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(21) International Application Number: PCT/GB93/01972 (22) International Filing Date: 17 September 1993 (17.09.93) (30) Priority data: <table border="0"> <tr> <td>9219743.3</td> <td>18 September 1992 (18.09.92)</td> <td>GB</td> </tr> <tr> <td>9220722.4</td> <td>1 October 1992 (01.10.92)</td> <td>GB</td> </tr> <tr> <td>9221578.9</td> <td>14 October 1992 (14.10.92)</td> <td>GB</td> </tr> <tr> <td>9224897.0</td> <td>27 November 1992 (27.11.92)</td> <td>GB</td> </tr> <tr> <td>9315991.1</td> <td>2 August 1993 (02.08.93)</td> <td>GB</td> </tr> <tr> <td>9315995.2</td> <td>2 August 1993 (02.08.93)</td> <td>GB</td> </tr> </table> (71) Applicant (for all designated States except US): GEC-MARCONI LIMITED [GB/GB]; The Grove, Warren Lane, Stanmore, Middlesex HA7 4LY (GB). (72) Inventor; and (75) Inventor/Applicant (for US only) : ABUKNESHA, Ramadan, A. [LY/GB]; 16 Burgess Park Mansions, Fortune Green Road, London NW6 1DP (GB).		9219743.3	18 September 1992 (18.09.92)	GB	9220722.4	1 October 1992 (01.10.92)	GB	9221578.9	14 October 1992 (14.10.92)	GB	9224897.0	27 November 1992 (27.11.92)	GB	9315991.1	2 August 1993 (02.08.93)	GB	9315995.2	2 August 1993 (02.08.93)	GB	(74) Agent: COCKAYNE, Gillian; GEC Patent Department, Waterhouse Lane, Chelmsford, Essex CM1 2QX (GB). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
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(54) Title: SEPARATION METHOD (57) Abstract The present invention relates to a separation method which finds application in immunological detection (e.g. in immunoassay and immunosensors), to a method suitable for use in detection, to a sensor and to a test-kit. The invention provides, <i>inter alia</i>, a separation method, suitable for use in an immunological method for the detection of an entity, which separation method includes effecting attachment of an auxiliary species to a support material by use of an additional auxiliary species and a reaction partner for the additional auxiliary species. The additional auxiliary species may be, for example, a ligand or a binder.																				

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Separation Method

The present invention relates to a separation method which finds application in immunological detection (e.g. in immunoassay and immunosensors), to a method suitable for use in detection, to a sensor, and to a test-kit.

According to one aspect of the present invention there is provided a separation method, suitable for use in an immunological method for the detection of an entity, which separation method includes effecting attachment of an auxiliary species to a support material by use of an additional auxiliary species and a reaction partner for the additional auxiliary species.

According to another aspect of the present invention there is provided a method, suitable for use in immunological detection of an entity, which method includes effecting attachment of an auxiliary species to a support material by use of an additional auxiliary species and a reaction partner for the additional auxiliary species.

According to a further aspect of the present invention there is provided a sensor, suitable for use in the detection of an entity, which sensor includes an additional auxiliary species provided on a support material, said additional auxiliary species being capable of interacting with a reaction partner for the additional

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auxiliary species thereby to effect attachment of an auxiliary species to a support material.

According to yet a further aspect of the present invention there is provided a test-kit, which test-kit includes an additional auxiliary species provided on a support material, said additional auxiliary species being capable of interacting with a reaction partner for the additional auxiliary species thereby to effect attachment of an auxiliary species to a support material.

An auxiliary species is one which is not itself capable of taking part in a primary immune binding reaction with an entity to be detected or with an authentic entity to be detected.

Also an additional auxiliary species and a reaction partner for the additional auxiliary species may be species which are not themselves capable of taking part in a primary immune binding reaction with an entity to be detected or with an authentic entity to be detected.

The additional auxiliary species and the auxiliary species are different species.

As is further disclosed hereinafter an additional auxiliary species and a reaction partner for the additional auxiliary species may be used, together with an auxiliary species and a reaction partner for the auxiliary species (i.e. an auxiliary species reaction partner) to bring about indirect attachment of a primary species to a support material. The present invention thus for example may be used to effect a separation (e.g. in an immunoassay method).

By way of example, a separation in accordance with the present invention may be effected by arranging for a primary species to be associated (in any suitable manner and at any suitable time) with an auxiliary species, arranging for the auxiliary species to be associated with an additional auxiliary species (in any suitable manner and at any suitable time) and arranging for the additional auxiliary species to be associated with a

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support material (in any suitable manner and at any suitable time) such that the primary species may become indirectly associated with the support material. An additional auxiliary species may be provided on a support material at any suitable time and in any suitable manner as is further disclosed hereinafter.

A primary species in accordance with the present invention is a species capable of taking part in a primary immune binding reaction. By way of example, a primary immune binding reaction in accordance with the present invention is one in which an entity to be detected undergoes a specific binding reaction, or an authentic entity to be detected (as hereinafter defined) undergoes a specific binding reaction, or an entity to be detected and an authentic entity to be detected undergo specific binding reactions. (It will be appreciated that the entity to be detected and the authentic entity to be detected undergo specific binding reactions with other species and not with each other.)

It is to be understood that an "authentic entity" is an entity which is capable of reacting in a substantially similar manner as an entity to be detected under similar conditions.

Further, it is to be understood that an authentic entity may, for example, have a chemically defined structure and/or a biologically defined activity.

It will be appreciated that an authentic entity to be detected may be used, for example, as a "standard" or "calibrator". Where, for example, the entity to be detected is itself an analyte species (as hereinafter disclosed) the authentic entity may be an authentic analyte species (or a suitable derivative thereof).

An auxiliary species and an auxiliary species reaction partner may be chosen so as to be capable of interacting with each other by means of specific binding. Thus, for example, if the auxiliary species is a ligand the auxiliary species reaction partner may be a binder

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for the auxiliary species; conversely, if the auxiliary species is a binder the auxiliary species reaction partner may be a ligand.

By way of example, an auxiliary species reaction partner may be a species which is not itself capable of taking part in a primary immune binding reaction with an entity to be detected, or an authentic entity to be detected.

Alternatively, by way of example, an auxiliary species reaction partner may be a species which has a part which is capable of undergoing a specific binding reaction with an auxiliary species and a part which is capable of undergoing a specific binding reaction with a primary species; the part which is capable of undergoing a specific binding reaction with an auxiliary species may be considered to be an "auxiliary function". An example of an auxiliary species reaction partner which has an "auxiliary function" and a part which is capable of undergoing a specific binding reaction with a primary species is a bifunctional antibody.

Upon specific binding occurring between the auxiliary species and the auxiliary species reaction partner, any species associated with the auxiliary species reaction partner may become associated with the auxiliary species. Any suitable species may be associated with the auxiliary species reaction partner. Thus, for example, a primary species may be associated with an auxiliary species reaction partner.

A primary species may be associated with an auxiliary species reaction partner in any suitable manner (e.g. by a non-specific link (such as covalent linking or adsorption) or specific binding (e.g. a ligand-binder link)); a primary species may be linked with an auxiliary species reaction partner directly (e.g. by a non-specific link or by a specific binding link) or indirectly (e.g. via other species); where a primary species is indirectly associated with an auxiliary species reaction partner a

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link may be used which link may have one, or more, non-specific links or one, or more, specific binding links or any suitable combination of non-specific links and specific binding links.

It will be appreciated that where the auxiliary species reaction partner is a species which has a part capable of undergoing a specific binding reaction with a primary species, a primary species may be associated with the auxiliary species reaction partner by direct specific binding therewith.

Also, it will be appreciated that where the auxiliary species reaction partner is a species and is not capable of undergoing specific binding with a primary species, the auxiliary species reaction partner may be associated with the primary species by a suitable link or links as hereinbefore disclosed.

