups either naturally occurring on the targeting or effector molecule, or introduced with an agent such as iminothiolane or S-acetyl mercaptosuccinic anhydride.

Procedure: 50 mg of microspheres produced as indicated in method 5 are suspended in 10 ml of 10 mM phosphate buffer, pH 7.4. 10 mg of GMBS dissolved in 500 ul of dimethyl formamide (DMF) are mixed with the spheres at 20°C for 0.5 hour. The spheres are washed 3 times in 20% DMF and then 3 times in phosphate buffer and resuspended in 5 cc of buffer.

500 ug of antifibrin antibody fragment (T2G1s Fab' Centocor, Malvern PA.) are suspended in 100 ul of N2 saturated distilled water and passed through a 1X5 cm Sephadex G25 column that has been pre-equilibrated with degassed, N_2 saturated phosphate buffer. The ml of eluate containing the peak concentration of antibody fragment is added directly to the washed microsphere suspension and reacted at room temperature for 1 hour.

Effector molecules that do not have free sulfhydryls may be coupled to the spheres in a fashion similar to the following: 500 ug of Streptokinase is suspended in 1 ml of 0°C 50 mM triethanolamine hydrochloride pH 8.0. 1 ml of 25 mM of 2-iminothiolane (Traut's reagent) in 50 mM triethanolamine buffer is added and the solution is mixed for 30 minutes at 0°C. The product is passed through a 1X5 cm Sephadex G25 column that has been pre-equilibrated with deaerated, N_2 saturated 10mM phosphate buffer, pH 7.4 and combined with the microsphere suspension and reacted at room temperature for 1 hour.

Method 8: Coupling effector and/or targeting molecules to the complex via disulfide bonding.

In this procedure, the amino group at the end of the complex spacer arm is reacted with iminothiolane or S-acetyl mercaptosuccinic anhydride to introduce a

sulfhydryl that is subsequently activated with a mercaptopyridine or thio-nitrobenzoic acid moiety. This prepares the end of the spacer molecule so that it may form a disulfide bond with free sulfhydryls on the targeting and/or effector molecule.

Procedure: 50 mg of microspheres produced as indicated in method 5 are suspended in 10 ml of 10 mM phosphate buffer, pH 7.4. As the pH is maintained at 7 to 7.5 with 1M NaOH, S-acetyl mercaptosuccinic anhydride is added with vigorous stirring until 100mM concentration is achieved. The reactants are maintained at pH 7.5 for 30 minutes and then the pH is raised to 11 with 1 M NaOH for 15 minutes. The microspheres are then washed in degassed, N_2 saturated 10 mM phosphate buffer, pH 7.4 three times. After the third wash they are resuspended and incubated for 10 minutes in a cold (4°C) solution of 10mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 100 mM phosphate buffer, pH 7.5. The microspheres are then washed with cold, degassed, N_2 saturated phosphate buffer until the washes show less than 0.020 absorbance at 412 nM due to the free DTNB monomer.

The Sephadex G25 column eluate containing antibody fragment or sulfhydryl containing effector molecule described in method 7 is added directly to the washed microsphere suspension and reacted at room temperature for 1 hour.

Tc-99m Labelling of Complex

The protein core is probably the primary site for Tc-99m tracer labelling. Although the site of Tc-99m attachment is unclear, the mass of albumin is relatively high in comparison to the antibody or

effector molecule and provides a greater sink for Tc-99m labelling. The microsphere complex can carry a large amount of tracer relative to a single antibody, which on a uCi/ug IgG basis, can easily exceed a factor of 10. Given the potential for rapid blood clearance, the microsphere complex should allow target imaging at an early enough time that a slow loss of Tc-99m from the microsphere in vivo will not be clinically evident.

The complex may be labelled in various manners depending on the radioisotope chosen. Labelling may be through an association with components of the complex directly or via linker molecules attached to the complex (e.g. a chelating molecule coupled to the spacer arm). Isotopes commonly available for this purpose include, for example, Tc-99m, In-111, and I-131. In the preferred embodiment, Tc-99m is used to label the complex by one of the three methods defined below:

Method 9: Stannous chloride in acidic solution and washed complex.

Low pH (<2.5) is required to dissolve $SnCl_2$ so that it can subsequently interact with the albumin microspheres. At pH levels below 2.5 glycosidic bonds may be hydrolyzed and antibodies can be damaged, hence complex microspheres are maintained just above this pH, soaked in stannous chloride solution to allow adsorption, washed free of tin and then labelled via tin mediated reduction of Tc-99m. In tests without the presence of complex, at the final pH of approximately 2.5 and a tin chloride concentration of 1 mg/ml, no colloid formation is detected after 4300 rcf centrifugation. The washing removes free stannous ions

that otherwise would be available to form colloid as the pH is raised back to the physiologic range.

Procedure: A nitrogen purged solution of stannous chloride 1 mg/ml is prepared by dissolving $SnCl_2$ in 12 N HCl and diluting it with distilled water, adjusting the pH to 2. 200 ul of this is added for each ml of complex microspheres (1-10 mg/ml) buffered with citrate buffer to pH 3. The particulates are allowed to incubate in this solution for one minute prior to centrifugation and are washed with sodium acetate buffer 0.1M, pH 3 and then pH 5.5. Particulates may be stored at this point or immediately combined with Tc-99m. Labeling efficiency studies using Tc-99m are performed by comparing pellet to supernatant counts after incubation for 5 minutes and 4300 rcf centrifugation. Greater than 90% labelling efficiencies are achieved with up to 90 mCi of Tc-99m per mg of microspheres.

