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(54) Title: IMMUNOREACTANT CARRIERS HAVING A NOVEL BIOCOMPATIBLE INTERMEDIATE COATING AND PROCESS OF MAKING SAME

(57) Abstract

An immunoreactant carrier having an antigen or antibody member of a single binding pair covalently bound to a biocompatible medium comprising a protein gel or polysaccharide coating the surface of a carrier body, and the method of producing the immunoreactant carrier, is provided. Where the biocompatible medium is a protein gel, it is coated on the surface of the carrier body by hydrophobic interaction or covalent binding. Where the biocompatible medium is a polysaccharide, it is coated on the surface of the carrier by covalent binding. The immunoreactant carrier can be used in an assay involving a member of a single binding pair such as an antigen or antibody member of a single binding pair in a biological test sample.

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Description

IMMUNOREACTANT CARRIERS HAVING A NOVEL BIOCOMPATIBLE INTERMEDIATE COATING AND PROCESS OF MAKING SAME

TECHNICAL FIELD

This invention relates generally to immunoreactant carriers, such as, microspheres or beads for use in immunoassays and more particularly, relates to improvements in immunoreactant carriers having a novel intermediate biocompatible protein or polysaccharide coating to which will be conjugated an antigen or antibody of a single binding pair for immunoassays and the process of making said carriers.

BACKGROUND ART

In the field of immunoassays, it is known to employ microspheres or beads as the carrier for one of the members of a single binding pair, such as an antibody or antigen of a single pair of binding members. A common immunoassay procedure is to coat the carrier with a specific labelled antibody selected to bind the antigen to be assayed and introduce the coated carrier to a sample of a biological fluid to be tested. The resultant complexing of the antibody coated carrier and bound antigen is detected and measured by known procedures to determine the assay resultant.

Another known procedure is to withdraw from a test sample of blood a selected class of blood cells by using magnetic microspheres coated with a monoclonal antibody which binds selectively to the selected class of cells, form the complex of the monoclonal antibody and bound cells and then remove the non-complexed cells from the test sample so as to isolate the bound cells. U.S. Patent No. 4,743,543 describes a procedure for removing red blood cells from a test sample which thereafter enables study of the remaining white blood cells.

These and other known procedures which use either magnetic or non-magnetic carriers coated with an immunoreactant member intended to bind a second member of a single binding pair require suitable and effective

coating of the carrier.

The adsorption of an inert protein such as serum albumin, egg albumin, and lactalbumin on solid carrier particles and the subsequent adsorption of an antigen or antibody on the coated carrier particles is described in U.S. Patent No. 3,551,555.

Cross-linking methods also have been employed to retain a protein on a carrier particle. For example, water-insoluble microcapsules whose circumferential wall contains a protein such as gelatin cross-linked to an antigen or antibody in an acidic solution is disclosed in U.S. Patent No. 4,590,170. This method requires the use of microcapsules negatively charged at an acidic pH.

U.S. Patent No. 4,123,396 describes a procedure for preparing metal containing polymeric microspheres which can be bonded covalently to proteins. Cross-linking of the protein and microspheres is preferred so that the stability and size of the microspheres both in aqueous solution and in organic solvents can be maintained.

U.S. Patent No. 4,478,946 teaches carriers such as roughened glass beads to which a film-forming "first layer" protein is cross-linked to the bead and to which a subsequent "second layer" antibody or antigen is covalently coupled. The first layer partially or totally may comprise a protein such as gelatin. Less antibody or antigen is required when the film-forming first layer is composed partially or totally of a protein such as gelatin. Crosslinking methods however are required to retain the first layer on the bead.

One desirable advantage in preparing such an immunoreactant microsphere is to achieve a desirable high ratio of bound antibody to bead surface.

Another desirable advantage is to be able to apply an intermediate biocompatible coating to the microspheres directly, which coating will enable direct conjugation thereto of the antibody or antigen.

Yet another desirable advantage is the ability to

use reduced quantities of the antibody or antigen to be coated to the microsphere or bead yet achieve the desired immunoassay determinations by means of the herein invention.

All of the desired advantages enumerated and others which will become apparent are derived from the methods embodying the invention as herein disclosed. An ancillary advantage is to produce the immunoreactant coated microspheres prepared by said methods of the invention for use in immunoassay kits and/or procedures.