By way of example, a primary species may be a primary antibody or a ligand (e.g. an antigen). It is to be understood that, for example, a primary species may be an antibody to an entity to be detected or an antibody to an authentic entity to be detected; it will be appreciated that, for a given immunoassay, the antibody to the entity to be detected and the antibody to the authentic entity to be detected will be the same antibody. It is also to be understood that a primary species may be, for example, an entity to be detected or an authentic entity to be detected. A primary species may be associated in any suitable manner with a detectable species as is further disclosed hereinafter.

An additional auxiliary species and an auxiliary species may be associated in any suitable manner and at any suitable time.

By way of example, an auxiliary species may be linked directly or indirectly with a reaction partner for the additional auxiliary species.

By way of example, an auxiliary species may be linked with a reaction partner for an additional

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auxiliary species by means of a non-specific link (e.g. a covalent link or adsorption) or a specific binding link (e.g. ligand-binder link) or by any suitable combination or selection of such links.

An additional auxiliary species and a reaction partner for an additional auxiliary species may be chosen so as to be capable of interacting with each other by means of specific binding.

Thus, for example, if the additional auxiliary species is a ligand the reaction partner for the additional auxiliary species may be a binder for the additional auxiliary species; conversely, if the additional auxiliary species is a binder the reaction partner for the additional auxiliary species may be a ligand.

Accordingly, for example, a reaction partner for the additional auxiliary species may be a species which is a binder or a species which is a ligand, which binder and ligand may be associated with any other species as appropriate in any suitable manner.

Alternatively, by way of example, a reaction partner for an additional auxiliary species may be a species which has a part capable of undergoing a specific binding reaction with an additional auxiliary species and a part which is capable of undergoing a specific binding reaction with an auxiliary species; an example of such a reaction partner for an additional auxiliary species is a bifunctional antibody.

By way of further example, where the additional auxiliary species is a binder, it is possible to choose an auxiliary species which may also act as a reaction partner for an additional auxiliary species. Thus, for example, if an auxiliary species is an antibody (the auxiliary species reaction partner for which may be a corresponding ligand) then the additional auxiliary species may be an antibody to the auxiliary species and the auxiliary species therefor will also constitute a

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reaction partner for the additional auxiliary species. It will be appreciated that a binder which is an additional auxiliary species will have a different specificity from that of a binder which acts as an auxiliary species and as a reaction partner for the additional auxiliary species.

An additional auxiliary species may be, for example, a ligand, examples of which are antigenic ligands (such as haptens) and non-antigenic ligands.

Examples of antigenic ligands are 2,4 dinitrophenol, fluorescein, digitoxin, coumarin and cibacron blue.

Examples of non-antigenic ligands are ligands of specific ligand-binder pairs (e.g. the ligand biotin in the case of the ligand-binder pair biotin-avidin).

Further examples of ligands which may be attached to a support material (e.g. by covalent linkage or non-covalent attachment (adsorption)), which ligands may find use as additional auxiliary species, are soluble materials for example soluble molecules (e.g. polymers) such as polypeptides, proteins, polysaccharides and conducting polymers; haptenic functions may be covalently coupled to such soluble materials (e.g. polymers). By way of example, a ligand may be provided as a coating of a polymer on a support material.

Where an additional auxiliary species is a ligand, a reaction partner for the additional auxiliary species may be a binder, such binder being a species capable of binding with the ligand; by way of example, the binder may be a binding protein (e.g. an antibody or a binding partner for a ligand).

Thus, for example, where the additional auxiliary species is a ligand which is an antigenic ligand, the binder therefor may be a binder which is an antibody to the ligand.

Where, for example, the additional auxiliary species is a ligand and the ligand is 2,4 dinitrophenol,

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fluorescein, digitoxin, coumarin or cibacron blue, the reaction partner for the additional auxiliary species may be a binder which may be, respectively, anti-2,4 dinitrophenol antibody, anti-fluorescein antibody, anti-digitoxin antibody, anti-coumarin antibody or anti-cibacron blue antibody.

Where, for example, an additional auxiliary species is a ligand which ligand is a non-antigenic ligand, the ligand may be, for example, such that the binder is a binder which is a binding partner that is a non-immunoglobulin (e.g. a naturally-occurring protein); the binding partner may be considered to be the binder of the ligand and the binding partner may be considered to be a reaction partner for the additional auxiliary species. An example of such a binding partner is avidin in the specific ligand-binder pair comprising a biotin-avidin complex.

Alternatively, the additional auxiliary species may be a binder. Thus, for example, the additional auxiliary species may be a binder (e.g. an antibody) for an antigenic ligand or a binder for a non-antigenic ligand such as a binder of a specific ligand-binder pair; an example of a binder for a non-antigenic ligand is avidin. By way of further example, the additional auxiliary species may be, for example, a binder (e.g. an antibody) for another binder (e.g. antibody).

Examples of binders for antigenic ligands which binders may be used as additional auxiliary species are anti-2,4 dinitrophenol antibody, anti-fluorescein antibody, anti-digitoxin antibody, anti-coumarin antibody and anti-cibacron blue antibody.

Where, for example, the additional auxiliary species is a binder and the binder is anti-2,4 dinitrophenol antibody, anti-fluorescein antibody, anti-digitoxin antibody, anti-coumarin antibody or anti-cibacron blue antibody, the reaction partner for the additional auxiliary species may be a ligand which may be,

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respectively, 2, 4 dinitrophenol, fluorescein, digitoxin, coumarin or cibacron blue.

Where, for example, an additional auxiliary species is a binder which is a binder for a non-antigenic ligand, the binder may be a binding partner which is a non-immunoglobulin (e.g. a naturally occurring protein); the binding partner may be considered to be the binder of the ligand and the ligand may be considered to be a reaction partner for the additional auxiliary ligand. An example of such a binding partner is avidin in the specific ligand-binder pair comprising a biotin-avidin complex.

An additional auxiliary species, in accordance with the present invention, may be provided on a support material in any suitable manner and at any suitable time, and it is to be understood that in this Specification "providing on a support material" and "provided on a support material" embrace, for example, situations where a support carries an additional auxiliary species and situations where the support material itself, or a part of the support material itself, provides an auxiliary species.

By way of example, an additional auxiliary species (e.g. a ligand or a binder) may be, for example, attached to the support material. Alternatively, by way of example, an additional auxiliary species (in the form of a ligand) may be provided by chemical groups or units of the support material.

Where an additional auxiliary species (e.g. a ligand or a binder) is attached to the support material the additional auxiliary species may be attached either directly or indirectly in any suitable manner. For example, a non-specific linking (covalent linkage or adsorption) or a specific binding linking may be utilised. Where attachment is indirect, an additional auxiliary species may be attached to a support material via another species (e.g. a carrier protein) or other species.

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By way of example, an auxiliary species may be attached to a support material via one additional auxiliary species (and a reaction partner therefor as appropriate); alternatively, by way of further example, if desired, a plurality of additional auxiliary species (and corresponding reaction partners), may be used to form a "bridge" between an auxiliary species and a support material. Thus, for example, a "chain" of additional auxiliary species (and corresponding reaction partners) may be formed via which an auxiliary species may be attached indirectly to a support material. Accordingly, by way of example, an auxiliary species may be associated with a first additional auxiliary species which may be associated with a second additional auxiliary species and so forth to form a "chain" containing as many additional auxiliary species as is appropriate.

It will be appreciated from the foregoing that any suitable link (e.g. non-specific link or specific binding link) or any suitable combination of links (e.g. a plurality of non-specific links, or a plurality of specific binding links, or any combination of non-specific and specific binding links) may be utilised as is appropriate.

Where, for example, a reaction partner for an additional auxiliary species has a part capable of specific binding with the additional auxiliary species and a part capable of specific binding with another species (e.g. a further additional auxiliary species) such a reaction partner may, optionally, be used in a "chain" in accordance with the present invention; thus, for example, a bifunctional species such as a bifunctional antibody may be used in a "chain".