Method 10: Low concentration stannous chloride without wash.

When low concentrations of stannous chloride are used it is not necessary to wash the complex to avoid tin colloid formation. It appears that in mildly acidic solutions colloid is not produced if a sufficient quantity of albumin is present.

Procedure: A nitrogen purged solution of stannous chloride 200ug/ml is prepared by dissolving $SnCl_2$ in 12 N HCl, diluting it with distilled water and adjusting the pH to 1.8. 100 ul of this is added for each mg of complex microspheres (1-10 mg/ml) buffered in nitrogen

purged 0.5 M sodium acetate, pH 4. The solution may be lyophilized or labelled immediately with Tc-99m (200 ul). Labelling efficiencies greater than 96% are routinely achieved.

Method 11: Stannous Chloride exposure via a transfer ligand and washing.

Complexing the stannous chloride with a transfer ligand of intermediate avidity appears to accomplish several important objectives. Although the mechanism of action is not established, it appears likely that when complexed with ligand, non-colloidal stannous ion is preserved at physiologic pH and precipitation does not occur in the presence of other moieties such as phosphate. Furthermore, it is likely that the ligand strength reduces transfer to weakly associating sites on the complex. Subsequent washing of the spheres removes the supernatant ligand-associated stannous ion so that Tc-99m is primarily reduced near stronger binding sites for multivalent cations within the complex. These considerations may explain the unexpectedly high efficiency of this simple, rapid and mild labelling procedure.

Procedure: $SnCl_2$ is dissolved in concentrated HCl and adjusted by dilution with N_2 saturated distilled water to pH 1. Sodium Saccharate is combined with the stannous ion in a 2:1 molar excess and the product is stored under nitrogen. Prior to use, the pH of the solution is raised to 7 with 1M NaOH and centrifuged at 16,000 rcf. Microsphere preparations to be labelled are exposed briefly (2-4 minute incubation) to 10 mM concentrations of stannous saccarate and are then

washed 2 times with degassed, N_2 saturated distilled water. The microsphere complex may be lyophilized or labelled immediately with Tc-99m (200 ul). Labelling efficiencies greater than 99% are routinely achieved.

EXAMPLE 1

Fixed Target Detection: Microsphere complexes are produced so as to provide activated DTNB protected sulfhydryls at the end of the complex spacer arm. After the foundation microsphere is linked to diamino-polyethylene glycol and the amino termini are derivatized with S-acetyl mercaptosuccinic anhydride, 5,5'-dithiobis(2-nitrobenzoic acid) is added to activate and protect the microsphere sulfhydryl moieties. 500 ug of antifibrin antibody fragment (T2G1s Fab' Centocor, Malvern PA.) are suspended in 100 ul of N2 saturated distilled water and passed through a 1X5 cm Sephadex G25 column that has been pre-equilibrated with degassed, N_2 saturated phosphate buffer. The ml of eluate containing the peak concentration of antibody fragment is added directly to the washed microsphere suspension and reacted at room temperature for 1 hour.

The washed complex is exposed to stannous saccarate, washed and lyophilized. Prior to use 1 ml of Tc-99m (5-10 mCi) is added in N_2 purged isotonic saline and the solution is administered intravenously. Fifteen to 30 minutes later, gamma camera images are obtained to identify sites of fibrin deposition as appropriate to a patient's presumed condition. For example, these images could be employed to locate venous thrombi, pulmonary emboli or fibrin deposition in association with arterial plaques as may be present in the coronary arteries in patients with acute angina.

Instead of anti-fibrin, other antibodies could be employed to identify sites of fixed endothelial antigens such as found in association with neovascular endothelium or endothelium containing inflammation-associated antigens.

EXAMPLE 2

Clearance of Target Antigen: 20 mg whole anti-murine antibody is desalted through Sephadex (PD-10 column). Antibody is exchanged into 0.1 M sodium acetate buffer, pH 4.0, and combined with 20 mM sodium periodate for 30 minutes in the dark at room temperature. The oxidized antibody is separated from periodate and reaction byproducts by desalting through a PD-10 column equilibrated with 0.1 M sodium acetate, pH 4.0. Oxidized antibody is coupled to complex in 0.1 M sodium acetate buffer, pH 5.5, overnight, and then stabilized with 50 mM borohydride for 1 hour. The complex is washed in 0.1 M acetate buffer, pH 4.5, made isotonic with 0.9% NaCl and lyophilized. A patient receives a Tc-99m or In-111 labelled murine antibody as appropriate to detect a tumor (e.g. 1 mg Tc-99m NR-Lu-10, NeoRx Corp, small cell or non-small cell carcinoma) and 14-17 hours thereafter, 5 mg of anti-murine antibody coupled complex is reconstituted in isotonic saline and administered intravenously to combine with any remaining intravascular Tc-99m murine antibody. The non-specific blood pool activity remaining on freely circulating murine antibody is thereby cleared with the complex by the patient's reticulo-endothelial system allowing greater detection efficiency of the specifically localized (tumor associated) extravascular antibody. Through the clearance of non-specific

antibody, the background activity is reduced and the target signal to noise ratio is enhanced.