DISCLOSURE OF THE INVENTION

The invention provides an immunoreactant carrier comprising a biocompatible coating medium retained directly on the surface of the carrier body without an intermediate binding agent and an immunoreactant covalently bound to said coating medium by means of a bifunctional reagent, and the method of producing the carrier.

One method of the invention comprises coating the peripheral surface of the carrier body with a biocompatible protein gel by hydrophobic interaction. After coating the carrier, an immunoreactant member of a single binding pair such as an antigen or antibody, is covalently bound to the coated carrier to form the immunoreactant carrier.

Another method of the invention provides coating the carriers with a biocompatible protein gel or a polysaccharide by covalent binding. The carrier coated with the biocompatible medium then is covalently bound to an immunoreactant member of a single binding pair to form the immunoreactant carrier.

BEST MODE FOR CARRYING OUT THE INVENTION

The term "biocompatible" as used herein to describe the intermediate coating medium is intended to mean a medium which does not interact nonspecifically and does not interfere with the immunological reaction or other components of the biological fluid test sample, and does not detrimentally influence the immunoreactants involved in the immunological reaction.

The term "immunoreactant" as used herein is intended to mean a substance capable of being one of the reaction members of a single binding pair, as in an antigen or antibody complex formation.

A glossary of reagents employed in practicing the invention is as follows:

Glossary of Reagents:

Gelatin Solution for Coating Microspheres by Hydrophobic Interaction:

Gelatin powder (available from Sigma Chemical Co.); or

Gelatin capsules (available from Eli Lilly & Co.): 10mg/ml dissolved in phosphate buffered saline (PBS), pH 7.3, containing 0.1% sodium azide

Gelatin Solution for Coating Microspheres by Covalent Binding:

Sodium Chloride 0.2M

EDAC (2 mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride in 0.2M NaCl)

Gelatin powder (available from Sigma Chemical Co.); or

Gelatin capsules (available from Eli Lilly & Co.): 20 mg/ml dissolved in PBS, pH 7.3, containing 0.1 sodium azide

Glycine (20 mg in 1 ml of water)

Water (containing 0.1% sodium azide)

Conjugation Procedure A:

Phosphate Buffered Saline (PBS) pH 7.3, containing 0.1% sodium azide

Bifunctional reagent, such as Sulfo-SMCC (2 mg/ml sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate in PBS), available from Pierce Chemical Co.

2-iminothiolane hydrochloride, (2 mg/ml in PBS), available from Pierce Chemical Co.

Immunoreactant >10 mg/ml

L-cysteine (5 mg/ml in PBS),

Iodoacetamide (30 mg/ml in PBS)

Bovine Serum Albumin (BSA) (in PBS containing 0.1% sodium azide) 1%

1M Borate pH 9.8

Conjugation Procedure B:

2-iminothiolane hydrochloride (2 mg/ml in PBS)

Bifunctional reagent, such as Sulfo-SMCC (1 mg/ml sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate in PBS), available from Pierce Chemical Co.

Immunoreactant >10 mg/ml

L-cysteine (5mg/ml in PBS),

Iodoacetamide (20 mg/ml in PBS)

Bovine Serum Albumin (BSA) (in PBS containing 0.1% sodium azide) 1%

1M Borate pH 9.8

Preparation of Polysaccharide Solution:

Dextran T-40 (Molecular weight 40,000 daltons), or,

Dextran T-110 (molecular weight 110,000 daltons), or,

Dextran T-500 (molecular weight 500,000 daltons), or,

Dextran T-2M (molecular weight 2,000,000 daltons), available from Fluka, Pharmacea; or

Ficoll (molecular weight 70,000 daltons), available from Sigma Chemical Co. 5g

Potassium Acetate pH 6.5 50 mM

Sodium Periodate (0.535 g in 6.25 ml of water) 85.6 mg/ml

1,3-diaminopropane (5 ml in 5 ml of water, adjust to pH 8.7 with glacial acetic acid) ~17ml

Sodium Borohydride (0.2 g in 2.5 ml of 0.1mM Sodium Hydroxide), available from Aldrich Chemical Co. 80 mg/ml

Covalent Binding of Polysaccharide to Microspheres:

