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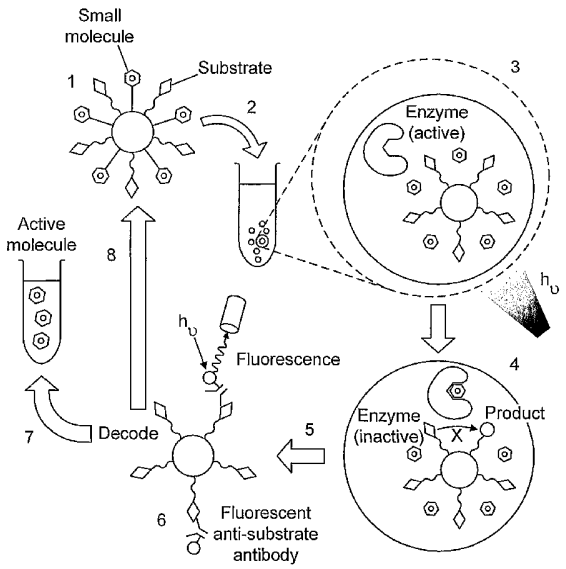
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(54) Title: SELECTION BY COMPARTMENTALISED SCREENING



(57) Abstract: The invention describes a method for the identification of compounds which bind to a target component of a biochemical system or modulate the activity of the target, comprising the steps of: a) compartmentalising the compounds into microcapsules together with the target, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule; and b) identifying the compound which binds to or modulates the activity of the target. The invention enables the screening of large repertoires of molecules which can serve as leads for drug development.

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SELECTION BY COMPARTMENTALISED SCREENING

The present invention relates to a method for selection of compounds from a library of compounds by screening in microcapsules. The method of the invention is particularly applicable to selection of low molecular weight compounds such as candidate drugs for potential activity against any desired drug target.

BACKGROUND TO THE INVENTION

The present invention relates to methods for use in the identification of molecules which bind to a target component of a biochemical system or modulate the activity of a target.

Over the past decade, high-throughput screening (HTS) of compound libraries has become a cornerstone technology of pharmaceutical research. Investment into HTS is substantial. A current estimate is that biological screening and preclinical pharmacological testing alone account for ~14% of the total research and development (R&D) expenditures of the pharmaceutical industry (Handen, Summer 2002). HTS has seen significant improvements in recent years, driven by a need to reduce operating costs and increase the number of compounds and targets that can be screened. Conventional 96-well plates have now largely been replaced by 384-well, 1536-well and even 3456-well formats. This, combined with commercially available plate-handling robotics allows the screening of 100,000 assays per day, or more, and significantly cuts costs per assay due to the miniaturisation of the assays.

HTS is complemented by several other developments. Combinatorial chemistry is a potent technology for creating large numbers of structurally related compounds for HTS. Currently, combinatorial synthesis mostly involves spatially resolved parallel synthesis. The number of compounds that can be synthesised is limited to hundreds or thousands but the compounds can be synthesised on a scale of milligrams or tens of milligrams, enabling full characterisation and even purification. Larger libraries can be synthesised using split synthesis on beads to generate one-bead-one compound libraries. This method is much less widely adopted due to a series of limitations including: the need for solid phase synthesis; difficulties characterising the final products (due to the shear numbers and small scale); the small amounts of compound on a bead being only sufficient for one

or a few assays; the difficulty in identifying the structure of a hit compound, which often relies on tagging or encoding methods and complicates both synthesis and analysis. Despite this split synthesis and single bead analysis still has promise. Recently there have been significant developments in miniaturised screening and single bead analysis. For example, printing techniques allow protein-binding assays to be performed on a slide containing 10,800 compound spots, each of 1 nl volume (Hergenrother et al., 2000). Combichem has so far, however, generated only a limited number of lead compounds. As of April 2000, only 10 compounds with a combinatorial chemistry history had entered clinical development and all but three of these are (oligo)nucleotides or peptides (Adang and Hermkens, 2001). Indeed, despite enormous investments in both HTS and combinatorial chemistry during the past decade the number of new drugs introduced per year has remained constant at best.

Dynamic combinatorial chemistry (DCC) can also be used to create dynamic combinatorial libraries (DCLs) from a set of reversibly interchanging components, however the sizes of libraries created and screened to date are still fairly limited ($\leq$40,000) (Ramstrom and Lehn, 2002).