Where the support material is, for example, a polymer, units of the polymer may act as additional auxiliary species in the form of ligands. Also, by way of example, surface groups present on a support material,

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such as polystyrene or modified silica, may act as additional auxiliary species in the form of a ligand.

By way of example, the support material may, if desired, provide oligomers or polymers of an additional auxiliary species in the form of ligands.

By way of further example, the surface of a support material may be activated thereby to permit attachment of an additional auxiliary species (e.g. a ligand or a binder); for example, the surface of a suitable support material may be activated by chemical treatment to provide free amino groups to which an additional auxiliary species (e.g. a ligand or a binder) may be linked.

Further, by way of example, an oligomer or oligomers of an additional auxiliary species, or a polymer or polymers of an additional auxiliary species may be attached directly or indirectly to a support material. Thus, for example, an oligomer or oligomers of a ligand, or an oligomer or oligomers of a binder, or a polymer or polymers of a ligand, or a polymer or polymers of a binder may be attached, directly or indirectly, to a support material.

Thus, for example, oligomers of an additional auxiliary species may be attached to free amino groups on a support material.

Also, by way of example, an additional auxiliary species may be linked (e.g. covalently or otherwise) to a further species (e.g. a carrier protein or a polymer) and the further species may be associated with the support material such that the additional auxiliary species may become indirectly provided on the support material.

By way of further example, it is possible to link (e.g. covalently or otherwise) oligomers or polymers of an additional auxiliary species to a further species (e.g. a carrier protein or a polymer) and the further species may be associated with the support material such that oligomers of the additional auxiliary species or

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polymers of the additional auxiliary species may become indirectly provided on the support material.

Where an additional auxiliary species is attached to a support material, attachment of the additional auxiliary species may be effected at any desired time. Thus, for example, an additional auxiliary species may be allowed to bind with an appropriate reaction partner therefor, before, or after, the additional auxiliary species is attached to the support material; however, in general, it may be more convenient to attach an additional auxiliary species to a support material before bringing together the additional auxiliary species and a reaction partner therefor.

It is to be understood that the use of oligomers or polymers of additional auxiliary species may be advantageous in certain circumstances. Thus, for example, by use of an oligomer or a polymer of an additional auxiliary species more additional auxiliary species may be provided than it is possible to provide when using only single "units" of an additional auxiliary species. Accordingly, for example, the provision of more additional auxiliary species offers the possibility of faster reactions since more additional auxiliary species is available to undergo reaction, with available reaction partner therefor.

In one embodiment the present invention provides a method which also includes the step of attaching, either directly, or indirectly, an additional auxiliary species (e.g. a ligand or a binder) to a support material.

It is to be understood that the examples of ligands and binders hereinbefore disclosed, with respect to ligands and binders which may be used as additional auxiliary species and reaction partners for additional auxiliary species, may also find application as auxiliary species and auxiliary species reaction partners.

Thus, for example, an auxiliary species may be a ligand or a binder selected from those hereinbefore

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exemplified and an auxiliary species reaction partner may be a binder or a ligand selected from those hereinbefore exemplified.

By way of example, if desired, auxiliary species and auxiliary species reaction partners may be provided by oligomers or polymers of ligands and/or binders; oligomers and polymers of ligands and binders are hereinbefore disclosed.

It is to be understood that ligands and binders as hereinbefore exemplified may be used as is appropriate in any "chain" which may be formed, as hereinbefore disclosed, to attach an auxiliary species to a support material.

It will be appreciated that ligands and binders for use respectively as additional auxiliary species and reaction partners for additional auxiliary species and for auxiliary species and auxiliary species reaction partners, may be chosen such that no unacceptable interference occurs between different ligands and different binders.

By way of further example, an auxiliary species in accordance with the present invention may be carried by a suitable carrier material, which carrier material may be attached to a support material.

Some carrier materials, such as dextran, may be suitable hydrophillic materials (e.g. polymers) to which an auxiliary species (e.g. a ligand or a binder) may be linked (either directly or indirectly).

However, some carrier materials (such as dextran) do not adsorb satisfactorily to some surfaces, such as polystyrene surfaces (e.g. those found on polystyrene microtitre plates); such carrier materials (e.g. dextran) may be, for example, attached to a surface (e.g. a polystyrene surface) by means of an additional auxiliary species and a reaction partner for the additional auxiliary species. Thus, for example, the reaction partner for the additional auxiliary species may be

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associated (either directly or indirectly) with a carrier material and the additional auxiliary species may be associated with a support material such that upon binding between the additional auxiliary species and the reaction partner for the additional auxiliary species, carrier material (and any auxiliary ligand linked thereto) may be attached to a support material.

By way of further example where, in accordance with the present invention, adsorption is used as a non-specific link, materials to be so linked may be each adsorbed onto a carrier material (such as latex particles).

Examples of support materials which may find application in accordance with the present invention are solid phase materials such as a reaction vessel wall, insoluble polysaccharides, microparticles (e.g. particulate microcellulose), polystyrene (e.g. in the form of wells, beads, microtitre plates, discs, sticks or tubes), cross-linked dextran (e.g. Sephadex), insoluble polymer structures, glass surfaces, derivatised silica surfaces (e.g. having silyl groups with chemical functions attached), soluble polymers attached to a suitable surface (e.g. a glass surface), microparticulate materials with entrapped ferrous oxide (magnetisable particles), nylon and polyamides.

It will be appreciated that some types of support materials may be inappropriate for use with some entities to be detected. Thus, for example, where the entity to be detected contains a metal, the use of some types of support materials may be inappropriate (e.g. support materials containing entrapped iron oxide may give rise to unacceptable interference).

Entities to be detected may be, for example, any suitable entities capable of undergoing a specific binding reaction.

Thus, for example, an entity to be detected may itself be an analyte species. For example, an entity to

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be detected may be an analyte species comprising a ligand (e.g. an antigenic ligand such as a hapten). By way of further example, entities to be detected may be analyte species comprising antibodies.

Examples of entities which are analyte species which may be detected in accordance with the present invention are:

- (a) steroid hormones such as progesterone, 17 α -hydroxy progesterone or estradiol (e.g. in a sample of blood, serum, saliva, urine or milk),
- (b) hormones such as thyroid hormone (e.g. thyroxine or triiodothyronine),
- (c) steroids in extracts (e.g. extracts of solids or liquids),
- (d) drugs such as drugs of abuse (e.g. phenobarbital) and therapeutic drugs (e.g. digoxin) (in for example, a sample of blood, serum, saliva or urine),
- (e) polypeptide hormones (e.g. hCG) in, for example, a sample of blood or urine,
- (f) tumour markers such as marker proteins (e.g. in a sample of blood or serum),
- (g) protein antigens,
- (h) blood proteins (e.g. human serum albumin, immunoglobulins (e.g. IgG), enzyme markers or receptors),
- (i) marker proteins in urine resulting from kidney diseases,
- (j) pesticides such as insecticides, or herbicides (e.g. in water or soil),
- (k) toxins (such as those extracted from feeds and food stuffs),
- (l) micro-organisms (e.g. viruses and bacteria), and
- (m) antibodies to micro-organisms.

Further examples of entities which are analyte species which may be detected in accordance with the present invention are complexes of metals.

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Thus, the present invention may find application in, for example, the detection of complexes of metals such as strong metal complexes which may be regarded as toxic (e.g. in biological terms when present in the environment).

An example of a metal complex which may be detected in accordance with the present invention is methyl mercury.

By way of further example, an entity to be detected may be an entity which carries or includes analyte species. Thus, for example, an entity to be detected may be a species formed by interaction of an analyte species with a suitable agent.

For example, where analyte species is a metal ion the agent may be a complexing agent capable of interacting with the metal ion to form an entity (e.g. a metal ion complex) for detection. Thus, for example, metal ions may be formed into a complex of a metal by use of a complexing agent and the complex thus formed may act as an entity to be detected in accordance with the present invention.