EXAMPLE 3

Clearance of Target Drug, Virus or Cell: As in examples 1 or 2, complex is prepared in lyophilized form containing a non-radiolabelled antibody specific for a toxin, drug (overdose), virus or undesired cellular element. This is administered to a patient to clear the target from the circulation to the reticuloendothelial system (primarily the liver) where it is inactivated or phagocytized and destroyed.

Although the specific product, methods of production and utilization of the invention have been described as preferred embodiments herein, it is apparent that the invention may be constructed and utilized in a variety of manners and means not specifically mentioned herein. Any modifications, additions, substitutions and the like which can be made without departing from the spirit of the invention are therefore considered to be within the scope of the invention as defined by the following claims.

What is Claimed is:

1. A macro-molecular complex for target localization comprising:

a bio-compatible, in-vivo circulatable central core; and

a bio-compatible, hydrophilic spacer arm greater than 20 Angstroms in length coupled to the central core.

2. The macro-molecular complex of claim 1, wherein the central core comprises albumin.

3. The macro-molecular complex of claim 1, wherein the spacer arm comprises a polysaccharide.

4. The macro-molecular complex of claim 3, wherein the polysaccharide comprises a polysaccharide selected from the group consisting of dextran and diamino dextran.

5. The macro-molecular complex of claim 1, wherein the spacer arm comprises a polymer.

6. The macro-molecular complex of claim 5, wherein the polymer comprises a polymer selected from the group consisting of polyethylene glycol and diamino polyethylene glycol.

7. The macro-molecular complex of claim 1 further comprising a specific targeting molecule coupled to the spacer arm.

8. The macro-molecular complex of claim 7, wherein the specific targeting molecule comprises an antibody.

9. The macro-molecular complex of claim 7, wherein the specific targeting molecule comprises a fragment of an antibody.

10. The macro-molecular complex of claim 8, wherein the antibody is specific to an antigenic

substance selected from the group consisting of a toxin, a drug, a virus, or a bacteria.

11. The macro-molecular complex of claim 9, wherein the fragment comprises the Fab' portion of the antibody.

12. The macro-molecular complex of claim 1, wherein the complex is labeled with a detectable marker.

13. The macro-molecular complex of claim 12, wherein the detectable marker comprises a radioisotope.

14. The macro-molecular complex of claim 13, wherein the radioisotope comprises Tc-99m.

15. The macro-molecular complex of claim 7 further comprising an effector molecule bound to the complex.

16. The macro-molecular complex of claim 15, wherein the effector molecule comprises a drug.

17. The macro-molecular complex of claim 16, wherein the drug comprises a thrombolytic agent.

18. A spacer arm for use in macro-molecular complexes, the spacer arm comprising a hydrophilic molecule greater than 20 Angstroms in length, the spacer arm having two ends, each end being capable of attachment so as to maintain the structural integrity of the spacer arm molecule.

19. The spacer arm of claim 18, wherein the molecule comprises a polysaccharide.

20. The spacer arm of claim 19, wherein the polysaccharide comprises a polysaccharide selected from the group consisting of dextran and diamino dextran.

21. The spacer arm of claim 18, wherein the molecule comprises a polymer.

22. The spacer arm of claim 21, wherein the polymer comprises a polymer selected from the group

consisting of polyethylene glycol and diamino polyethylene glycol.

23. A composition comprising the macro-molecular complex of 1 and a physiologically acceptable carrier.

24. The composition of claim 23, wherein the composition is in lyophilized form.

25. A composition comprising the spacer arm of claim 18 and a suitable carrier.

26. The composition of claim 25, wherein the composition is in lyophilized form.

27. A method of producing a stabilized albumin, bio-compatible, in-vivo circulatable central core for use in a macro-molecular complex, which method comprises:

a) dissolving albumin in an aqueous solution;

b) raising the pH of the resulting solution to about pH 9-11;

c) heating the then-resulting solution having a pH of about 9-11 at about 70-90°C for about 15-25 minutes;

d) cooling the heated solution to about 20-30°C;

e) lowering the pH of the cooled solution to about 6-8;

f) adjusting the pH of the resulting solution having a pH of about 6-8 to about 5.8-6.6 so as to produce albumin microspheres;

g) heating the then-resulting solution having a pH of about 5.8-6.6 to 70-120°C until the albumin microspheres are stabilized; and

h) recovering the resulting stabilized albumin microspheres.

28. A method of stabilizing a microsphere in an aqueous solution, which method comprises adjusting the pH of the solution to about pH 5.8-6.6 and heating the resulting solution to about 70-120°C in less than 5 minutes.

29. A method of claim 28, wherein the pH comprises about 6.2 and the resulting solution is heated to about 85°C.

30. A method of labeling the macro-molecular complex of claim 1 with Tc-99m which comprises:

a) dissolving $SnCl_2$ in concentrated hydrochloric acid so as to form a first solution;

b) adjusting the pH of the first solution to 1 so as to form a second solution;

c) adding a suitable amount of sodium saccharic acid to the second solution so as to obtain a third solution having about a 2:1 molar excess of sodium saccharate to tin ion;

d) adjusting the pH of the third solution to about 7 so as to form a stannous saccharate solution having a pH of about 7;

e) exposing the macro-molecular complex to the stannous saccharate solution;

f) washing the resulting macro-molecular complex in an oxygen-free solution so as to remove the stannous saccharate; and

g) exposing the then-resulting macro-molecular complex to Tc-99m so as to label the macro-molecular complex.