Sodium Chloride	200mM
Aminoderivatized polysaccharide	3.75 mg/ml
EDAC (1-ethyl-3-[3-dimethylamino-propyl) carbodiimide hydrochloride, in 0.2M NaCl)	10 mg/ml
Water (containing 0.1% sodium azide)	

Conjugation of Immunoreactant to Polysaccharide Coated Microsphere:

The aminopolysaccharide coated beads may be conjugated to immunoreactants by using a bifunctional reagent. Some examples of bifunctional reagents are as follows:

Glutaraldehyde

Bis (succinimidyl) suberate

SPPD (succinimidyl 3-[2-pyridyldithio]propionate

SMCC (succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate)

SIAB (succinimidyl [4-iodoacetyl] aminobenzoate)

SMPB (succinimidyl 4-[1-maleimidophenyl]butyrate)

The biocompatible intermediate coating medium can be a protein or a polysaccharide. In one preferred embodiment, the intermediate coating medium is a protein, such as gelatin. Commercially available gelatins are graded according to their viscoscity and gel strength (or Bloom level) of their aqueous solutions. Various commercially available porcine and bovine gelatins with Bloom levels between 60 and 300 were tested during preliminary experiments. Gelatin capsules also were tested. It was found that increased Bloom levels of gelatin enabled higher quality coating of the microspheres. Also, bovine gelatin appeared to be preferable to porcine gelatin. Gelatin from capsules was the preferred gelatin of all gelatins tested.

The biocompatible intermediate coating medium can also be a polysaccharide. Polysaccharides tested included Dextran T-40 (molecular weight 40,000 daltons), Dextran T-110 (molecular weight 110,000 daltons), Dextran T-500 (molecular weight 500,000 daltons), Dextran

T-2M (molecular weight 2,000,000 daltons), Fluka, Pharmacea, and Ficoll (molecular weight 70,000 daltons), Sigma Chemical Co.

Commercially available microspheres tested included divinyl-benzene/polystyrene magnetic microspheres (0.7 micron, 20% magnetic; 0.7 micron, 42% magnetic; and 1.5 micron, 13% magnetic), Seragen Corporation; Non-magnetic microspheres also may be employed. Although the diameter size of the microspheres tested ranged from 0.7 to 1.5 microns, diameter sizes larger or smaller than those tested may be employed.

The immunoreactant may be labelled or unlabelled, depending upon the immunoassay system to be utilized. Suitable labels include enzymes, radioactive elements or dyes.

The preparation of the immunoreactant carriers involves four basic steps. The carriers are washed in Step I. The washing reagent and procedure will depend upon both the biocompatible medium and the coating method to be used to apply the biocompatible medium on the carrier.

The solution of biocompatible medium is prepared in Step II. Again, reagents and methods will vary depending upon which coating medium is used and which coating method is employed.

In Step III the solution of the biocompatible medium is coated on the carrier and excess coating medium is removed. The coating method may be by either hydrophobic interaction or covalent binding if gelatin is the biocompatible coating medium used. The coating method is by covalent binding if a polysaccharide is used.

The carrier coated with the biocompatible coating medium can be sterilized by known irradiation techniques prior to Step IV.

In Step IV the immunoreactant is covalently bound to the carrier which has been coated with the biocompatible medium by use of a bifunctional reagent.

PREPARATION OF IMMUNOREACTANT CARRIERS:

In Step I, 1 milliliter (ml) of a 10% suspension comprised of 0.7 micron, 42% magnetic microspheres is diluted to 4 ml with PBS and 0.1% azide and washed three (3) times using 4 ml of Phosphate Buffered Saline (PBS) containing a bacteriostatic agent such as 0.1% sodium azide and recovered in the conventional manner.

Then, Step II, the gelatin solution is prepared. Gelatin powder or capsules is weighed and then combined with PBS to obtain a concentration of 10 mg/ml. The solution is heated gently to approximately 50°C on a magnetic stir plate until the gelatin solution is clear. The gelatin solution is cooled to room temperature by stirring before further use.

