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(21) International Application Number: PCT/GB96/02919 (22) International Filing Date: 28 November 1996 (28.11.96) (30) Priority Data: 9524393.7 29 November 1995 (29.11.95) GB (71) Applicant (for all designated States except US): THE MINISTER OF AGRICULTURE, FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall Place, London SW1A 2HH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): PINDER, Andrew, Charles [GB/GB]; Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UA (GB). CLARKE, Rosemary, Georgina [GB/GB]; Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UA (GB). CUNLIFFE, David [GB/GB]; Institute of Food Research, Reading Laboratory, Earley Gate, Reading, Berkshire RG6 6BZ (GB).		(74) Agent: SKELTON, S., R.; D/IPR, Formalities Section (Procurement Executive), Poplar 2 MOD Abbey Wood #19, P.O. Box 702, Bristol BS12 7DU (GB). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: EXTRACTION AND LABELLING OF BIOLOGICAL MATERIALS WITH MAGNETIC AND FLUORESCENT BEADS OR LIPOSOMES (57) Abstract A detection agent for detecting target materials, especially biological materials such as bacterial cells, present in a sample material (e.g. a foodstuff) the agent comprising binding means (e.g. an antibody) for binding to the target, magnetic means for extraction of the target and a fluorescent dye for labelling the target. The agents are less than 1 μ m, preferably around 100 nm, in diameter. Methods of use are also disclosed wherein the cells, each labelled by several polymeric-bead agents, are detected by fluorescence-detected flow cytometry. Also disclosed are kits for use in such methods, and processes for manufacturing the kits and agents.		

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EXTRACTION AND LABELLING OF BIOLOGICAL MATERIALS WITH MAGNETIC AND FLUORESCENT BEADS OR LIPOSOMES

TECHNICAL FIELD

The present invention relates to methods having utility for the extraction of target materials present as part of a larger source material, and their fluorescent labelling for detection purposes. The invention further relates to agents for use in these methods, methods for preparation of these agents, and kits employing them.

BACKGROUND ART

It is well known in the art that target materials, e.g. biological materials such as pathogenic organisms, can be fluorescently labelled in order to increase the ease by which they can be detected and quantified when present in a larger sample from which they could otherwise not be readily distinguished. For instance, target DNA can be labelled specifically using a fluorescent nucleotide probe. Another commonly used technique is 'immunofluorescence' whereby antibodies conjugated to a fluorescent agent may be specifically bound to a target antigenic material

Such specific labelling techniques are particularly useful when the target material is initially present in only small quantities in a sample material e.g. for detecting specific bacterial contaminants in food.

Once labelled, the target can be more readily detected and quantified. In the case of cellular materials, one useful technique is immunofluorescence flow cytometry. Using this technique it is possible to detect specific organisms in a sample material down to levels of 10^4 /ml (Pinder & McClelland 1994; J Microscopy 176: 17-22).

One drawback with this technique is that in order to detect organisms present at very low initial concentrations in a sample, it may be necessary to perform a pre-enrichment culturing step. Thus a 6 hour non-selective pre-enrichment can increase sensitivity 1000-fold (Pinder & McClelland 1994, supra). This

second step inevitably adds to the cost and delay involved in getting a test result.

An alternative to pre-enrichment would be the use of magnetic particles coated with bacteria-specific antibodies. This extraction technique is well established (see e.g. Skjerve & Olsvik (1991) *Int J Food Microb* **14**: 11-18). Problems arise, however, with the low recovery of the bacteria from the beads after separation from the sample. Separation from the beads is necessary in order to allow quantification of the bacteria - for instance because the beads are generally much larger than the bacteria, there may be a variable number present on each one. Nor is it possible to use light-scatter flow cytometry to count the bacteria on the beads because light scattering can not sufficiently differentiate between beads with no bacteria attached and beads with one or more attached. Thus, as with pre-enrichment, the second step adds to the cost and delay associated with the test.

In PCT patent application WO 91/09141 published in 1991 there is disclosed large (1-100 μm) fluorescent, magnetically-responsive polymeric particles. The beads are intended to be used as a removable solid phase for various types of immunoassays. Cells or other target analytes bound to the surface of the particles can then be labelled with a further reactive marker and visualised e.g. by fluorescence spectroscopy.

Japanese patent application JP 04285857 A published in 1992 discloses ferromagnetic particles having fluorescently labelled antibody coupled thereto. Suitable antigens can be assayed by contacting them with the antibody and detecting the fluorescence intensity.

Similarly Griwatz et al (1995) in "An immunological enrichment method for epithelial cells from peripheral blood" *J Immunol Methods* **183**:251-265 used a so-called "immune paramagnetic separation complex" comprising fluorescein isothiocyanate-conjugated mouse anti-human antibody to label epithelial cells,

the mouse antibody being itself bound by a rat anti-mouse antibody coupled to superparamagnetic particles.

The present invention provides, methods and kits with one or more improved properties compared with any of these prior art technologies.

DISCLOSURE OF THE INVENTION

According to a first aspect of the present invention there is provided a detection agent for detecting target biological material present in a sample material, the agent comprising: (a) a core material; (b) binding means peripherally disposed away from the surface of the core material for binding to the target; (c) magnetic means present within the core material (d) and a fluorescent dye present within the core material characterised in that the agent is less than 1 μm in diameter.