Thus, for example, metals may be detected in accordance with the present invention.

Examples of complexing agents are chelating agents.

The present invention may find application, for example, in the detection of an entity to be detected, in any suitable sample. Thus, for example, samples of water, soil, living species (such as plants (e.g. vegetables) or animals) or air may provide an entity to be detected, for detection in accordance with the present invention. Examples of biological samples in which an entity to be detected may be detected in accordance with the present invention are blood, plasma, serum, urine, saliva and milk. An entity to be detected may be, for example, present in water, an aqueous preparation or a fluid extract (e.g. one prepared by solvent extraction). An entity to be detected may be, for example, a hapten.

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The present invention may be utilised, for example, in any suitable configuration, examples of which are given immediately hereinafter:

(i) In one configuration an additional auxiliary species is provided on a support material, said additional auxiliary species being a ligand.

A reaction partner for the additional auxiliary species, said reaction partner being a binder for the additional auxiliary species, is linked by covalent linking to an auxiliary species (e.g. a ligand or a binder). If desired, the reaction partner for the additional auxiliary species may be linked to a plurality of auxiliary species (of the same type).

An auxiliary species reaction partner (e.g. a binder or a ligand depending upon whether the auxiliary species is a ligand or a binder) may be arranged to be associated with a primary species (e.g. a primary antibody or an authentic entity to be detected) in any suitable manner (as hereinbefore disclosed).

Indirect attachment of primary species to the support material may be effected by binding between the auxiliary species and the auxiliary species reaction partner and by binding between the reaction partner for the additional auxiliary species and the additional auxiliary species.

Any detectable species associated with the primary species which primary species is indirectly associated with the support material may be detected or determined in any manner suitable to the detectable species used;

(ii) In another configuration, a configuration similar to that hereinbefore disclosed in (i) may be used, with the exception that the additional auxiliary species provided on the support material is a binder and the reaction partner for the

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additional auxiliary species is a ligand; the reaction partner for the additional auxiliary species may be linked to an auxiliary species (e.g. a ligand or a binder) or a plurality of auxiliary species (for the same type).

Indirect attachment of a primary species to the support material and detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(iii) In a further configuration, an additional auxiliary species is provided on a support material, said additional auxiliary species being a ligand.

A reaction partner for the additional auxiliary species is a binder for the ligand.

An auxiliary species (e.g. a ligand or a binder) is, or a plurality of auxiliary species (of the same type) are, attached (e.g. covalently) to an antibody species which antibody species is capable of undergoing specific binding with the reaction partner for the additional auxiliary species; it will be appreciated that where the reaction partner for the additional auxiliary species is an antibody, the antibody species capable of binding with it may be regarded as an anti-antibody antibody species. More than one of the antibody species (each having one or a plurality of auxiliary species attached) may become attached to the reaction partner for the additional auxiliary species.

Indirect attachment of primary species to the support material may be effected in a manner similar to that hereinbefore disclosed in (i) with the exception that the auxiliary species is indirectly attached to the reaction partner for the additional auxiliary species (by means of binding between the antibody species and the reaction partner for the auxiliary species).

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Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(iv) In a further configuration an additional auxiliary species is provided on a support material, said additional species being a binder.

A reaction partner for the additional auxiliary species is provided in the form of a plurality of substantially similar ligands linked together.

An auxiliary species is (or a plurality of auxiliary species, of the same type, are) linked (e.g. covalently) to a binder for the reaction partner for the additional auxiliary species, which binder may be substantially the same type of species as the additional auxiliary species provided on the support material.

Indirect attachment of primary species to the support material may be effected in a manner substantially similar to that hereinbefore disclosed in (i) with the exception that the auxiliary species may be associated with the additional auxiliary species via reaction partner for the additional auxiliary species and the binder therefor. Where the reaction partner for the additional auxiliary species provides more than two ligands, more than one binder therefor (associated with one or more auxiliary species) may become attached to the reaction partner for the additional auxiliary species.

Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(v) In a further configuration, a configuration similar to that hereinbefore disclosed in (iv) may be used with the exceptions that (a) the reaction partner for the additional auxiliary species is provided in the form of a ligand (of a first type),

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capable of binding with the additional auxiliary species, linked to one ligand of a second type, or a plurality of ligands of a second type, (said second type being different from said first type) and (b) an auxiliary species is, or a plurality of auxiliary species (of the same type) are, linked to a binder for the ligand or ligands of the second type.

Indirect attachment of primary species to the support material may be effected in a manner similar to that hereinbefore disclosed in (iv) with the exception that the auxiliary species may be associated with the additional auxiliary species (via the reaction partner for the additional auxiliary species) by means of ligand of the second type and binder for ligand of the second type.

Where the reaction partner for the additional auxiliary species provides more than one ligand of the second type, more than one binder therefor (associated with one or more auxiliary species) may become attached to the reaction partner for the additional auxiliary species.

Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(vi) In a further configuration an additional auxiliary species, said additional auxiliary species being a ligand, is provided on a support material.

A reaction partner for the additional auxiliary species is provided in the form of a bifunctional binding species having two different types of binding function (one capable of binding with the additional auxiliary species and one capable of binding with a ligand of a different type).

An auxiliary species is, or a plurality of auxiliary species (of the same type) are, attached (e.g. covalently) to said ligand of a different type.

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Indirect attachment of a primary species to the support material may take place in a manner similar to that hereinbefore disclosed in (i) with the exception that the auxiliary species is indirectly attached to the reaction partner for the additional auxiliary species by means of said ligand of a different type.

Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(vii) In a further configuration an additional auxiliary species is provided on a support material, such additional auxiliary species being a ligand.

A reaction partner for the additional auxiliary species, said reaction partner being a binder for the additional auxiliary species, is linked by covalent linking to a ligand of a different type from that provided on the support material; if desired the binder may be linked to a plurality of said ligands of a different type.

An auxiliary species is, or a plurality of auxiliary species (of the same type) are, linked to binder for the said ligand of a different type.

Indirect attachment of a primary species to the support material may take place in a manner similar to that hereinbefore disclosed in (i) with the exception that auxiliary species is indirectly attached to the reaction partner for the additional auxiliary species via said ligand of a different type and binder therefor.

Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(viii) In a further configuration an additional auxiliary species is provided in the form of a binder and a reaction partner for the additional

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auxiliary species is a ligand which may be bound by the binder.

The reaction partner for the additional auxiliary species is linked, by covalent linking, to an auxiliary species (which may be a binder or a ligand).

A support material is used upon which there is provided a binder for the additional auxiliary species. It will be appreciated that where the additional auxiliary species is a binder, the binder for the additional auxiliary species may be an antibody which may be considered to be an anti-antibody antibody.

An auxiliary species reaction partner may be arranged to be associated with primary species as hereinbefore disclosed in (i).

Indirect attachment of a primary species may be effected by binding between the auxiliary species reaction partner and the auxiliary species, binding between the reaction partner for the additional auxiliary and the additional auxiliary species and binding between the binder provided on the support material and the additional auxiliary species.

It is to be understood that any of the binding reactions involved in the indirect attachment of primary species to a support material may be arranged to take place in any appropriate or convenient manner or time and in any appropriate or convenient order.

Detection or determination of any detectable species may be effected substantially as hereinbefore disclosed in (i);

(ix) In a further configuration an additional auxiliary species is provided on a support material which additional auxiliary species is a binder for a reaction partner for the additional auxiliary species; in this configuration the reaction partner

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for the additional auxiliary binder is also a binder. It will be appreciated that in this configuration the additional auxiliary species may thus be, for example, an anti-binder antibody.

The reaction partner for the additional auxiliary species may, in this configuration, also constitute an auxiliary species in that it may be a binder capable of binding with a ligand, which ligand is an auxiliary species reaction partner.