Next, Step III, the gelatin solution is coated onto the microspheres by hydrophobic interaction. 4 ml of the gelatin solution is combined with the washed microspheres, sonicated in a water bath at room temperature for 1 minute and roller-mixed for 3 to 16 hours. Excess gelatin is removed by washing the suspension in the conventional manner four (4) times with 4 ml of PBS and 0.1% azide each wash. The microspheres are resuspended to a volume of 4 ml in PBS and 0.1% azide and stored at 4°C until used.

0.7 micron, 20% magnetic microspheres and 1.5 micron, 13% magnetic microspheres also were prepared in this manner. Microspheres thus prepared are ready for Step IV, covalent binding to an immunoreactant by Conjugation Procedure A or Procedure B.

Conjugation Procedure A:

2 ml of a 2.5% suspension of microspheres coated with gelatin by hydrophobic interaction are resuspended to 2 ml in PBS. 27 microliter (μl) of sulfosuccinimidyl-4-[N-maleimido-methyl] cyclohexane-1-carboxylate (sulfo-SMCC) is added to the microsphere suspension and this mixture is roller-mixed for 1 hour at room temperature. Then, the mixture is washed four (4) times with 2 ml of PBS each wash in the conventional manner, resuspended to a volume of 2 ml in PBS

and stored at 4°C until used.

Next, the immunoreactant is thiolated using 2-imino-thiolane hydrochloride according to the method of R. Jue et al., Biochemistry 17:5399 (1978). In this method, immunoreactant (2mg) at >10 mg/ml concentration is reacted with 13 µl of 2-iminothiolane hydrochloride for 1 hour at 22°C. The thiolated immunoreactant is separated by gel filtration and the protein concentration determined by absorbance at 280 nanometers (nm) in a spectrophotometer. Then, 1 mg of the thiolated immunoreactant is added to 2 ml of the sulfo-SMCC activated microspheres and reacted for 2 hours at room temperature. The reaction is quenched by adding 240 µl of cysteine to the suspension for 15 minutes followed by treatment with 240 µl of Iodoacetamide followed by 240 µl of 1M borate buffer. After 1 hour the microspheres then are blocked for at least 1 hour with 1% bovine serum albumin (BSA), washed 4 times in the conventional manner with 4 ml of 1% BSA each wash and resuspended to a volume of 2 ml in 1% BSA to obtain 2.5% suspension.

Conjugation Procedure B:

Alternatively, 4 ml of a 2.5% suspension of coated microspheres is treated with 40 µl of 2-iminothiolane hydrochloride at room temperature for 1 hour, washed 4 times in the conventional manner with 4 ml of PBS each wash, resuspended to 3.8 ml in PBS and stored at 4°C until used. Then, 1 mg of immunoreactant is treated with 40 µl sulfo- SMCC at 10 mg/ml protein concentration in PBS for 1 hour at room temperature. This treated immunoreactant then is passed through a Sephadex G-50 column in PBS, the protein peak collected and the protein concentration calculated from the A_{280} value. 0.4 mg of the modified immunoreactant is added to 4 ml of the thiolated microspheres. The suspension is roller-mixed at room temperature for 2 hours. The reaction is quenched by adding 120 µl of cysteine per ml of reaction volume to the suspension for 15 minutes. The free sulphydral groups are capped by adding 120 µl

each of Iodoacetamide and 1M Borate per ml of reaction volume to the suspension, and roller-mixing for 30 minutes at room temperature. The microspheres then are blocked with 1% BSA for 1 hour at room temperature, washed 4 times with 4 ml of 1% BSA each wash, resuspended to 4 ml in 1% BSA and stored at 4°C until used.

Alternatively, gelatin is covalently bound on the microspheres. In Step I of this procedure, 1 ml of a 10% suspension of 0.7 micron, 42% magnetic microspheres is diluted with 3 ml of 0.2M Sodium Chloride (NaCl), washed once in the conventional manner with 4 ml of 0.2M NaCl and suspended to a volume of 4 ml with 0.2M NaCl. The microspheres then are treated with 15 μl of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) (2 mg/ml) for 15 minutes.

During this treatment period, the gelatin solution is prepared (Step II). Gelatin powder or capsules is weighed and then combined with PBS to obtain a concentration of 20 mg/ml. The solution is heated gently to approximately 50°C on a magnetic stir plate until the gelatin solution is clear. The gelatin solution is cooled to room temperature by stirring before further use.