By use of these small detection agents, which are at the same time fluorescent and magnetic, the efficiency of extraction and sensitivity of detection by flow cytometry of, for example, pathogenic bacteria from food, may be much improved.

By 'diameter' is meant size along the longest axis of the core, irrespective of the shape of the core, although preferably it is spherical or approximately spherical. In preferred forms of the invention, using the processes made available below, the agents may be produced as monodispersed particles having uniform properties (size, fluorescence, magnetic content).

Preferably the target material is a cellular organism, and more preferably it is a single cell e.g. a bacterium. Preferably the invention is applied to sample materials wherein the target material is present only in a relatively small concentration e.g. as a contaminant, or as a minor component of a larger population. Most preferably the sample material is a foodstuff, which may be prepared such as to increase the ease of extraction e.g. by liquefaction or dilution.

The size of the agent is important in determining whether it will be suitable for flow cytometry. Thus preferably the size of agent is approximately 10% of the size of the target.

Thus for most (e.g. bacterial) targets, it is preferable that the agent's diameter is less than 500 nm, more preferably less than 200 nm or less than 100 nm, but most preferably approximately 100nm (i.e. within 20%, plus or minus). Methods for producing agents down to this size are disclosed in the Examples below.

The size of the agent is important because it is one factor which will determine how many agents will bind to the target. Preferably the diameter of the core material of the agent is selected such that in use at least 2 agents are bound per target, and more preferably at least 5 agents are bound per target. This will allow the easy discrimination between the high accumulated fluorescence of the target bacteria and the much weaker signal from an unbound bead in solution. Essentially the size and the fluorescence loading of the core of the agent is such that a single target having one (but preferably more) agents bound thereto is readily distinguishable from a single agent, or from an unlabelled target, using fluorescence-detection and, where required, light scatter flow cytometry.

It should be noted that this system is quite different to existing magnetic polymer beads which are generally larger than their bacterial targets, and where several bacteria can thus bind to a single bead. The bead/bacterial complexes are not readily distinguished from beads alone using fluorescence-detection and/or light scatter flow cytometry. Submicroscopic fluorescent spheres are known; however they do not contain a magnetic element and therefore can not be used for extraction as in the present invention.

The fluorescent dyes and magnetic particles may be present as a dispersion within the core. Alternatively they may be present in a void within the core. The use of a labelled core has the advantage that a high loading of fluorescent dye can be achieved compared to direct fluorescent labelling of antibodies, and background leakage of the fluorescent label can be virtually

eliminated: this provides a high contrast system when the beads and complexes are detected. In each case the binding means must be suitably disposed away from the core in order to be able to effectively bind the target e.g. in the form of one or more peripherally attached antibodies.

In one embodiment of the first aspect of the invention the core of the detection agent is composed of a polymeric material, e.g. latex, for retaining the magnetic and fluorescent elements of the agent, and for attachment of the binding means.

In an alternative embodiment the core is a liposome core. Although polymer cores may be more robust to storage and more widely applicable than liposomes, under some circumstances liposomes may be easier to prepare and load with fluorescent dye.

In each case, because of their potential for high fluorescent-dye loading, the agents of the invention can be readily detected individually by flow cytometry.

Preferably the binding means is an antibody capable of specific binding to the target material or an antigen thereon; antibodies thus used could be polyclonal but are preferably monoclonal in order to aid consistency of binding.

Preferably several target-binding antibodies are attached to each particle such as to increase the chances of successful binding of the particle to the target.

By synthesizing polymer particles which have a surface of carboxyl groups, target-binding antibodies may be attached directly to the particles by conjugation methods well known to those skilled in the art e.g. by using carbodiimide activation. It may be desirable to attach a further connecting group between the two, such as Protein A. For liposome cores, biotin may be incorporated into the lipid bilayer (e.g. in the form of Biotin-X-dipalmityl phosphatidyl ethanolamine). This can then be used to anchor target-binding antibodies

It may be also be desirable, in order to reduce steric interactions between the antibody groups, to have an extra spacer

group between the targeting antibodies and the core e.g. in the form of another antibody bridging the gap between the two.

It may also be desirable, in order to reduce non-specific binding to the particle surface, that the surface and/or sample material are 'blocked' with a suitable agent e.g. serum proteins.

The magnetic means allows extraction of the bound target from the sample material by use of magnetic force e.g. by methods analogous to those presently used in non-fluorescent magnetic beads such as Dynabeads (TM). Preferably the magnetic means comprises a paramagnetic material such as magnetite.

By fluorescent dye is meant any fluorescent material or functional group. Such materials and groups are well known to those skilled in the art e.g. dansyl chloride (UV excitable), fluorescein isothiocyanate.

In a second aspect of the invention there is disclosed a method for detecting a target material present in a sample material, comprising the steps of: (a) combining agent as described above with the sample under conditions whereby the agent can bind the target, (b) extracting agent and target/agent complex from the sample using magnetic force, (c) detecting the target/agent complex by means of the fluorescent dye associated therewith.

Preferably the target/agent complex is detected by fluorescence-detection flow cytometry, for instance by methods analogous to those described in Pinder & McClelland 1994, supra. It should be noted that because the fluorescence intensity of targets bound with the agents of the present invention will generally be higher than that associated with targets labelled immunofluorescently, a less sophisticated optical system is required to detect and quantify the targets. This will allow the use of cheaper more rugged detection instruments, thereby increasing the attractiveness of the method to industry and encouraging 'pro-active' intervention in manufacturing processes. Such intervention is presently difficult because the widely used detection techniques e.g. culture techniques, take too long to perform.