The auxiliary species reaction partner may be arranged to be associated with a primary species in any suitable manner.

Indirect attachment of primary species to the support material may be effected by binding between the additional auxiliary species and the binder which constitutes both an auxiliary species and a reaction partner for the additional auxiliary species.

Determination of any detectable species may be effected substantially as hereinbefore disclosed in (i).

By way of example, in a competitive immunoassay method which includes a separation method in accordance with the present invention the following may take place:

- (a) an additional auxiliary species is introduced in high concentration to a support material directly (e.g. by covalent linkage), or indirectly, or a support material itself is arranged to provide an additional auxiliary species;
- (b) a reaction partner for the additional auxiliary species is linked (e.g. covalently) with an auxiliary species;
- (c) an auxiliary species reaction partner is linked (e.g. by covalent linking) to an authentic entity of interest;
- (d) an anti-entity antibody (which may be termed, for example, a primary antibody), is labelled with

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appropriate detectable species capable of giving rise to a signal (e.g. fluorescent substances or enzymes);

- (e) in what may be termed a primary immune reaction, an entity to be detected (if present), suitable amounts of labelled anti-entity antibody, and authentic entity linked to the auxiliary species reaction partner are allowed to mix in solution.

The primary immune reaction may be expected to attain or approach equilibrium in a short period of time (e.g. at a temperature of 37°C to 42°C) thereby to produce a primary immune reaction mixture. It will be appreciated that the primary reaction mixture may contain, *inter alia*, bound and unbound fractions.

Auxiliary species linked to reaction partner to the additional auxiliary species is subsequently introduced to the primary immune reaction mixture such that binding may occur between the auxiliary species and the auxiliary species reaction partner.

Subsequently, the support material provided with additional auxiliary species is used to effect attachment, indirectly, of primary species to the support material by binding between the additional auxiliary species and the reaction partner for the additional auxiliary species.

It is possible to arrange for auxiliary species to be present in very large excess with respect to the corresponding auxiliary species reaction partner, such that the binding reaction between them may be expected to occur rapidly and efficiently with a high proportion of the auxiliary species reaction partner becoming bound with the auxiliary species.

Thus, depending upon the amount of entity to be detected, some of the labelled primary antibodies may be retained on the support material. The proportions of retained primary antibodies carrying detectable species will depend upon the concentration of "competing" entity,

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if any, in a given sample; the competing entity may be a standard or an unknown quantity.

Washing of the support material with appropriate buffers may be used to remove unbound materials and detectable species activity associated with the support material may be measured by any suitable method such as those known in the art.

Optionally, detectable species activity may be eluted into solution using appropriate buffers to facilitate measurement of such species.

In some embodiments of the present invention (e.g. embodiments involving the use of an optical immunosensor based on the surface plasmon resonance principle or on evanescent wave fluorescence) a washing step may not be required; thus, detectable species activity associated with the support material (which may be, for example, a sensor surface) may be measured in the presence of unbound material.

It will be understood that, by use of standard quantities of authentic entity, calibration may be effected such that, subsequently, unknown quantities of entity may be determined. It will also be appreciated that, in a competitive immunoassay, as an amount of a given entity in a sample increases, the amount of authentic entity corresponding thereto retained on the support material decreases (and hence the amount of detectable species retained on the support material decreases); it will also be appreciated that, conversely, as the amount of a given entity in a sample decreases, the amount of authentic entity corresponding thereto retained on the support material increases (and hence the amount of detectable species retained on the support material increases), reaching a maximum when no entity is present in a sample.

By way of example, in a non-competitive immunoassay method which involves a separation method in accordance with the present invention the following may be utilised:

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- (a) an anti-entity antibody [(which may be termed, for example, a primary antibody) which anti-entity antibody is to act as a "signal" antibody] is conjugated to a detectable species which is capable of giving rise to a signal (e.g. a fluorescent substance or an enzyme);
- (b) a hybrid complex which comprises an "immobilising" antibody (which is also an antibody to an entity to be detected) which is conjugated to an auxiliary species reaction partner;
- (c) an entity to be detected (which may be, for example, antigenic substances of high molecular weight);
- (d) an auxiliary species conjugated to a reaction partner for an additional auxiliary species;
- (e) a support material which itself provides or has attached thereto an additional auxiliary species.

Also, by way of example, in a non-competitive immunoassay method which includes a separation method in accordance with the present invention the following may take place:

- (i) an entity of interest and the antibody conjugated to a detectable species are mixed in solution whereupon the entity present is bound by the signal antibody;
- (ii) to the reaction mixture formed in (i) in solution a moderate "excess" of the hybrid complex is added whereupon an immune complex is formed in which the entity is "sandwiched";
- (iii) the reaction mixture formed in (ii) is brought into contact with an "excess" of auxiliary species conjugated to a reaction partner for the additional auxiliary species such that binding occurs between the auxiliary species reaction partner and the auxiliary species;
- (iv) the reaction mixture formed in (iii) is brought into contact with support material upon which is provided additional auxiliary species. By virtue of

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binding between the reaction partner for the additional auxiliary species and the additional auxiliary species, only "signal" antibody bound to an entity to be detected becomes attached to the support material.

Washing the support material with appropriate buffer may be used to remove unattached "signal" antibody.

Detectable species left on the support material, the amount of which is directly proportional to the concentration of the entity, may be measured by any suitable method such as those known in the art.

Optionally, detectable species activity may be eluted into solution using appropriate buffers to facilitate measurement of such species.

In some embodiments of the present invention (e.g. embodiments involving the use of an optical immunosensor based on the surface plasmon resonance principle or on evanescent wave fluorescence) a washing step may not be required; thus, detectable species associated with the support material (which may be, for example, a sensor surface) may be measured in the presence of unbound material.

It will be understood that by use of standard quantities of authentic entities calibration may be effected such that, subsequently, unknown quantities of entities may be determined.

It will be appreciated that any suitable configuration may be used in accordance with the present invention, examples of which are given below:

(I) an additional auxiliary species may be associated with a support material and an antibody to a reaction partner for the additional auxiliary species may be used to link the reaction partner for the additional auxiliary species to an auxiliary species, said antibody being an antibody both to the reaction partner to the additional auxiliary species and to the auxiliary species;

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(II) an additional auxiliary species may be provided on a support material and an antibody to a reaction partner for the additional auxiliary species may be linked (e.g. covalently) to an auxiliary species;

(III) an additional auxiliary species in the form of a ligand may be provided on a support material and a reaction partner for the auxiliary species may be a species which has a part capable of undergoing a specific binding reaction with an additional auxiliary species and a part which is capable of undergoing a specific binding reaction with an auxiliary species which auxiliary species is in the form of a ligand (being a different type of ligand from the ligand constituting the additional auxiliary species);

(IV) an additional auxiliary species in the form of a ligand may be provided on a support material, a reaction partner for the additional auxiliary species is provided, an auxiliary species is provided and a bifunctional species is used to link the reaction partner for the additional auxiliary species and the auxiliary species, said bifunctional species having a first part capable of specific binding with the reaction partner for the additional auxiliary species and a second part, which is a different function from the first part, capable of binding with the auxiliary species;

(V) an additional auxiliary species in the form of a binder may be provided on a support material. A reaction partner for the additional auxiliary species may then be a ligand and may be covalently linked to an auxiliary species which is a different ligand. An auxiliary species reaction partner may then be a binder for the auxiliary species;

(VI) an additional auxiliary species in the form of a binder may be provided on a support material, a reaction partner for the additional auxiliary ligand

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may then be a ligand and may be covalently linked to another, different, binder which then may constitute an auxiliary species. An auxiliary species reaction partner may then be another, different, ligand;
(VII) an additional auxiliary species in the form of a binder may be provided on a support material.

A reaction partner for the additional auxiliary species may be provided in the form of a ligand and this may be linked to an auxiliary species which is a ligand of the same type as the reaction partner for the additional auxiliary species.