Then, Step III, the microspheres are bath-sonicated for 30 seconds, treated with 500 μl of the gelatin solution, bath-sonicated again for 30 seconds, and roller-mixed overnight at room temperature. The reaction is stopped by adding 100 μl of glycine to the treated microspheres for 30 minutes. The microspheres then are washed in the conventional manner four (4) times with 4 ml of water each wash, resuspended to a volume of 4 ml in water containing 0.1% sodium azide to achieve a concentration of 2.5% and stored at 4°C until used.

In Step IV, these coated microspheres are covalently bound with an immunoreactant by Conjugation Procedure A.

The carrier also may be coated with a polysac-

charide. In Step I of this coating procedure, 18 ml of a 10% suspension of 0.7 micron, 42% magnetic microspheres is diluted to 72 ml with 200 mM NaCl and washed once with 200 ml of 200 mM NaCl.

Then, Step II, the polysaccharide solution is prepared. The method of R. S. Molday and L. L. Molday (FEBS Letters 170, No.2:232 [1984]) for T-40 was adapted as herein described. 5 g of dextran is dissolved in 37.5 ml of Potassium Acetate, treated with prepared solution of Sodium Periodate, vigorously stirring during 10 minutes at 20-25°C, and allowed to react at 20-25°C for 1 hour and 30 minutes. This solution is dialized extensively 5 times against 1 liter of water each time for 1 to 2 hours at 4°C. This solution then is added dropwise, with vigorous stirring, to the prepared solution of 1, 3-diaminopropane. The mixture is stirred on a magnetic stir plate at room temperature for 2 hours and 15 minutes and then reduced with prepared solution of Sodium Borohydride for 15 minutes at room temperature. The mixture is extensively dialyzed against water at 4°C, filtered through a 0.22 micron filter and stored at 4°C.

Aminoderivatives of the following polysaccharides were prepared in this manner: Dextran T-40, Dextran T-110, Dextran T-500, Dextran-2M and Ficoll.

Next, Step III, the aminoderivatized polysaccharide is covalently bound to the microspheres by use of a carbodiimide reagent. The washed microspheres are resuspended to 72 ml in 200 mM NaCl containing the aminoderivatized polysaccharide at 3.75 mg/ml. 450 μl of EDAC then is added to the suspension and the suspension roller-mixed overnight at room temperature. The microspheres are washed 3 times with 72 ml of water and resuspended to 72 ml in water containing 0.1% sodium azide.

Other microspheres coated with a polysaccharide by this method included 1.5 micron, 13% magnetic microspheres and 0.7 micron, 20% magnetic microspheres.

In Step IV, the polysaccharide coated microspheres

are covalently bound to an immunoreactant by either Conjugation Procedure A or Conjugation Procedure B. A preferred method is by using thiolated immunoreactant and SMCC-treated microspheres as previously described in Conjugation Procedure A.

CLAIMS

1. An immunoreactant carrier comprising a biocompatible coating medium retained directly on the surface of a carrier body without an intermediate binding agent, and an immunoreactant covalently bound to said coating medium by means of a bifunctional reagent.

2. The carrier according to claim 1 in which said coating medium is selected from the group consisting of a protein gel or a polysaccharide

3. The carrier according to claim 1 wherein said coating medium consists essentially of a protein gel.

4. The carrier according to claim 1 wherein said coating medium consists essentially of a polysaccharide.

5. The carrier according to claim 4 wherein said polysaccharide is dextran or Ficoll.

6. The carrier according to claim 2 wherein said carrier body is solid.

7. The carrier according to claim 6 wherein said carrier body is a microsphere or a bead.

8. The carrier according to claim 7 wherein said carrier body is magnetic.

9. The carrier according to claim 7 wherein said carrier body is polystyrene.

10. The carrier according to claim 1 wherein said immunoreactant is an antigen or antibody of a single binding pair of members.

11. The carrier according to claim 9 wherein said imunoreactant is labelled with a detector member selected from the group consisting of an enzyme, a radioactive element or a dye.

12. An immunoreactant carrier for use in an immunoassay to bind thereto either the antigen or antibody of a single binding pair, said carrier comprising, a carrier body having either a protein gel or polysaccharide coated to its peripheral surface by hydrophobic interaction and one of the members of the binding pair is covalently bound to said coating.