The use of fluorescence-detection flow cytometry in conjunction with the agents of the present invention may allow not only detection of target, but also estimation or quantification of it, thus allowing the severity of contamination to be assessed.

In a third aspect of the invention there is disclosed a kit suitable for use in the methods of third aspect comprising 'cores' of a size as discussed above in relation to the agents of the present invention, containing magnetic means and a fluorescent dye, and being adapted to be attached to an antibody capable of binding a target material in a sample material.

The beads may be adapted for target antibody attachment by having a surface of activatable carboxyl groups, or by having an intermediate antibody capable of specifically binding a target-specific antibody, or by other means discussed above or such as may occur to those skilled in the art.

Such kits will allow users to readily prepare the agents of the present invention using target antibodies of their choice.

Processes for the manufacture of the agents discussed above form a fourth aspect of the invention. For instance the polymer beads can be prepared by the emulsion polymerisation of monomers in the presence of an aqueous dispersion of colloidal magnetite. The fluorescent dye can be incorporated in the polymerisation process as discussed in more detail below.

Thus the invention makes available agents, methods, processes and kits for the detection of target materials which address many, and preferred forms all, of the problems of the prior art. The relative cheapness and simplicity of the agents, and the methods of their use, means that they can be used as part of regular quality control or investigative testing programmes. The invention is particularly suited to the food industry wherein the facility for rapid testing for bacterial contaminants will reduce the possibility of any infected foodstuffs leaving the factory.

The agents, processes, methods and kits of the present invention will now be described, by way of illustration only, through

reference to the following examples and figures. Other embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

FIGURES

Fig 1 Shows the size distribution of the latex beads prepared in accordance with Example 1 as judged by light scatter.

Fig 2 Shows a schematic scheme for the conjugation of Protein A to the beads.

EXAMPLES

EXAMPLE 1: PROCESS FOR MANUFACTURE OF MAGNETIC/FLUORESCENT POLYMER PARTICLES

Polymer particles were prepared by the emulsion polymerisation of monomers in the presence of an aqueous dispersion of colloidal magnetite and fluorescein.

The magnetite ('ferrofluid') was prepared by the co-precipitation of iron(II) and iron (III) hydroxides. These ultra-fine magnetite particles were then coated in a double layer of surfactant: the primary layer was a long chain fatty acid (oleic) chemically adsorbed on to the magnetite surface; the secondary layer was either a fatty acid salt, long chain sulphonic acid or sulphate acid salt, (sodium dodecyl sulphate and sodium dodecylbenzene sulphonate were investigated). Agents were selected such as to avoid later extensive demulsification (creaming) in the presence of the monomer (styrene or styrene/methacrylic acid).

PREPARATION OF FERROFLUID: The detailed method was based on that of Wooding et al (1991) J. Colloid. Interface Sci. 144, 236 and (1992) J. Colloid. Interface Sci. 149, 98, and was as follows:

100 ml of 0.6036 M iron (II) chloride and 100 ml of 0.8879 M iron (III) chloride were mixed under nitrogen with a further 100 ml of water. To this, an excess of concentrated ammonia solution was added with rapid stirring to flocculate the magnetite particles. The sediment was washed with water/ammonia solution (95:5) (2 x

250 ml) and water (200 ml). Water (200 ml) and oleic acid (21 g) were added and the mixture heated to 90°C for 1 hour with rapid stirring. It was found that oleic acid reduced creaming and improved incorporation of magnetite compared with saturated fatty acids. Upon cooling, hydrochloric acid was added to lower the pH to 4, to flocculate the magnetite. The liquor was decanted off and the precipitate washed with acetone (200 ml) and water (100 ml). Water (150 ml) and sodiumdodecylbenzenesulfonate (20 g) was added to redisperse the magnetite.

PREPARATION OF AGENTS: The fluorescent dye fluorescein was incorporated during the latex polymerisation process. This dye, unlike rhodamine dyes, does not leech out significantly. Dansyl chloride was also used, being prepared by the method of Rembaum et al (1976) *Macromolecules* 9, 238, but it was found that the resulting dye had a relatively low solubility in the monomer mixture. The detailed polymerisation method was based on that of Naguchi et al (1993) *J. Appl. Poly. Sci.* 48, 1539 and Yanase et al (1993) *J. Appl. Polym. Sci.* 50, 765 and was as follows:

In a typical synthesis, a 500 ml round bottomed, flanged reactor was charged with water (300 ml), ferrofluid (20 g = 21% magnetite), sodium acrylate (NaAA) (0.3 g), methylmethacrylate (MAA) (27 g), ethyleneglycoldimethacrylate (EGDME) (3 g), aminostyrene (0.05 g) and fluorescein isothiocyanate (50 mg), and left stirring at 200 or 300 r.p.m. at 70°C under nitrogen for 1 hour. An initiator, 4'-Azobis-4-cyanopentanoic (ACVA) (0.6 g), was then added and the mixture left for 27 hours, then cooled in air. Upon cooling, the latex was filtered through well-washed glass wool to remove any coagulum.