31. In a method of tin-reduction labeling of microspheres with Tc-99m, the method comprising dissolving $SnCl_2$ at low pH to form a solution, exposing microspheres to the resulting $SnCl_2$ solution to form a microsphere/$SnCl_2$ solution, removing free tin from the

resulting microsphere/$SnCl_2$ solution, and exposing the then-resulting microsphere/$SnCl_2$ solution to Tc-99m so as to label the microspheres, the improvement comprising combining the $SnCl_2$ solution with a transfer ligand and neutralizing the pH of the resulting $SnCl_2$/transfer ligand solution prior to exposing the microspheres to the resulting $SnCl_2$/transfer ligand solution, so as to bind the $SnCl_2$ to the microspheres, and then removing the $SnCl_2$ and the transfer ligand that is not bound to the microspheres prior to exposing the then-resulting microsphere/$SnCl_2$ solution to Tc-99m.

32. The method of claim 31, wherein the transfer ligand comprises sodium saccharate.

33. The method of claim 31, wherein the labeling efficiency of the microsphere with Tc-99m is about ninety-nine percent.

34. A method of producing the macro-molecular complex of claim 1 which comprises:

a) forming the bio-compatible, in-vivo circulatable central core; and

b) coupling the spacer arm to the central core.

35. A method of producing the macro-molecular complex of claim 7 which comprises:

a) forming the bio-compatible, in-vivo circulatable central core;

b) coupling the spacer arm to the central core; and

c) coupling the specific targeting molecule to the coupled spacer arm.

36. A stabilized albumin, bio-compatible, in-vivo circulatable central core for use in a macro-molecular complex produced by the method of claim 27.

37. A stabilized microsphere produced by the method of claim 28.

38. A microsphere labeled by the method of claim 31.

39. A method of detecting a target which comprises:

labeling the macro-molecular complex of claim 7 with a detectable marker;

contacting the complex with the target under conditions such that the complex binds to the target; and

detecting bound complex, thereby detecting the target.

40. A method of removing a target which comprises:

contacting the macro-molecular complex of claim 7 with the target under conditions such that the complex binds to the target; and

removing the macro-molecular complex, thereby removing the target bound thereto.

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FIG 1

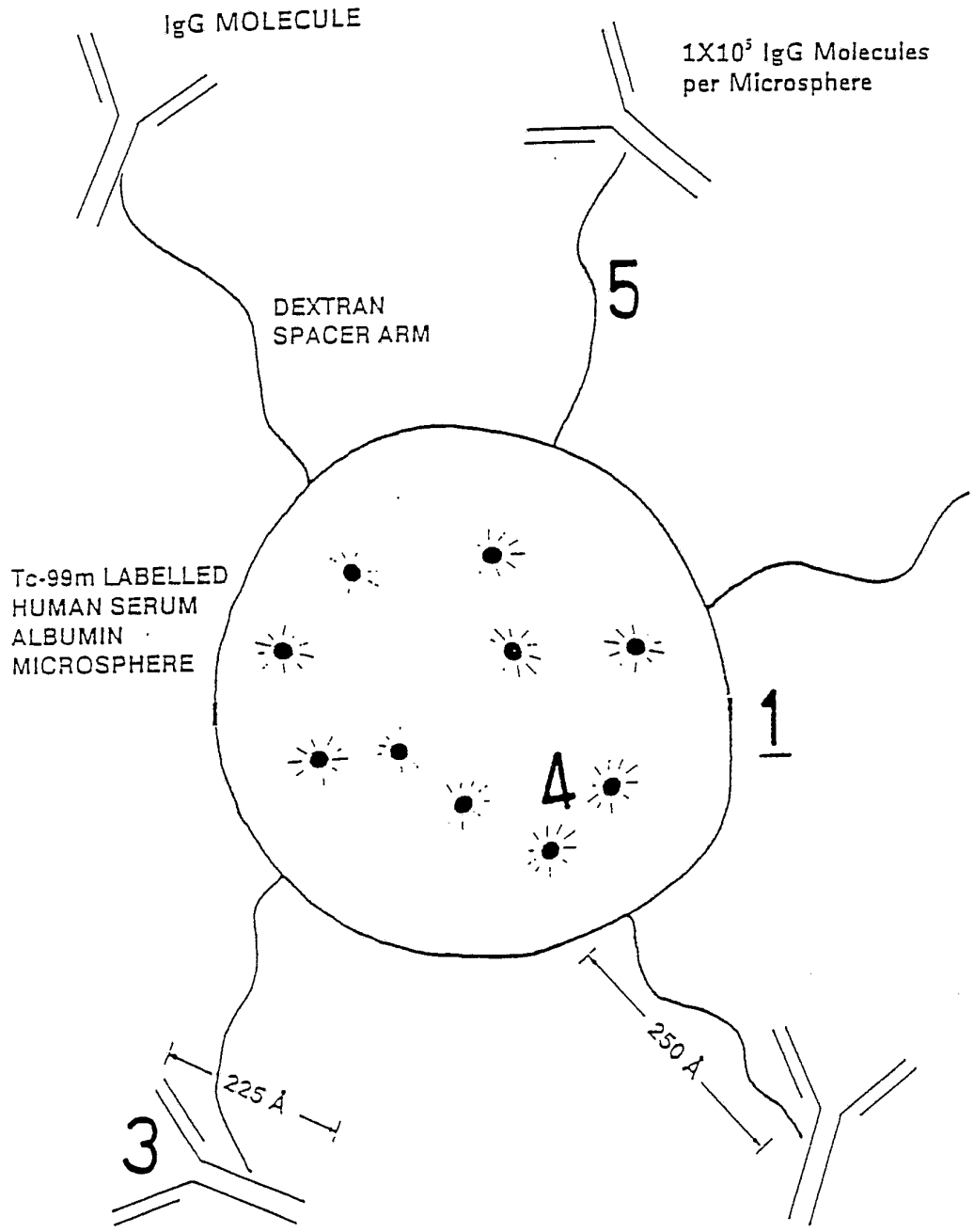
IgG MOLECULE
1X10⁵ IgG Molecules per Microsphere
DEXTRAN SPACER ARM
5
Tc-99m LABELLED HUMAN SERUM ALBUMIN MICROSPHERE
1
4
225 Å
250 Å
3