An auxiliary species reaction partner may then be provided in the form of a binder which may be of substantially the same type as the binder which is the additional auxiliary species. Thus, for example, where the additional auxiliary species is a binder, it is possible to choose a type of ligand which can act as a reaction partner for the additional auxiliary species and as an auxiliary species;

(VIII) an additional auxiliary species is provided on a support material. Also there is provided a reaction partner for the additional auxiliary species and a bifunctional species having a part capable of specific binding with the reaction partner for the additional auxiliary species and a part capable of specific binding with a primary species.

Indirect attachment of a primary species may be effected by binding between the additional auxiliary species and the reaction partner for the additional auxiliary species and by binding between the bifunctional species and the reaction partner for the additional auxiliary species (which reaction partner for the additional auxiliary species is also serving as an auxiliary species);

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(IX) an additional auxiliary species is provided on a support material. A reaction partner for the additional auxiliary species is also provided. The reaction partner may be capable of also acting as an auxiliary species, in the form of a ligand, with which an auxiliary species reaction partner comprising a binder may undergo specific binding.

It will be appreciated that in heterogenous immunochemical analysis (immunoassay) there is a requirement for the separation of bound and unbound fractions of detectable species in order to permit assessment of the distribution of detectable species between bound and unbound states. It may be considered that this separation step in an immunoassay method is one of the most important features of such a method.

The practical importance of a separation step is based upon the fact that the efficiency of the step, the simplicity (or otherwise) thereof, and the speed thereof may influence the general properties of an assay method and may influence the performance of an assay method.

Separation of bound and unbound fractions of detectable species may be effected using a solid phase immobilised antibody technique in which primary antibody is adsorbed onto a surface of a reaction vessel (e.g. polystyrene tubes) or covalently linked to a micro-particulate material such as microcellulose, Sephadex or micro-particulate material with entrapped ferrous oxide (magnetisable particles).

The use of immobilised primary antibodies in separation in an immunoassay may suffer from several disadvantages; for example interaction between primary antibody and an entity (or an authentic entity) may be considerably slower (e.g. due to diffusion considerations) than that which takes place with the same primary antibody and entity (or authentic entity) in solution (i.e. a longer time may be required to reach equilibrium).

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Also, the immobilisation of antibody on a solid phase is not a problem-free procedure; thus, for example, it can be difficult to control or reproduce and also serious protein leakage may occur.

The present invention may offer the advantages of reactions in solution with the convenience of a rapid solid-phase separation technique. Thus, for example, a primary immune reaction may be allowed to proceed in solution and then separation may be effected.

The present invention may also offer for assays and for sensors, for example immunosensors such as optical immunosensors and electrochemical immunosensors, the possibility that a surface carrying an additional auxiliary species in accordance with the present invention may be the same for a variety of, or all, entities to be detected thereby providing a generic assay or sensor construction.

A combination comprising a sensor surface carrying an additional auxiliary species in accordance with the present invention may also, for example, be such as to permit regeneration for multiple reuse; for example, such a combination of a sensor surface carrying additional auxiliary species may be regenerated by removal of bound substances by washing with a suitable buffer preparation (e.g. 0.1M glycine-HCl at pH 2.5).

Also, by way of further example, additional auxiliary species may be selected so as to be stable to multiple treatment with low or high pH organic solvents and/or chaotropic reagents thereby to permit regeneration for multiple reuse.

The present invention thus offers the possibility of constructing a non-dedicated sensor such as an immunosensor or immunoelectrode.

Thus, by way of example, an electrochemical immunosensor may be provided in which an electrode surface is coated with a conducting polymer (e.g. polypyrrole polymer) which may be arranged to act as an

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additional auxiliary species (in the form of a ligand). For use in such a sensor antibodies to the conducting polymer (e.g. polypyrrole polymer), or segments thereof, may be generated by suitable techniques such as those known in the art; a combination comprising an electrode surface and coatings of a conducting polymer may be, for example, such as to permit regeneration for multiple reuse.

The methods of competitive and non-competitive immunoassay, hereinbefore described by way of example, may be used in an electrochemical immunosensor as immediately hereinbefore described. A detectable species for use in such an electrochemical immunosensor may be arranged to provide an electroactive species.

In conventional electrochemical immunosensors known in the art specific antibodies may be immobilised on an electrode surface thereby to provide a dedicated immunosensor; such immunosensors may have inherent constraints when multiple electrodes with the same properties are required. Thus, disadvantages may become apparent since regeneration of the sensor may be difficult or regeneration may lead to deterioration of antibody activity.

The present invention may offer the possibility of avoiding such disadvantages in that a sensor in accordance with the present invention may not need to be a dedicated sensor.

It will be appreciated from the foregoing that, by way of further example, detection of entities in accordance with the present invention may use an immunosensor technique.

For example, an additional auxiliary species may be immobilised on a surface of a sensor device (e.g. on a glass surface, on a quartz surface, or on the surface of an electrode).

For example, in the use of direct optical immunosensors no detectable species is used and the

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binding of an entity to be detected and antibodies may give rise to signal generation to permit detection (e.g. by surface plasmon resonance).

By way of further example, in the use of a detectable species labelled antibody immunosensor, signal generation may be detected by a suitable method appropriate to the nature of the signal generated by the detectable species (e.g. a means for measuring fluorescent emission may be used when a detectable species is a fluorescence producing species).

By way of example, if desired, the present invention may make use of an inactive species which may be attached as required to be utilised in accordance with the present invention.

Thus, for example, an active species may be such as to be reconstitutable to an active form. An inactive form of a species may be prepared by drying and/or freeze-drying (lyophilising).

Thus, for example, any species or combination of species suitable for use in accordance with the present invention may be rendered inactive (e.g. by drying and/or freeze-drying (lyophilising)) so as to produce a reconstitutable reagent system or reconstitutable reagent material.

Reconstitution may be effected in any suitable manner, for example, by addition of a reconstituting agent such as a suitable solvent (e.g. water), or a suitable solution (e.g. an aqueous solution) or a suitable sample (e.g. an aqueous sample).

By way of further example, if desired, the present invention may make use of a "switchable" species. Thus, for example, where an additional auxiliary species, or a reaction partner for the additional auxiliary species, or an auxiliary species, or an auxiliary reaction partner is a ligand, the ligand may be provided in a first 'inactive' form which may be formed into an 'active' form capable of interacting with another species.

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The present invention may find application in, for example, label-free or detectable species-dependent (e.g. tracer species-dependent) assay methods such as enzyme-immunoassay, fluoro-immunoassay and radio-immunoassay. By way of example, the present invention may find application in antibody-labelled or antigen-labelled assays.

Any suitable detectable species (e.g. tracer species) may be used as a label in accordance with the present invention.

Examples of detectable species are enzymes (e.g. alkaline phosphatase, β -galactosidase and horse-radish peroxidase), species capable of giving a fluorescent signal (e.g. fluorophores (or polymeric fluorophores)), chemiluminescent compounds, bioluminescent compounds, radioisotopes, dyes, and detectable structures (such as ligand species (or polymers or a ligand species), and binder species (or polymers of a binder species)). Examples of fluorophores are fluoresceins, coumarins and rhodamine.

Detection or determination of a signal from a detectable species may be effected in any suitable manner such as those known in the immunochemical field.

It will be appreciated that "at any suitable time" as used in this Specification, with respect to the performance of a method in accordance with the present invention, indicates at a suitable time during the performance of a method in accordance with present invention.

The terms "antibody" as used in this Specification embraces whole antibody or antibody fragments such as Fab and (Fab)₂, and, accordingly, the term "antibodies" used herein embraces whole antibodies and antibody fragments.

It will be appreciated that an antibody for use in accordance with the present invention may be prepared by any suitable method, such as those known for raising

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polyclonal and monoclonal antibodies; thus, antibodies may be raised, for example, by immunising animals.