Further preparations are shown in Table 1 at the end of the description. The Ferrofluid used in the table is as follows: FC-80= 21% magnetite; FC-195= 30% magnetite; FC-205= 24% magnetite.

The use of EGDME and MAA generally encouraged smaller more uniform particles. The use of NaAA reduced the likelihood that the carboxylic acid groups being buried within the matrix.

CHARACTERISATION OF THE PARTICLE SIZE: The particle size as judged by laser light scattering measurements is shown in Fig 1 and was optimised at around 120 nm diameter.

More detailed results are shown in Table 2 at the end of the description. Note that this table gives the figures for the average radii; thus the figure must be doubled to give the diameter. For a number of samples the standard deviation (γ) and coefficient of variation (CV) has been calculated indicating a fairly uniform distribution of sizes.

More detailed characterisation of individual particles was performed by electron microscopy. This confirmed that the light scattering measurements gave a very slight overestimate (within 10% or so) of the particle size owing to the presence of a surface film of water molecules. Thus the radii figures in Table 2 demonstrate that uniform particles with a mean diameter of between 50 and 200 nm are readily obtainable using the methods of the present invention.

CHARACTERISATION OF THE PARTICLE SURFACE: The particle surface was characterised by a method was based on that of Vanderhoff et al (1970) In 'Clean Surfaces: Their Preparation and Characterisation for Interfacial Studies', ed. G Goldfinger. Marcel Dekker, New York. To determine the carboxyl content of the polymer particles, the latex was cleaned by contact with a bed of mixed ion exchange resins (IR-120 PLUS and IRA-410 - Aldrich Chemical Co) for 12 hours. This removed surfactant molecules from the latex and converted the carboxyl groups on the particles' surface into the acid form. The solids content of the latex was determined by taking a sample of known mass and evaporating to dryness. Another sample was then taken and titrated with sodium hydroxide solution (0.5 mM) while purging with nitrogen. The end point was determined by measuring the conductivity as the sodium hydroxide was added.

As shown in Table 2, different preparations of particles were found to have between 0.4 and 13 $\mu\text{mol g}^{-1}$ carboxyl groups/particle. This is broadly comparable with commercially available beads.

EXAMPLE 2 - ANTIBODY CONJUGATION

The active carboxy groups of the synthesised particles were used for covalent Protein A and antibody attachment, as shown schematically in Fig 2. Alternative 'bridging' molecules to Protein A were also tried (e.g. intermediate antibodies) - however such molecules lead to beads which were more prone to clumping and therefore Protein A was adopted for the standard preparation.

The beads as prepared have carboxyl groups exposed on their surface. These groups were activated using the carbodiimide (CDI) activation procedure (Lea et al (1990) in "Flow Cytometry and Sorting" Eds. Melamed, Lidmo and Mendelsohn; Wiley-Liss, New York).

To 1 ml of 1 mg/ml beads in 0.01 M Hepes (pH 4.5), 100 µl of 1mg/ml 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ICN Biomedicals) was added. The mixture was left for 5 minutes to allow activation of the carboxyl groups, and the particles were washed and resuspended in 0.01 M Hepes, pH 7.0. Meanwhile, a Protein A solution was made to 1 mg/ml in Hepes, pH 6.0. 1 ml of this sample was then added to the bead/CDI mixture. After gentle mixing, the sample was reacted overnight 4°C. This allowed a slow removal of the surfactant from the beads and covalent attachment of the Protein A. Beads were washed three times in 0.01 M Hepes, pH 7.0 °C and stored in the same containing 0.1% BSA.

In a second method, Pierce Slide-A-Lyzer cassettes were used for dialysis. To 100 µl of 1 mg/ml beads in distilled water, 100 µl of 1mg/ml 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ICN Biomedicals) was added. The mixture was left for 30 minutes to allow activation of the carboxyl groups. Meanwhile, a Protein A solution was made to 1 mg/ml in 0.01 M Hepes, pH 6.0. 1 ml of this sample was then added to the bead/CDI mixture. The sample was dialysed overnight against 0.01 M Hepes, pH 6.0 for 16 hours. Beads were stored at 4°C in PBS containing 0.1% BSA.

ATTACHMENT OF ANTIBODY: A solution of ammonium sulphate-precipitated tissue culture supernatant containing Salmonella-

specific IgG antibody was added to the beads. After mixing, absorption of the IgG to the protein A was allowed overnight at 4°C with gentle rotation. The supernatant was then removed and the beads washed twice in PBS pH 7.0, containing 0.1 % BSA and stored in the same. In order to minimise clumping, 0.05% v/v Tween-20 (TM) was sometimes used.

QUANTIFICATION OF BOUND ANTIBODY: The amount of antibody binding to the particles was determined by protein isotherms.

EXAMPLE 3 - METHOD FOR TARGET EXTRACTION

The overall target-binding performance evaluated by plate counting following magnetic separation of a *Salmonella typhimurium*/E. Coli mixture. The detailed protocol was as follows:

Samples containing bacteria were prepared in phosphate buffered saline at pH 7.4 (PBS). To each 1 ml of sample, 50 µl of prepared particles were added. This mixture was then rotated gently for 20-30 minutes at room temperature to allow attachment of the beads to the antigen.

Samples were then placed in a magnetic particle concentrator (Dynal Ltd) for 5 minutes. The supernatant was then removed and the beads were resuspended in PBS or PBS + 0.05% Tween-20 (TM).