FIG 2A 2/5

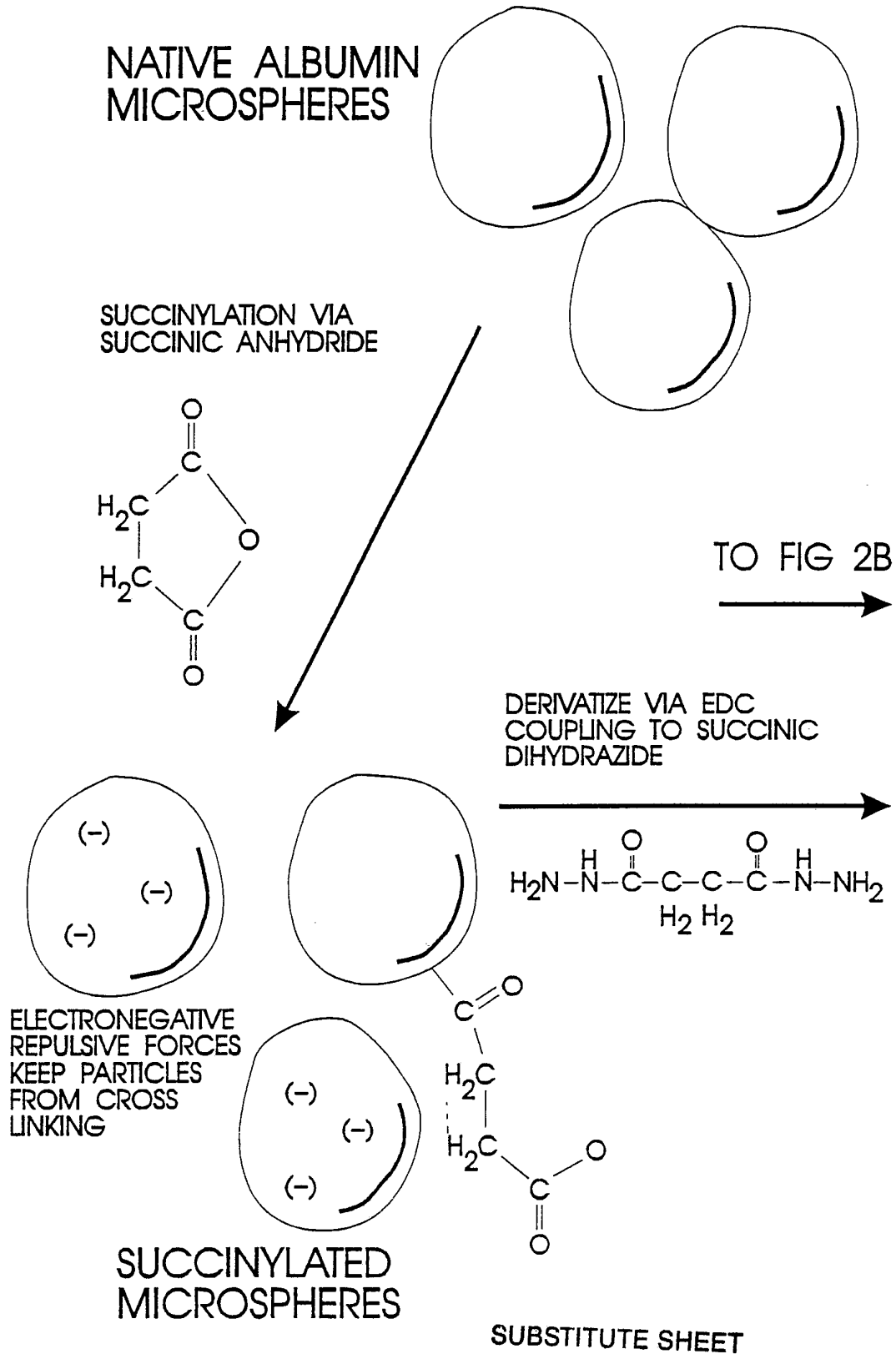
NATIVE ALBUMIN MICROSPHERES
SUCCINYLATION VIA SUCCINIC ANHYDRIDE
TO FIG 2B
DERIVATIZE VIA EDC COUPLING TO SUCCINIC DIHYDRAZIDE
ELECTRONEGATIVE REPULSIVE FORCES KEEP PARTICLES FROM CROSS LINKING
SUCCINYLATED MICROSPHERES