Virtual screening (VS) (Lyne, 2002), in which large compound bases are searched using computational approaches to identify a subset of candidate molecules for testing may also be very useful when integrated with HTS. However, there are to date few studies that directly compare the performance of VS and HTS, and further validation is required.

Despite all these developments, current screening throughput is still far from adequate. Recent estimates of the number of individual genes in the human genome (~30,000) and the number of unique chemical structures theoretically attainable using existing chemistries suggests that an enormous number of assays would be required to completely map the structure-activity space for all potential therapeutic targets (Burbaum, 1998).

Hence, the provision of a method which permits screening vast numbers ($\geq 10^{10}$) of compounds quickly, using reaction volumes of only a few femtolitres, and at very low cost would be of enormous utility in the generation of novel drug leads.

Tawfik and Griffiths (1998), and International patent application PCT/GB98/01889, describe a system for *in vitro* evolution using compartmentalisation in microcapsules to link genotype and phenotype at the molecular level. In Tawfik and Griffiths (1998), and in several embodiments of International patent application PCT/GB98/01889, the desired activity of a gene product results in a modification of the genetic element which encoded it (and is present in the same microcapsule). The modified genetic element can then be selected in a subsequent step.

SUMMARY OF THE INVENTION

We have now developed a methodology for selection of compounds, not encoded by genetic elements, using a compartmentalised microcapsule system based on that described in Griffiths & Tawfik (1998). The novel method according to the present invention permits the rapid, high-throughput screening of compounds for activity against a target at low cost in a manner compatible with modern HTS approaches.

In a first aspect, there is provided a method for identifying a compound or compounds in a repertoire of compounds, which compound or compound(s) possess(es) a desired activity, comprising the steps of:

a) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;

b) identifying the compounds which possess the desired activity.

Preferably, the desired activity is selected from the group consisting of a binding activity and the modulation of the activity of a target. The target is advantageously compartmentalised into microcapsules together with the compound(s), allowing the activity of the compound(s) on the target to be measured within the microcapsule which links the target and the compound together.

Preferably, the subset of the repertoire present in any one microcapsule is a single compound. Each microcapsule contains multiple molecules of the subset of the repertoire, which is advantageously multiple copies of a single compound.

Compounds can be screened in accordance with the invention by screening for a change in a microcapsule containing a compound. In a preferred embodiment, the microcapsules are modified by the action of the compound(s) such as to change their optical properties.

The change in optical properties of the microcapsule can be due to a change in the optical properties of the compound when bound to target or to a change in the optical properties of the target when bound by the compound. Moreover, the change in optical properties of the microcapsule can be due to a change in the optical properties of both target and compound on binding.

The change in the optical properties of the microcapsule may be due to modulation of the activity of the target by the compound. The compound may activate or inhibit the activity of the target. For example, if the target is an enzyme, the substrate and the product of the reaction catalysed by the target can have different optical properties. Advantageously, the substrate and product have different fluorescence properties.

It is to be understood that the detected change in the microcapsule may be caused by the direct action of the compound, or indirect action, in which a series of reactions, one or more of which involve the compound having the desired activity leads to the detected change.

The compounds in a microcapsule can be identified using a variety of techniques familiar to those skilled in the art, including mass spectroscopy, chemical tagging or optical tagging. Advantageously, the compounds are contained in optically tagged microcapsules to enable the identification of the microcapsule and the compound contained in it.

Advantageously, the microcapsules are analysed by detection of a change in their fluorescence. For example, microcapsules can be analysed by flow cytometry and, optionally sorted using a fluorescence activated cell sorter (FACS). The different fluorescence properties of the target and the product can be due to fluorescence resonance energy transfer (FRET).

In a further embodiment, the internal environment of the microcapsules can be modified by the addition of one or more reagents to the oil phase. This allows reagents to be diffused in to the microcapsules during the reaction, if necessary.

According to a preferred implementation of the present invention, the compounds may be screened according to an activity of the compound or derivative thereof which makes the microcapsule detectable as a whole. Accordingly, the invention provides a method wherein a compound with the desired activity induces a change in the microcapsule, or a modification of one or more molecules within the microcapsule, which enables the microcapsule containing the compound to be identified. In this embodiment, therefore, the microcapsules are either: (a) physically sorted from each other according to the activity of the compound(s) contained therein, and the contents of the sorted microcapsules analysed to determine the identity of the compound(s) which they contain; or (b) analysed directly without sorting to determine the identity of the compound(s) which the microcapsules contain.