Table 3: The extraction efficiency of the beads

	Salmonella	Non-salmonella
10^3 - 10^4 cells/ml:	60%	6%
10 - 10^2 cells/ml:	40%	0%

The extraction efficiency of the beads at different cell concentrations is shown in Table 3. The target-binding specificity of the particles was found to be improved when the surface of the particles was 'blocked' with mouse serum (5% BSA for 3 hours). This was found to reduce (relatively) the non-specific binding of

non-target organisms to the latex beads (Table 4).

Table 4: Effect of blocking procedures on the percentage extraction of bacteria at 10^4 cells/ml.

	Salmonella montevideo	E. coli
No blocking	54	7
Beads only blocked	156	11
Cells only blocked	37	6

Also beneficial was washing the beads after target extraction to remove the non-specifically (more weakly bound) bacteria. At low test cell concentrations (10^3 - 10^4 cells/ml) 3 washes was sufficient to remove the unbound or weakly bound bacteria.

EXAMPLE 4 - METHOD FOR DETECTION OF LABELLED TARGET FROM
FOODSTUFFS

BINDING OF TARGET: Solid/viscous food samples were diluted 1 gm/10 mls PBS and placed in a sterile stomacher bag. The sample was stomached for 1 minute and filtered through a muslin filter to remove very large food particles. 50 μ l of prepared beads were then added per ml of sample. It was found that a 30 minute exposure allowed significant binding to occur without unduly slowing the overall detection process down. The small, magnetic-fluorescent, beads of the present invention not only bound a higher % of the target organism than commercially available (non-fluorescent) magnetic beads using the same antibody, but also had a far lower degree of non-specific binding to E. coli cells.

FLOW CYTOMETRY: This was carried out essentially as described in Pinder & McClelland 1994 (supra) the content of which document is incorporated herein by reference. In this study samples were analysed on the basis of narrow angle (<20°) forward light

scatter, wide angle (90°) light scatter, and red and green fluorescence. The sample was introduced into the flow cytometer at speeds of between 5 and 30 l/min, depending on the exact concentration of the sample (resuspended beads). From the data it is possible to distinguish between unlabelled bacteria, bacteria labelled with beads and unassociated beads. It is also possible to obtain a value for the concentration of each population.

ADDITIONAL STAINING OF BACTERIA: fluorescent antibody labels do not distinguish between viable and non-viable target organisms. Viability was assessed with a secondary staining procedure using proprietary ChemChrome stain (Chemunex). Essentially only bacteria with intact outer membranes and active intracellular esterases fluoresce green as a result of the procedure. The use of this stain, in conjunction with green fluorescence flow cytometry, can increase the certainty of the results obtained using the fluorescein/light scatter detection alone.

EXAMPLE 5 - LIPOSOME BASED AGENTS

As an alternative to the latex polymer agents, liposome based agents were also tested. Liposomes were produced using the reverse-phase separation technique, a modification of the procedures described in Szoka and Papahadjopoulos (1978) Proc Natl Acad Sci USA **75**(9): 4194-4198, and Siebert et al (1995) Anal Chim Acta **311**: 309-318. The ratio of dipalmityl phosphatidyl choline: cholesterol: dipalmityl phosphatidyl glycerol: Biotin-X-dipalmityl phosphatidyl ethanolamine was 5 : 5 : 0.5 : 0.01. Carboxyfluorescein (2.5 mM) was encapsulated within the liposome. Magnetite may also be encapsulated using methods analogous to those above, or as used in De Cuyper and Joiau (1988) Eur Biophys J **15**: 311-319.

Liposomes were filtered sequentially through 3, 0.4 and 0.22 µm pore size filters. Antibodies were conjugated to mouse anti-S typhimurium antibody (43 µg/ml) using streptavidin (27 µg/ml) and goat anti-mouse IgM-biotin (42 µg/ml). This was mixed with liposomes (diluted 1:10). Conjugation of the antibodies was confirmed using a test strip containing (first) strips of bound S.

typhimurium lipopolysaccharide and then (second) goat anti-biotin. Binding of the liposomes to these strips was confirmed using UV to visualise the liposomes. The protocol used produced optimum binding (i.e. almost all liposomes localised in the first strip). The liposomes were found to be stable (no fluorescence leakage) to the shear forces induced by passage through the flow cytometer. In target binding experiments, bacteria with bound liposomes could be readily distinguished from unbound liposomes by their different light scatter characteristics, and from unlabelled bacteria by their different fluorescent characteristics.

Table 1.