The present invention may find application in any suitable form of immunological detection or immunoassay examples of which are heterogeneous immunoassay methods, competitive immunoassay methods, non-competitive immunoassay methods, sandwich immunoassay methods and direct immunoassay methods.

By way of example, the present invention may find application in multi-entity (e.g. multi-analyte species) detection and in ratiometric detection.

It is to be understood that the present invention may find application in qualitative detection of an entity or entities, which may also be considered to be qualitative analysis, or in quantitative detection of an entity or entities, which may also be considered to be quantitative analysis (i.e. measurement or determination of an entity or entities).

It will be appreciated that, for example, the present invention may be utilised with samples which contain an entity to be detected and with samples which contain substantially no entity to be detected (e.g. such as in the case of a "zero" standard sample or an "unknown" sample, which upon subjecting to detection in accordance with the present invention, is found to contain substantially no entities to be detected). Thus, it will be appreciated that the present invention may be used to "probe" for an entity, whether or not a particular entity is present in a particular sample.

Further it will be appreciated, by way of example, that by use of standard quantities of authentic entity to be detected, calibration may be effected such that, subsequently, unknown quantities of entity to be detected may be determined.

The present invention will now be further described, by way of example only, as follows:

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Example 1 Provision of additional auxiliary species on a support material

5(6)-carboxy fluorescein (CF) was selected as an example of an additional auxiliary species and was conjugated to bovine serum albumin (BSA) by reacting 5(6)-carboxy fluorescein-N-hydroxy succinimide ester (6.0 mg in 200 μ l of dimethyl formamide (DMF)) with BSA (50 mg in 10 ml of 50mM NaHCO₃ solution, pH 8.6).

The resulting conjugate was purified by dialysis and treatment with 1% activated Norit A charcoal.

The resulting purified BSA-CF conjugate (being a conjugate of a carrier protein and additional auxiliary species) was attached to the inner walls of polystyrene microtitre plate wells by adsorption.

Thus, 200 μ l volumes of BSA-CF solution (20 μ g/ml in 1% NaHCO₃) were added to each well and left to stand at 4°C for 24 hours. The wells were subsequently emptied and washed twice with plate washing buffer (5mM NaHCO₃, 100mM NaCl 0.05% Tween 20).

Example 2 Preparation of reaction partner for the additional auxiliary species

Anti-fluorescein antiserum was raised by injecting sheep with BSA-CF conjugate (see Example 1), according to known immunological procedures, at monthly intervals for 9 months.

Purified antibodies were isolated, from antiserum thus produced, by immunoaffinity chromatography using 5(6)-carboxy-2',7'-diochlorofluorescein coupled to AH-Sepharose (Pharmacia) as an affinity matrix. Bound antibodies were eluted from the affinity matrix by 20% acetonitrile and 1% propionic acid in distilled water.

Example 3 - Linking of auxiliary species to reaction partner for the additional auxiliary species

7-amino-4-methyl coumarin-3-propionic acid (AMCP) was chosen as an example of an auxiliary species and was

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covalently coupled to purified anti-fluorescein antibodies (prepared as in Example 2) by reacting AMCP-N hydroxy succinimide ester (0.5 mg in 20 μ l DMF) with IgG antibody (20 mg in 2.5 ml of 50mM NaHCO₃, pH 8.6, 100mM NaCl). The resulting conjugate was purified by gel filtration on a Sephadex G50 (Pharmacia) column.

The presence of AMCP in the purified conjugate was confirmed by UV absorption.

Example 4 Preparation of auxiliary species reaction partner

Anti-AMCP antiserum was raised in sheep by injecting key-hole limpet haemocyanin (KLH)-AMCP conjugate, in accordance with known immunological procedures, at monthly intervals for 9 months.

Purified antibodies were isolated, from the antiserum thus produced, by immunoaffinity chromatography using 7-hydroxy-4-methyl coumarin-3-propionic acid coupled to AH Sepharose as affinity matrix. Bound antibodies were eluted from the affinity matrix by a mixture of 20% acetonitrile and 1% propionic acid in distilled water.

Example 5 Linking of a primary species to the auxiliary species reaction partner

Atrazine was selected as an entity to be detected (i.e. in this Example the entity is itself an analyte species) to illustrate determination of an entity in accordance with the present invention.

Accordingly, an atrazine derivative(*) was selected to act as an authentic entity to be detected (in this case an authentic analyte species).

The atrazine derivative was covalently linked to anti-AMCP IgG (see Example 4) by reacting an N-hydroxy succinimide ester of the atrazine derivative (0.35 mg) with the IgG (20 mg) in a manner similar to the conjugating procedure hereinbefore disclosed in Example 3.

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The resulting conjugate was purified by gel filtration on a Sephadex G50 column.

*The atrazine derivative for use in this Example was prepared according to published methods. The derivative was

6-{{4-chloro-6-[(methyl ethyl) amino]-1,3,5-triazine-2-yl}thio}-propionic acid.

Example 6 Preparation of primary antibody

Anti-atrazine antiserum was raised in rabbits by injecting atrazine derivative(*)-KLH conjugate according to known immunological procedures for 9 months.

The antiserum thus produced was used for the development of enzyme immunoassay methods for the measurement of atrazine.

*The atrazine derivative for use in this Example was produced according to published methods. The derivative was

6-{{4-chloro-6-[(methyl ethyl) amino]-1,3,5-triazine-2-yl} amino}-hexanoic acid.

Example 7 Enzyme Immunoassay for atrazine

- (a) Microtitre plates (coated with additional auxiliary species as disclosed in Example 1) were treated to attach to the inner walls of the wells, auxiliary species thereby to immobilise auxiliary species on the plates.

Thus, to each well 200 μ l of a solution containing reaction partner for the additional auxiliary species-auxiliary species conjugate (see Example 3) were added and left to react for 1 hour at 37°C; the solution contained 2 μ g/ml of the conjugate in assay buffer (50mM Tris, pH 7.2, 100mM NaCl, 0.2% gelatine, 50 μ g ml rhodamine base B and 0.03% thimerosal. The wells were subsequently emptied and washed three times.

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Auxiliary species was now attached to the plates (support material) by being conjugated to reaction partner for additional auxiliary species which reaction partner was bound, by specific binding, to the additional auxiliary species attached to the plates.

- (b) Suitably diluted solutions of auxiliary species reaction partner-primary species conjugate (see Example 5) in assay buffer (200 μ l) were added to each well of the plates to which auxiliary species was attached (see immediately preceding paragraph (a)) and left to react for 1 hour at 37°C. The wells were emptied and washed three times.
- (c) A "zero" standard solution and atrazine standards solutions were prepared (in 50mM Tris, pH 7.0, 100mM NaCl, 0.25 mg/ml ovalbumin at 0.03% thimerosal). The atrazine content of the standards was respectively zero, 0.05, 0.1, 0.25, 0.5 and 1.0 ng/100 μ l.

The standard solutions were added to duplicate wells (100 μ l), followed by 100 μ l of primary antibody ((anti-atrazine antibody) prepared as disclosed in Example 6) suitably diluted in assay buffer ($1/6000$).

- (d) After standing for 15 minutes at 24°C the wells were emptied and washed three times. 200 μ l of commercially available goat anti-rabbit-horse radish peroxidase conjugate (Sigma) were added to each well and left to incubate for 1 hour at 37°C.
- (e) Finally the wells were emptied and washed three times and the activity of bound horse radish peroxidase (HRP) was assessed using H₂O₂ and ABTS chromophore according to standard conditions.

A calibration graph for atrazine was constructed by plotting Optical Density (OD) at 405 nm against atrazine concentrations added to the

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assay wells. The figures obtained for the assays using the standard solution are shown in Table I.