Preferably, microencapsulation is achieved by forming a water-in-oil emulsion.

Compartmentalisation of a subset of a repertoire in multiple copies may be achieved in a number of ways. For example, compounds may be attached to beads, and the emulsion formed such that substantially only a single bead is included in each compartment. Step (a) above is thus modified, such that it comprises

a) attaching the repertoire of compounds onto microbeads, such that only a subset of the repertoire is represented on any one microbead;

b) compartmentalising the microbeads into microcapsules;

such that a subset of the repertoire is represented in multiple copies in any one microcapsule.

Thus, in a further aspect, there is provided a method for identifying a compound or compounds in a repertoire of compounds, which compound or compound(s) possess(es) a desired activity, comprising the steps of:

4. attaching the repertoire of compounds onto microbeads, such that only a subset of the repertoire is represented on any one microbead;
5. compartmentalising the microbeads into microcapsules;
6. optionally, releasing compounds from the microbeads; and
7. identifying the compounds which possess the desired activity.

Preferably, the subset of the repertoire present on any one microbead is a single compound. Advantageously, each microbead has attached thereto multiple molecules of a single compound.

Preferably, compounds are attached to microbeads by means of cleavable linkers, for example photocleavable linkers, which permit the release of the compound from the microbead if desired.

Compounds can be screened in accordance with the invention by screening either for a change in a microcapsule containing a compound or a change in or on a microbead to which a compound is attached.

The compounds on beads can be identified using a variety of techniques familiar to those skilled in the art, including mass spectroscopy, chemical tagging or optical tagging. Advantageously, the compounds are coupled to optically tagged microbeads to enable the identification of the bead and the compound coupled to it in step (d).

Repertoires may also be emulsified in different ways to achieve encapsulation of multiple copies of a single compound.

For example, an aqeous solution of each compound can be compartmentalised in the microcapsules of a water-in-oil emulsion and then aliquots of the emulsions containing the different compounds mixed. Microcapsules can normally also contain a target and the activity of the target can be suppressed by keeping the microcapsules cold.

In a further embodiment, small aliquots of an aqueous solution of each compound can be deposited into an oil phase (advantageously containing surfactants and/or other stabilising

molecules) whilst applying mechanical energy, thereby dispersing each compound into multiple aqueous microcapsules, each of which contains (for the most part) a single sort of compound but multiple copies thereof. Advantageously, the compounds can be deposited into the oil phase in the form of droplets generated using inkjet printing technology (Calvert, 2001; de Gans et al., 2004), and more advantageously by piezoelectric drop-on-demand (DOD) inkjet printing technology. Inkjet printing technology can also be used to mix reagents (e.g. the compound, the target and reagents to assay target activity) immediately prior to forming the emulsion. Advantageously, multiple compounds can be mixed with multiple targets in a combinatorial manner. This approach achieves a similar result, forming individual emulsions in situ in the oil phase from the aqueous droplets, which are subsequently mixed.

Thus, step (a) above can be modified such that it comprises forming separate emulsion compartments containing individual compounds and mixing the emulsion compartments to form an emulsified compound repertoire wherein a subset of the repertoire is represented in multiple copies in any one microcapsule.

Moreover, compound libraries can be be compartmentalised in highly monodisperse microcapsules produced using microfluidic techniques. For example, aliquots of each compound can be compartmentalised into one or more aqueous microcapsules (with less than 3% polydispersity) in water-in-oil emulsions created by droplet break off in a co-flowing steam of oil (Umbanhowar et al., 2000). Advantageously, the aqueous microcapsules are then transported by laminar-flow in a stream of oil in microfluidic channels (Thorsen et al., 2001). These microcapsules containing single compounds can, optionally, be split into two or more smaller microcapsules using microfluidics (Link et al., 2004; Song et al., 2003). The microcapsules containing single compounds can, optionally be fused with other microcapsules (Song et al., 2003) containing a target. A single microcapsule containing a target can, optionally, be split into two or more smaller microcapsules which can subsequently be fused with microcapsules containing different compounds, or compounds at different concentrations. Advantageously, a compound and a target can be mixed by microcapsule fusion prior to a second microcapsule fusion which delivers the necessary to assay the activity of the target (e.g. the substrate for the target if the target is an enzyme). This allows time for the compound to bind to the target. The

microcapsules can be analysed and, optionally, sorted using microfluidic devices (Fu et al., 2002).