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FIG 2B

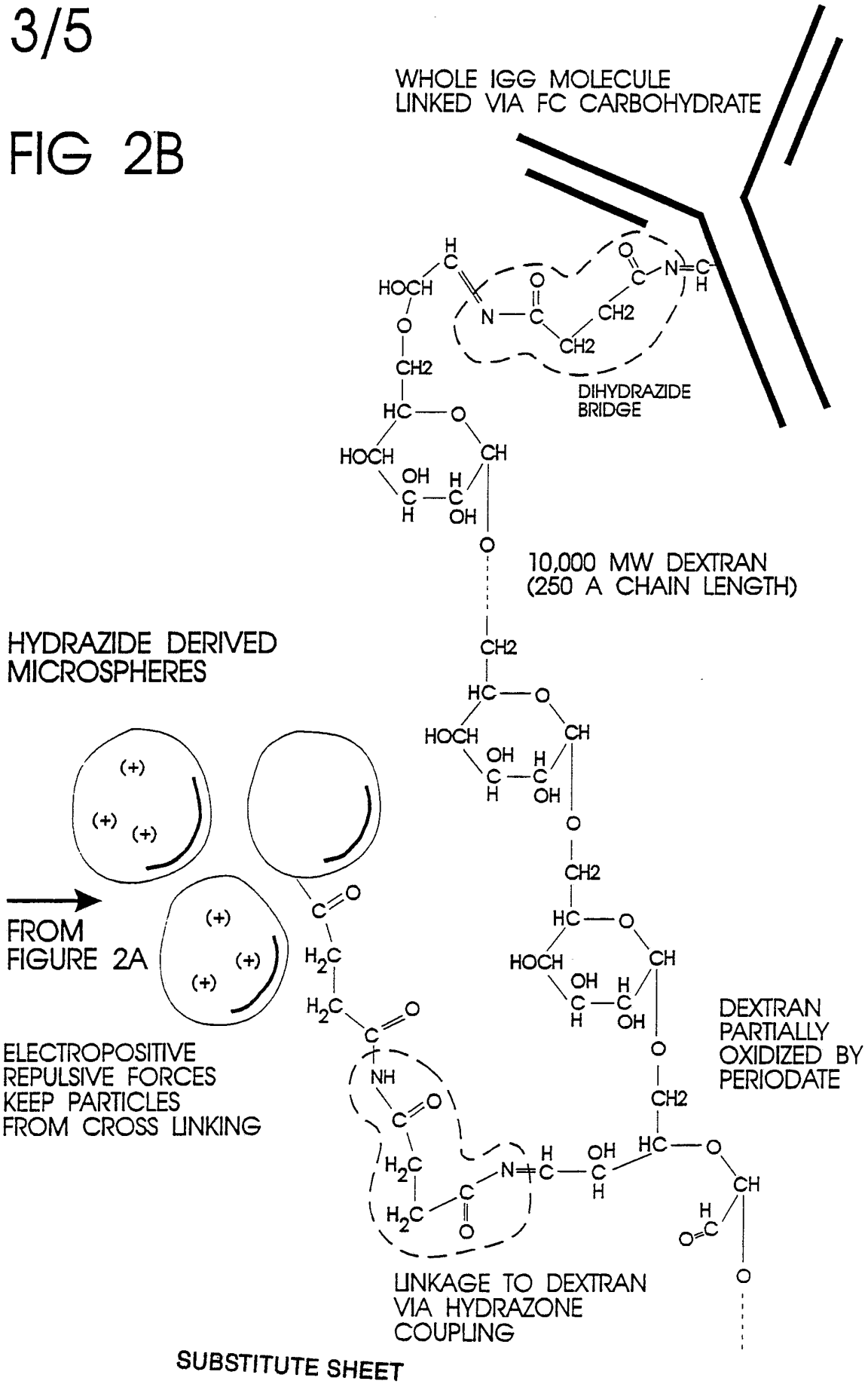
WHOLE IGG MOLECULE
LINKED VIA FC CARBOHYDRATE
DIHYDRAZIDE
BRIDGE
10,000 MW DEXTRAN
(250 A CHAIN LENGTH)
HYDRAZIDE DERIVED
MICROSPHERES
FROM
FIGURE 2A
ELECTROPOSITIVE
REPULSIVE FORCES
KEEP PARTICLES
FROM CROSS LINKING
DEXTRAN
PARTIALLY
OXIDIZED BY
PERIODATE
LINKAGE TO DEXTRAN
VIA HYDRAZONE
COUPLING