Sample	Monomers	(g)	Ferro-fluid	(g)	Dye	(mg)	ACVA (g)	Water (g)	Time (hours)
EN-97	MMA EGDMA MAA	6 1 3	FC-80	1	E96	1	0.2	100	24
EN-100	MMA EGDMA NaAA	6 1 0.1	FC-80	1	E96	1	0.2	100	24
EN-101	MMA EGDMA MAA	7 1 2	FC-80	1	E96	1	0.2	100	24
EN-103	MMA EGDMA NaAA	9 1 0.1	FC-80	1	E96	2	0.2	100	24
EN-105	MMA EGDMA NaAA	9 1 0.1	FC-80	1.5	E96	1	0.2	100	24
EN-106	MMA EGDMA NaAA	9 1 0.1	FC-80	1.5	E96	1.9	0.2	100	24
EN-107	MMA EGDMA NaAA	9 1 0.1	FC-80	2	E96	1.07	0.2	100	24
EN-108	MMA EGDMA NaAA	9 1 0.1	FC-80	1.5	E96	2.52	0.2	100	24
EN-110	MMA EGDMA NaAA	9 1 0.1	FC-80	2	E96	2.5	0.2	100	24
EN-141	MMA EGDMA NaAA Aminostyrene	9 1 0.1 0.015	FC-80	2	FTTC	6.05	0.2	100	24
EN-154	MMA EGDMA NaAA Aminostyrene	18 2 0.2 0.051	FC-80	5	FTTC	11.8	0.2	100	24
EN-155	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.056	FC-80	6.1	FTTC	21.4	0.4	300	24
EN-156	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.066	FC-80	8	FTTC	23.6	0.6	300	27

Table 1 (continued)

Sample	Monomers	(g)	Ferro-fluid	(g)	Dye	(mg)	ACVA (g)	Water (g)	Time (hours)
EN-157	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.064	FC-80	15	FITC	20.2	0.6	300	26
EN-158	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.067	FC-80	18	FITC	22.2	0.6	300	26
EN-159	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.067	FC-80	20	FITC	23.2	0.65	300	26
EN-190	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.03	FC-80	20	FITC	51.2	0.65	300	26
EN-191	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.022	FC-80	20	FITC	60.5	0.315	300	27
EN-196	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.037	FC-195	13.9	FITC	70.3	0.6	300	27
EN-197	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.044	FC-19	13.9	FITC	80.3	0.6	300	27
EN-198	MMA EGDMA NaAA Aliylamine	27 3 0.3 0.019	FC-195	13.9	FITC	50	0.7	300	27
EN-199	MMA EGDMA NaAA Aliylamine	27 3 0.3 0.024	FC-195	14	FITC	60.2	0.6	300	26
EN-206	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.044	FC-205	17.4	FITC	73	0.61	300	38
EN-207	MMA EGDMA NaAA Aminostyrene	27 3 0.3 0.052	FC-205	17.4	FITC	83	0.62	300	38

Table 2.

Sample	[CO ₂ H] (μmol g ⁻¹)	Mean Radius (nm) ¹
EN-97	8.468	48 and 272
EN-100	1.287	54.5
EN-101	2.024	50 and 216.7
EN-103	1.1089	61.8
EN-105	1.0259	40
EN-106	0.6882	48
EN-107	0.4218	40
EN-108	1.4302	51
EN-110	0.5779	50
EN-141	0.8975	41 (γ= 1.01, CV= 0.3181)
EN-154	0.9606	40 (γ= 1.165, CV= 0.4068)
EN-155	5.7206	38 (γ= 1.018, CV= 0.1358)
EN-157	11.567	
EN-158	10.294	30 (γ= 1.014, CV= 0.1205)
EN-159	3.313	30 (γ= 1.112, CV= 0.3349)
EN-190	-	25 (γ= 1.114, CV= 0.337)
EN-191	-	24 (γ= 1.089, CV= 0.2899)
EN-197	-	44 (γ= 1.551, CV= 0.7148)
EN-198	2.966	61 (γ= 1.047, CV= 0.2168)
EN-199	0.3665	60 (γ= 1.0599, CV= 0.2448)
EN-206	0.30115	30 (γ= 1.026, CV= 0.1621)
EN-207	1.642	74 (γ= 1.2356, CV= 0.4853)
EN-208	13.675	90 (γ= 1.0124, CV= 0.1114)
FC-80	-	20
FC-195	-	24 (γ= 1.053, CV= 0.2302)

CLAIMS

1. A detection agent for detecting target biological material present in a sample material, the agent comprising:

(a) a core material

(b) binding means peripherally disposed away from the surface of the core material for binding to the target.

(c) magnetic means present within the core material

(d) and a fluorescent dye present within the core material characterised in that the agent is less than 1 μm in diameter.

2. An agent as claimed in claim 1 wherein the target is a cell.

3. An agent as claimed in claim 2 wherein the target is a bacterium.

4. An agent as claimed in any one of the preceding claims wherein the sample material is a foodstuff.

5. An agent as claimed in any one of the preceding claims wherein the size of agent is approximately 10% of the size of the target.

6. An agent as claimed in any one of the preceding claims wherein the agent is less than 500 nm in diameter.

7. An agent as claimed in claim 6 wherein the agent is less than 200 nm in diameter.

8. An agent as claimed in claim 7 wherein the agent is approximately 100 nm in diameter.

9. An agent as claimed in any one of the preceding claims wherein the diameter of the core material is selected such that in use at least 2 agents are bound per target.

10. An agent as claimed in claim 9 wherein the size of the agent is selected such that in use at least 5 agents are bound per target.