According to a preferred implementation of the present invention, the screening of compounds may be performed by, for example:

(I) In a first embodiment, the microcapsules are screened according to an activity of the compound or derivative thereof which makes the microcapsule detectable as a whole. Accordingly, the invention provides a method wherein a compound with the desired activity induces a change in the microcapsule, or a modification of one or more molecules within the microcapsule, which enables the microcapsule containing the compound to be indentified. In this embodiment, therefore, the microcapsules are either: (a) physically sorted from each other according to the activity of the compound(s) contained therein, the contents of the sorted microcapsules optionally pooled into one or more common compartments, and the microcapsule contents analysed to determine the identity of the compound(s); or (b) analysed directly without sorting to determine the identity of the compound(s) which the microcapsules contained. Where the microcapsule contains microbeads, the microbeads can be analysed to determine the compounds with which they are coated.

(II) In a second embodiment, microbeads are analysed following pooling of the microcapsules into one or more common compartments. In this embodiment, a compound having the desired activity modifies the microbead which carried it (and which resides in the same microcapsule) in such a way as to make it identifiable in a subsequent step. The reactions are stopped and the microcapsules are then broken so that all the contents of the individual microcapsules are pooled. Modified microbeads are identified and either: (a) physically sorted from each other according to the activity of the compound(s) coated on the microbeads, and the sorted microbeads analysed to determine the identity of the compound(s) with which they are/were coated; or (b) analysed directly without sorting to determine the identity of the compound(s) with which the microbeads are/were coated. It is to be understood, of course, that modification of the microbead may be direct, in that it is caused by the direct action of the compound, or indirect, in which a series of reactions, one or more of which involve the compound having the desired

activity, leads to modification of the microbead. Advantageously, the target is bound to the microbead and is a ligand and the compound within the microcapsule binds, directly or indirectly, to said ligand to enable the isolation of the microbead. In another configuration, a substrate for the target is and is bound to the microbead, and the activity of the compound within the microcapsule results, directly or indirectly, in the conversion of said substrate into a product which remains part of the microbead and enables its isolation. Alternatively, the activity of the compound may prevent or inhibit the conversion of said substrate into product. Moreover, the product of the activity of the compound within the microcapsule can result, directly or indirectly, in the generation of a product which is subsequently complexed with the microbead and enables its identification.

(III) In a third embodiment, the microbeads are analysed following pooling of the microcapsules into one or more common compartments. In this embodiment, a compound with a desired activity induces a change in the microcapsule containing the compound and the microbead which carries it. This change, when detected, triggers the modification of the microbead within the compartment. The reactions are stopped and the microcapsules are then broken so that all the contents of the individual microcapsules are pooled. Modified microbeads are identified and either: (a) physically sorted from each other according to the activity of the compound(s) coated on the microbeads, and the sorted microbeads analysed to determine the identity of the compound(s) with which they are/were coated; or (b) analysed directly without sorting to determine the identity of the compound(s) with which the microbeads are/were coated.

The microcapsules or microbeads may be modified by the action of the compound(s) such as to change their optical properties. For example, the modification of the microbead can enable it to be further modified outside the microcapsule so as to induce a change in its optical properties.

In another embodiment, the change in optical properties of the microcapsule or microbead is due to binding of a compound with distinctive optical properties to the target.

Moreover, the change in optical properties of the microcapsule or microbead can be due to binding of a target with distinctive optical properties by the compound.

The change in optical properties of the microcapsule or microbead can be due to a change in the optical properties of the compound when bound to target or to a change in the optical properties of the target when bound by the compound. Moreover, the change in optical properties of the microcapsule or microbead can be due to a change in the optical properties of both target and compound on binding.

The change in the optical properties of the microcapsule may be due to modulation of the activity of the target by the compound. The compound may activate or inhibit the activity of the target. For example, if the target is an enzyme, the substrate and the product of the reaction catalysed by the target can have different optical properties. Advantageously, the substrate and product have different fluorescence properties. In the case where the microcapsules contain microbeads, both the substrate and the product can have similar optical properties, but only the product of the reaction, and not the substrate, binds to, or reacts with, the microbead, thereby changing the optical properties of the microbead.

In a further configuration, further reagents specifically bind to, or specifically react with, the product (and not the substrate) attached to or contained in the microcapsule or microbead, thereby altering the optical properties of the microcapsule or microbead.