FIG 3A

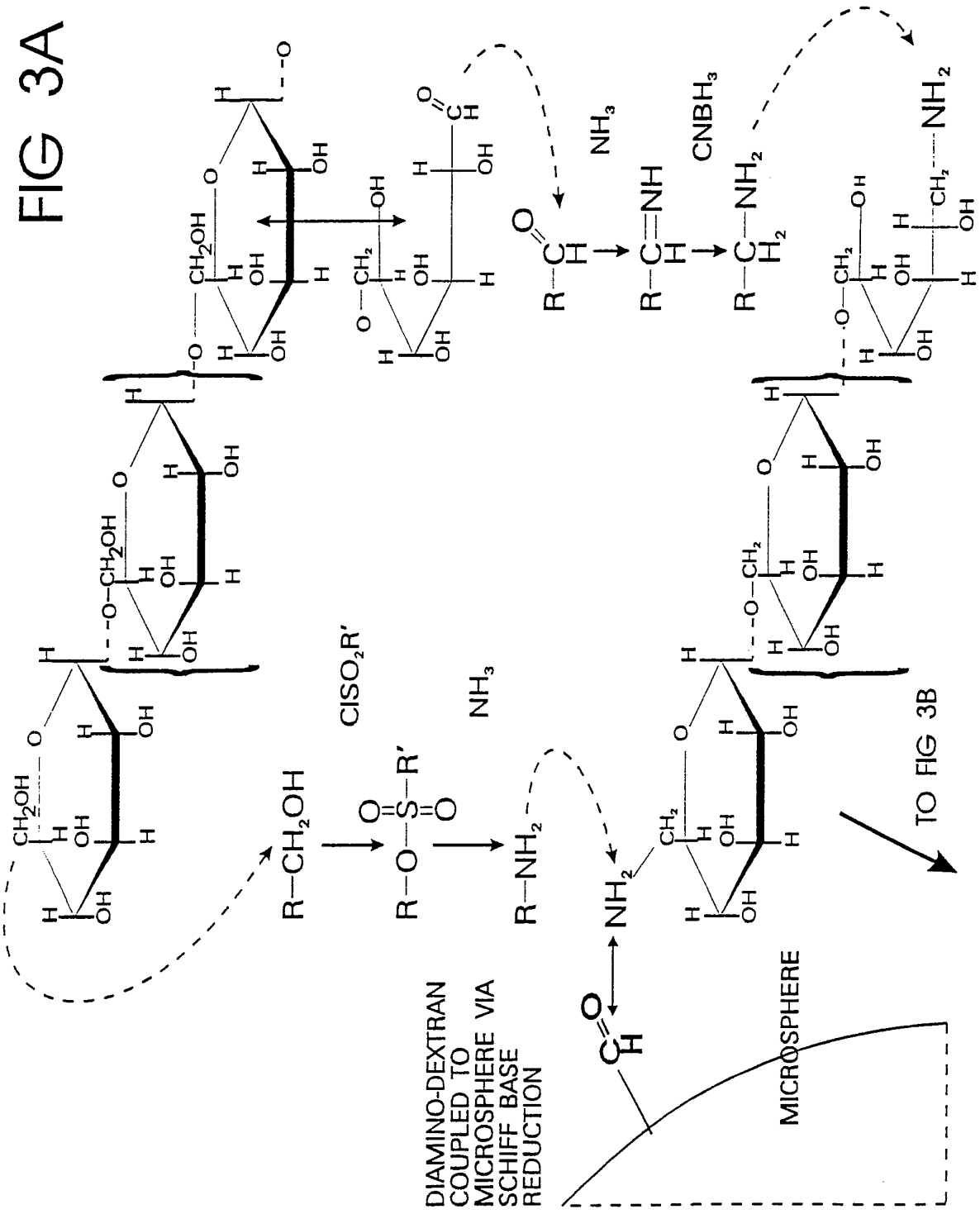
ClSO2R'
NH3
NH3
CNBH3
TO FIG 3B
DIAMINO-DEXTRAN COUPLED TO MICROSPHERE VIA SCHIFF BASE REDUCTION
MICROSPHERE

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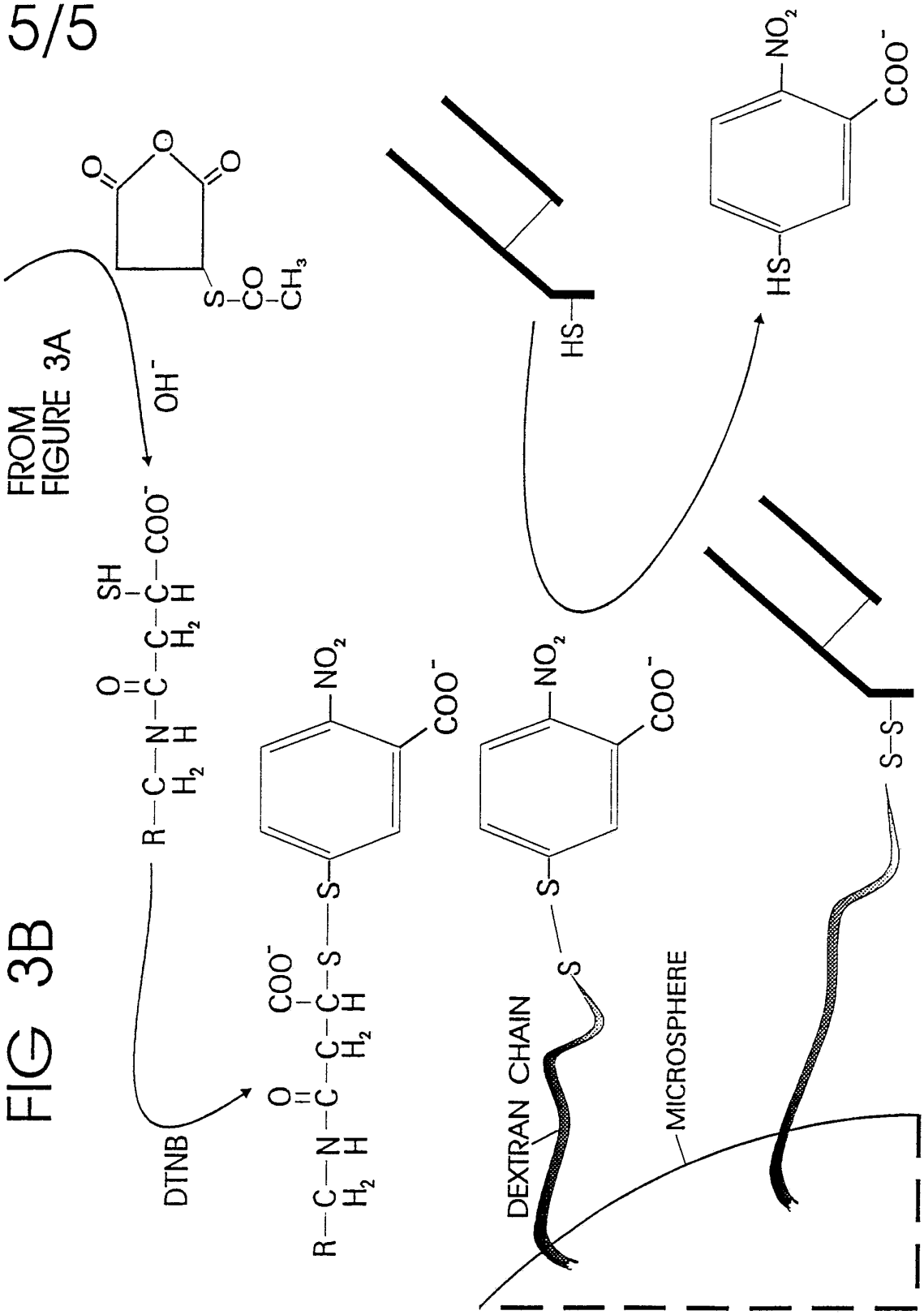
FIG 3B