Atrazine concentration (ng/well)	O.D. (405 nm) (average)
zero	1.75
0.05	1.69
0.10	1.55
0.25	1.41
0.50	1.00
1.00	0.80

Example 8 Enzyme immunoassay of atrazine

Suitably diluted solutions of auxiliary species reaction partner-primary species conjugate (see Example 5) in assay buffer (50 μ l) were added to wells of a microtitre plate having additional auxiliary species attached thereto (prepared as in Example 1).

Volumes (100 μ l) of standard solutions of atrazine and of a zero standard (see Example 7, paragraph (c)) were added to duplicate wells, followed by 50 μ l of anti-atrazine antibody (see Example 6) suitably diluted in assay buffer ($1/3000$) and the plate was left to stand at 24°C for 10 min. Subsequently 50 μ l of suitably diluted reaction partner for the additional auxiliary species-auxiliary species conjugate (see Example 3) were added to each well.

After 10 min. standing the microtitre plate was treated as described in Example 7 paragraph (e).

The results obtained were substantially similar to those obtained in Example 7.

In this present Example the delay in addition of the reaction partner for the additional auxiliary species-auxiliary species conjugate allowed primary immune binding reactions to take place with all reagents (except of course the additional auxiliary species) in solution.

Example 9 Enzyme immunoassay of atrazine

A microtitre plate was prepared as in Example 7 paragraph (a) to give a plate having auxiliary species

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attached thereto via a reaction partner for an additional auxiliary species and an additional auxiliary species.

Volumes (100 μ l) of standard solutions of atrazine and of zero standard (see Example 7 paragraph (c)) were added to duplicate wells of the microtitre plate.

Subsequently volumes (50 μ l) of anti-atrazine antibody (see Example 6), (suitably diluted in assay buffer ($1/3000$)), were added and left to stand at 24°C for 10 minutes before volumes (50 μ l) of auxiliary species reaction partner-primary species conjugate (see Example 5) were added to each well (suitably diluted). After further incubation for 10 min. the plate was processed as in Example 7 paragraph (e).

The results obtained were substantially similar to those obtained in Examples 7 and 8, but with some increase in assay sensitivity; the calibration graph of this present Example was steeper.

In this present Example the delay in addition of the auxiliary species reaction partner-primary species conjugate allowed the binding of primary antibody and entity to be detected (in this Example the entity itself being an analyte species) to take place in solution and delayed competition of the authentic entity until the binding of entity to be detected by the primary antibody had substantially taken place.

This delay resulted in a more sensitive assay.

[In the above Examples, N-hydroxy succinimide ester derivatives of carboxylic acid functions were prepared by reacting 1 mmole of a carboxylic acid function containing compound in dimethyl formamide and 1.1 mmole of N-hydroxy succinimide and 1.1 mmole of dicyclohexyl carbodiimide at 4°C for 72 hours. The esters thus prepared were able to be used for conjugation to proteins without further treatment.

It will be appreciated that in the Examples "suitably diluted" indicates diluted to optimal dilution as may be determined by dilution response experiments in accordance with known practice in immunotechnology.]

From the foregoing it will be appreciated that in accordance with the present invention it is possible, for example, to effect a separation at any convenient point in an immunoassay. This may be achieved, for example, by delaying the addition, to a reaction mixture, of a "bridging" auxiliary species until a chosen time in an immunoassay.

Thus, for example, the addition of a conjugate, said conjugate being a reaction partner for the additional auxiliary species - auxiliary species conjugate, may be delayed until a selected point in an immunoassay.

Accordingly, it possible, for example, to attach a selected fraction of an immunoassay reaction mixture to a support material at any convenient point during an array.

Claims

1. A separation method, suitable for use in an immunological method for the detection of an entity, which separation method includes effecting attachment of an auxiliary species to a support material by use of an additional auxiliary species and a reaction partner for the additional auxiliary species.
2. A method, suitable for use in immunological detection of an entity, which method includes effecting attachment of an auxiliary species to a support material by use of an additional auxiliary species and a reaction partner for the additional auxiliary species.
3. A method as claimed in Claim 1 or Claim 2 wherein an additional auxiliary species and a reaction partner for the additional auxiliary species are used together with an auxiliary species and an auxiliary species reaction partner, to bring about indirect attachment of a primary species to a support material.
4. A method as claimed in any one of the preceding claims wherein the additional auxiliary species is a ligand.
5. A method as claimed in any one of Claims 1 to 3 wherein the additional auxiliary species is a binder.
6. A method as claimed in any one of Claims 1 to 4 wherein the reaction partner for the additional auxiliary species is a binder.
7. A method as claimed in any one of Claims 1 to 3 or in Claim 5 wherein the reaction partner for the additional auxiliary species is a ligand.
8. A method as claimed in any one of Claims 1 to 3 wherein the reaction partner for the additional auxiliary species is a species which has a part capable of undergoing a specific binding reaction with an additional auxiliary species and a part which is capable of undergoing a specific binding reaction with an auxiliary species.

9. A method as claimed in any one of Claims 1 to 3 wherein an auxiliary species is a ligand.
10. A method as claimed in any one of Claims 1 to 3 wherein an auxiliary species is a binder.
11. A method as claimed in any one of Claims 1 to 3 wherein the auxiliary species reaction partner is a ligand.
12. A method as claimed in any one of Claims 1 to 3 wherein the auxiliary species reaction partner is a binder.
13. A method as claimed in any one of Claims 1 to 3 wherein the auxiliary species reaction partner is a species which has a part which is capable of undergoing a specific binding reaction with an auxiliary species and a part which is capable of undergoing a specific binding reaction with a primary species.
14. A method as claimed in any one of Claims 4, 7, 9 or 11 wherein the ligand is an antigenic ligand or a non-antigenic ligand.
15. A method as claimed in any one of Claims 5, 6, 10 or 12 wherein the binder is a binder for an antigenic ligand or a binder for a non-antigenic ligand.
16. A method as claimed in any one of the preceding Claims wherein the additional auxiliary species is provided on a support material.
17. A method as claimed in Claim 16 wherein the support material is a reaction vessel wall, an insoluble polysaccharide, a microparticle, polystyrene, cross-linked dextran, an insoluble polymer structure, a glass surface, a derivatised silica surface, a polymer attached to a surface, a microparticulate material with entrapped ferrous oxide, nylon, or a polyamide.
18. A method as claimed in any one of the preceding Claims wherein a plurality of additional auxiliary species are used to form a bridge between an auxiliary species and a support material.

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19. A method as claimed in any one of the preceding Claims wherein an entity to be detected is itself an analyte species or an entity to be detected may carry or include an analyte species.

20. A method as claimed in Claim 19 wherein the entity to be detected is a steroid hormone, a thyroid hormone, a steroid in an extract, a drug, a polypeptide hormone, a tumour marker, a protein antigen, a blood protein, a marker protein, a pesticide, a herbicide, a toxin, a micro-organism, an antibody to a micro-organism, or a metal complex.

21. A sensor, suitable for use in the detection of an entity, which sensor includes an additional auxiliary species provided on a support material, said additional auxiliary species being capable of interacting with a reaction partner for the additional auxiliary species thereby to effect attachment of an auxiliary species to a support material.

22. A test-kit, which test kit includes an additional auxiliary species provided on a support material, said additional auxiliary species being capable of interacting with a reaction partner for the additional auxiliary species thereby to effect attachment of an auxiliary species to a support material.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01972

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/537 G01N33/547

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	EP,A,0 177 191 (SERONO DIAGNOSTICS PARTNERS) 9 April 1984 see the whole document ---	1-22
Y	EP,A,0 077 671 (ORTHO DIAGNOSTICS SYSTEMS INC.) 27 April 1983 see the whole document ---	1-22
Y	WO,A,87 04794 (MICROBIOLOGICAL ASSOCIATES INC.) 13 August 1987 see the whole document ---	1-22
P,Y	WO,A,92 16838 (GEC-MARCONI LTD) 1 October 1992 see the whole document --- -/--	1-22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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- *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
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- *&* document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/01972

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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PCT/GB 93/01972

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