11. An agent as claimed in any one of the preceding claims wherein the size and fluorescence loading of the core of the agent is such that a single target having one or more agents bound thereto is distinguishable from a single agent and/or from an unlabelled target, using fluorescence-detection and/or light scatter flow cytometry.
12. An agent as claimed in any one of the preceding claims wherein the magnetic means and the fluorescent dye are present as a dispersion within the core material
13. An agent as claimed in any one of claims 1 to 11 wherein magnetic means and the fluorescent dye are within a void within the core material
14. An agent as claimed in any one of the preceding claims wherein the core is composed of a polymeric material.
15. An agent as claimed in claim 14 wherein the polymeric material is latex.
16. An agent as claimed in any one of claims 1 to 13 wherein the core is a liposome
17. An agent as claimed in any one of the preceding claims wherein the binding means is an antibody.
18. An agent as claimed in claim 17 comprising more than one antibody.
19. An agent as claimed in any one of the preceding claims wherein the binding means is attached to the core via carboxyl groups present on the surface of the core.
20. An agent as claimed in any one of the preceding claims wherein the binding means are attached to the core via Protein A which is attached to the surface of the core
21. An agent as claimed in any one claims 1 to 18 wherein the binding means are attached to the core via biotin groups present on the surface of the core.

22. An agent as claimed in any one of the preceding claims wherein the surface of the agent has been blocked in order to reduce non-specific binding.

23. A method for detecting a target material present in a sample material, comprising the steps of: (a) combining the agent of any one of the preceding claims with the sample under conditions whereby the agent can bind the target, (b) extracting the agent from the sample using magnetic force, (c) detecting the target/agent complex by means of the fluorescent dye associated therewith.

24. A method as claimed in claim 23 wherein the target/agent complex is detected by fluorescence-detection flow cytometry

25. A method as claimed in claim 24 wherein the target agent is quantified by fluorescence-detection flow cytometry

26. A kit for use in the method of any one of claims 23 to 24 comprising agent cores less than 1 μm in diameter containing magnetic means and a fluorescent dye, and being adapted to be attached to an antibody capable of binding a target material in a sample material.

27. A kit as claimed in claim 26 wherein the surface of the cores comprises at least one of the following: activatable carboxyl groups, an intermediate antibody capable of specifically binding a target-specific antibody, protein A, biotin.

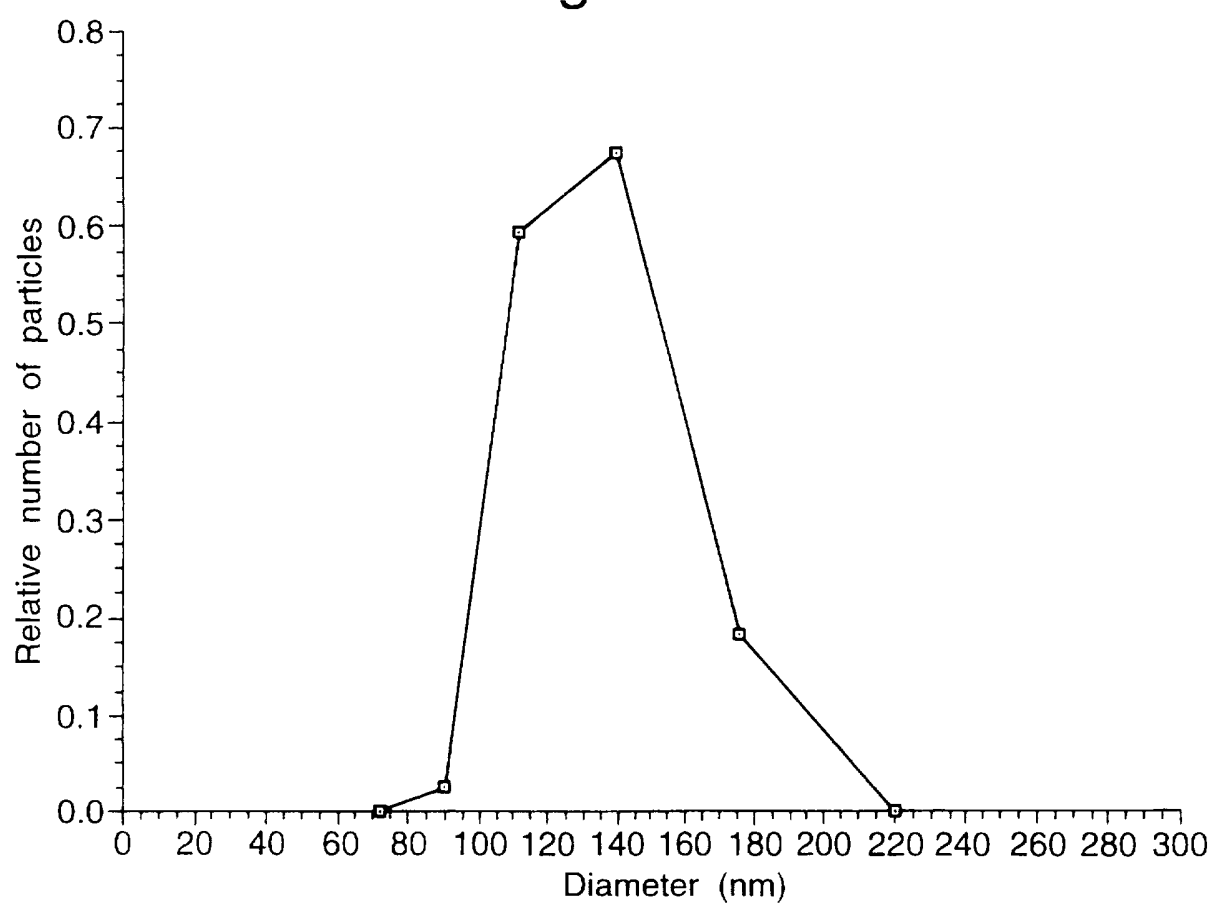
28. A process for preparing the cores of claim 26 or claim 27 comprising the following steps: (a) mixing magnetic means, fluorescent dye and monomers, and (b) carrying out in-situ emulsion polymerisation.