FROM FIGURE 3A
OH⁻
DTNB
DEXTRAN CHAIN
MICROSPHERE
HS
S-S
NO2
COO⁻

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03512

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

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

See attachment.

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.:	530/362,363,388,391,409; 514/21,59,668,715,722; 424/1.1,9, 85.91,94.3; 536/51,112; 568/589,672; 435/261;534/14;564/505; 436/504,512,536.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, file CA, file biosis

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,452,773 (MOLDAY) 05 June 1984, see columns 3, 5, 13 and 14.	1,3,4,7-12, 18-20,23-26,40
Y	EP,A, 0,357,401 (Feijen) 07 March 1990, see columns 4, 6, 8, 10 and claim 13.	17,26-29, 36,37

See attachment.

* Special categories of cited documents: [10]

"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	Date of Mailing of this International Search Report
02 September 1991	30 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Kay Kim, Ph.D.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . ..., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to form PCT/ISA/210
Continuation in part I.
Classification of Subject Matter

IPC(5): C07K 15/14, 17/08, 17/10; A01N 31/14; A61K 31/075, 31/715, 37/04, 39/44, 43/00, 49/00; C03B 37/02; C07C 43/11, 217/42; C12N 1/02; C07F 13/00; G01N 33/534

U.S.Cl.: 530/363, 388, 391, 409; 514/21, 59, 668, 715, 722; 424/1.1, 9, 85.91; 536/51, 112; 568/589, 672; 435/261; 534/14.

Attachment to form PCT/ISA/210
Continuation in part III.
Documents Considered to be
Relevant

X Y	The Society of Nuclear Medicine, 36th Annual Meeting, St. Louis, MO, presented on 13 June 1989-16 June 1989, Line et al.; "Technecium-99m Albumin Immunospheres for intravascular Targeting," see abstract no. 16363.	1-4,7-9,12-14,8-20,23, 25,34,35 5,6,10,11, 15-17,21,22, 24,26-33, 36-40
X Y	Methods in Euzymology, Volume 112, issued 1985, L. Illum et al., "Attachment of Monoclonal Antibodies to Microspheres," pages 67-84, see particularly pages 68, 70, 73, 81, and 82.	1-4,7,8, 23,34,35 5,6,9-22,24-33,36-40
Y	Methods in Enzymology, Volume 112, issued 1985, J.J. Burger et al., "Technetium-99m Labeling of Albumin Microspheres Intended for Drug Targeting," pages 43-56, see particularly pages 43-45.	12-17,23,27-33,36-38
Y	US,A, 4,877,868 (RENO et al.) 31 October 1989, see columns 6 and 7.	30-34
X Y	M. Windholz et al. eds., "The Merck Index", Ninth ed., published 1976, by Merck & Co., Inc. (NJ), see pages 983-984, product no. 7349.	18,21,22 25,26
X Y	M. Windholz et al. eds., "The Merck Index", ninth edition, published 1976 by Merck & Co., Inc. (NJ), see pages 386-387, product no. 2904.	18-20 25,26
Y	Journal of Nuclear Medicine, Volume 13, No. 7, issued 1972, U. Scheffel et al., "Albumin microspheres for study of the Reticulo endothelial system", pages 498-503, particularly page 499.	27-33
X Y	CA,A, 1,079,268 (ROWLAND) 10 June 1980, see page 2, lines 18-21, page 4, line 19 through page 5, line 5 and page 16.	18-20 25,26

Attachment to form PCT/ISA/210
Continuation in part III.
Documents Considered to be
Relevant

Y	US,A, 4,101,380 (RUBINSTEIN et al.) 18 July 1978, see columns 8 and 9 and abstract.	18,21,22
Y	WO,A, 88/07,365 (RANNEY) 06 October 1988, see pages 16,21,26-29,46.	1-4,7-11, 27-29
Y	Journal of Biomedical Materials Research, Volume 18, issued 1984, L. Marcus et al., "Extracorporal removal of Specific antibodies by hemoperfusion through the immunosorbent agarose polyacrolein microsphere beads: Removal of anti-bovine serum albumin in animals", pages 1153-1167, see pages 1160 and 1161.	40