13. The carrier according to claim 12 wherein

said carrier body is a solid microsphere.

14. The carrier according to claim 13 wherein said carrier body is magnetic in character.

15. The carrier according to claim 13 wherein said carrier body is polystyrene.

16. The immunoreactant carrier according to claim 12 wherein said immunoreactant is labelled with a member of a detector group consisting of an enzyme, a radioactive element or a dye.

17. The carrier according to claim 12 wherein said polysaccharide is dextran or Ficoll.

18. The carrier according to claim 17 wherein said polysaccharide is aminoderivatized.

19. A method for preparing an immunoreactant carrier for use in an immunoassay to bind thereto either the antigen or antibody of a single binding pair, said method comprising, coating a protein gel to the peripheral surface of a carrier body by means of hydrophobic interaction and covalently binding one member of a single binding pair to said protein gel coating said surface of said carrier body.

20. The method according to claim 19 wherein the protein gel is in solution for coating.

21. The method according to claim 19 wherein said antigen or antibody is covalently bound to the protein gel coating by means of a bifunctional reagent.

22. The method according to claim 19 wherein said protein gel is bound to the peripheral surface of the carrier body by mixing the protein gel and the carrier body for 3-16 hours at ambient temperature.

23. A method for preparing an immunoreactant carrier for use in an immunoassay to bind thereto either the antigen or antibody of a single binding pair, said method comprising, coating a protein gel or polysaccharide on the peripheral surface of a carrier body by means of covalent binding and covalently binding one member of a single binding pair to said protein gel or polysaccharide coating said carrier body.

24. The method according to claim 23 wherein said

polysaccharide is dextran or Ficoll.

25. The method according to claim 23 wherein said protein gel or polysaccharide is in solution.

26. The method according to claim 23 wherein said polysaccharide is aminoderivatized prior to covalent binding with said carrier body.

27. A method of preparing an immunoreactant carrier for use in an immunoassay to form a detectible complex with the antigen or antibody of a single binding pair, said method comprising:

A. coating the peripheral surface of the carrier directly with a biocompatible medium consisting of a protein gel or polysaccharide;

B. covalently binding to said medium the immunoreactant comprising either the antigen or antibody of the single binding pair.

28. The method of claim 27 in which the carrier is labelled with a member of a detector group consisting of an enzyme, a radioactive element or a dye to form the detectible complex.

29. The method of claim 27 in which the polysaccharide is dextran or Ficoll.

30. The method of claim 29 in which said polysaccharide is aminoderivatized.

31. The method of claim 27 in which said biocompatible medium comprising protein gel is coated by means of hydrophobic interaction.

32. The method claim 27 in which said biocompatible coating medium is coated by means of covalent binding.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04467

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

IPC (5) G01N 33/53,534,535,543-548,553

U.S. Cl. 435/7; 436/518,525,529,531,532,534,547; 422/57,58

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/7; 436/518,525,529,531,532,534,547; 422/57,58

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

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]
X Y	U.S., A, 4,452,773 (MOLDAY), 5 June 1984, see Abstract, columns 3-4, and column 5, lines 12-36.	1-2,4-8,10,12-14,16-18,27-31 23-26
X Y	U.S., A, 4,253,844 (LIMET), 3 March 1981, see column 1, line 55 to column 2, line 65.	1 and 10 2-3,6-9,12-16,19-23,25,27
X Y	U.S., A, 4,210,418 (BROWN), 1 July 1980, see column 1, line 63 to column 2 line 26, column 2, lines 40-55, column 3, lines 29-39, column 4, lines 50-56, column 5, lines 31-67, and column 6, lines 7-26.	1 and 10 2-3, 6-7, 9,11-13,15-16,19-23,25,27-28,31-32
Y	U.S., A, 3,639,558 (CSIZMAS), 1 February 1972, see Abstract, column 2, lines 40-48,column 3, lines 15-30, column 4, lines 31-41, column 7, lines 20-47 and lines 56-63.	2-3,6-16,19-23,25,27-28,31-32

* Special categories of cited documents: [10]

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"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.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 February 1990	07 MAR 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jack Spiegel Jack Spiegel

Form PCT/ISA/210 (second sheet) (Rev.11-87)