29. A process for preparing the agents of claim 14 or claim 15 comprising the following steps: (a) mixing magnetic means, fluorescent dye and monomers, (b) carrying out in-situ emulsion polymerisation, and (c) attaching binding means to the resulting polymer.

30. An agent, method or process substantially as described herein with reference to Examples 1 to 5 and Figures 1 and 2.

1/2

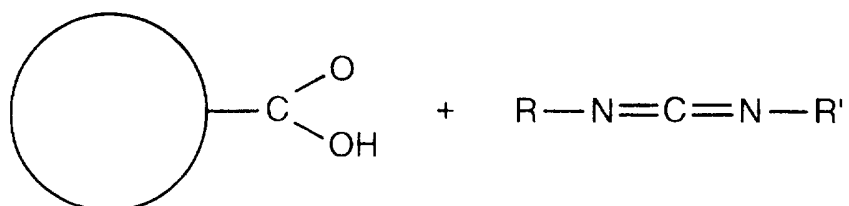
Fig.1.



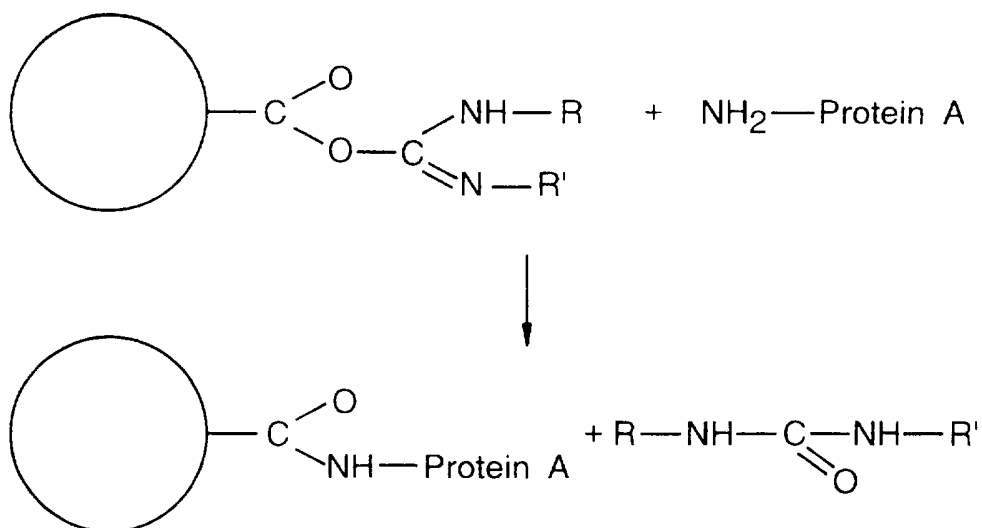
2/2

Fig.2.

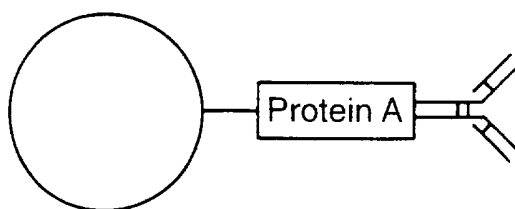
Carboxy groups on the beads are activated by a carbodiimide



The modified groups then react with amine groups on Protein A



Protein A then binds the FC fragment of an IgG molecule



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 96/02919

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 G01N33/543 G01N33/58

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 6 G01N C08F

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	see example 17	17-25, 27-29
Y	US 5 393 527 A (MALICK ADRIEN ET AL) 28 February 1995 see claim 3; example 3	13, 16
Y	US 5 320 906 A (ELEY CRISPIN G S ET AL) 14 June 1994 see the whole document	13, 16
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

14 March 1997

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Wells, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/02919

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	JOURNAL OF APPLIED POLYMER SCIENCE, 1993, NEW YORK US, XP002027479 NOGUCHI H. ET AL: "Preparation and Characterisation by Thermal Analysis of Magnetic Latex Particles" cited in the application	1-12,14, 15,26,30
Y	see page 1541; table I	17-25, 27-29
Y	--- US 4 230 685 A (SENYEI ANDREW E ET AL) 28 October 1980 see claim 1	20
Y	--- US 5 395 688 A (WANG CHAO-HUEI J ET AL) 7 March 1995 cited in the application & WO-A-91/09141 see examples 7,29	19
Y	--- PATENT ABSTRACTS OF JAPAN vol. 015, no. 345 (C-0864), 3 September 1991 & JP 03 137022 A (TDK CORP;OTHERS: 01), 11 June 1991, see abstract	13,16
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A	--- JOURNAL OF APPLIED POLYMER SCIENCE, vol. 50, no. 5, 5 November 1993, pages 765-776, XP000464169 YANASE N ET AL: "PREPARATION OF MAGNETIC LATEX PARTICLES BY EMULSION POLYMERIZATION OF STYRENE IN THE PRESENCE OF A FERROFLUID" cited in the application	1
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national Application No

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