Advantageously, microcapsules or microbeads are modified directly or indirectly by the activity of the compound are further modified by Tyramide Signal Amplification (TSA™; NEN), resulting directly or indirectly in a change in the optical properties of said microcapsules or microbeads thereby enabling their separation.

Where the compounds are attached to beads, the density with which compounds are coated onto the microbeads, combined with the size of the microcapsule will determine the concentration of the compound in the microcapsule. High compound coating densities and small microcapsules will both give higher compound concentrations which may be advantageous for the selection of molecules with a low affinity for the target. Conversely, low compound coating densities and large microcapsules will both give lower compound

concentrations which may be advantageous for the selection of molecules with a high affinity for the target.

The microbead can be nonmagnetic, magnetic or paramagnetic.

Advantageously, the microcapsules or microbeads are analysed by detection of a change in their fluorescence. For example, microbeads can be analysed by flow cytometry and, optionally sorted using a fluorescence activated cell sorter (FACS). The different fluorescence properties of the target and the product can be due to fluorescence resonance energy transfer (FRET).

The invention also provides for a product when identified according to the invention. As used in this context, a "product" may refer to any compound, selectable according to the invention.

Further embodiments of the invention are described in the detailed description below and in the accompanying claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Examples of PTP1B inhibitors. Compounds with a *bis*-difluoromethylene phosphonate moiety (e.g. **2**) have significantly more potency than those with a single moiety (e.g. **1**).

Figure 2. Screening PTP1B inhibitors using microencapsulation. Polystyrene beads with surface carboxylate groups, died with orange or red fluorochromes (Fulton et al., 1997), are derivatised with a phosphopeptide PTP1B substrate, and either PTP1B inhibitors or non-inhibitory compounds attached *via* a cleavable linker (1). After mixing the beads, single beads and target enzyme (PTP1B) are colocalised in a microcompartment by forming a water-in-oil emulsion (2). The compound is released photochemically (3). Inhibitors reduce the amount of substrate converted to product (dephosphorylated peptide) (4). The enzyme reaction is stopped and the emulsion is broken (5). After labelling with green fluorescent anti-substrate antibodies, beads are

analysed by 3-colour flow cytometry to simultaneously determine extent of inhibition and the compound on the beads (6). Ultimately, compound libraries will be coupled to optically tagged beads (see below) and rapidly decoded by flow cytometry (at up to 100,000 beads s-1). Hit compounds can be re-synthesised for further characterisation (7) or elaborated and rescreened in a process of synthetic evolution (8).

Figure 3. Compartmentalisation of small molecules in water-in-fluorocarbon emulsions. Water-in-perfluorooctyl bromide emulsions were made containing texas red (1 mM) and calcein (1 mM) in the aqueous phase by homogenisation as described in example 9. The two emulsions were mixed by vortexing and imaged by epifluorescence microscopy after 24 hours. No exchange of texas-red (red fluorescence) and calcein (green fluorescence) between microdroplets could be observed.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "microcapsule" is used herein in accordance with the meaning normally assigned thereto in the art and further described hereinbelow. In essence, however, a microcapsule is an artificial compartment whose delimiting borders restrict the exchange of the components of the molecular mechanisms described herein which allow the identification of the molecule with the desired activity. The delimiting borders preferably completely enclose the contents of the microcapsule. Preferably, the microcapsules used in the method of the present invention will be capable of being produced in very large numbers, and thereby to compartmentalise a library of compounds. Optionally, the compounds can be attached to microbeads. The microcapsules used herein allow mixing and sorting to be performed thereon, in order to facilitate the high throughput potential of the methods of the invention. Arrays of liquid droplets on solid surfaces, and multiwell plates, are not microcapsules as defined herein.

The term "microbead" is used herein in accordance with the meaning normally assigned thereto in the art and further described hereinbelow. Microbeads, are also known by those skilled in the art as microspheres, latex particles, beads, or minibeads, are available in

diameters from 20 nm to 1 mm and can be made from a variety of materials including silica and a variety of polymers, copolymers and terpolymers. Highly uniform derivatised and non-derivatised nonmagnetic and paramagnetic microparticles (beads) are commercially available from many sources (e.g. Sigma, Bangs Laboratories, Luminex and Molecular Probes) (Fornusek and Vetvicka, 1986).

Microbeads can be "compartmentalised" in accordance with the present invention by distribution into microcapsules. For example, in a preferred aspect the microbeads can be placed in a water/oil mixture and emulsified to form a water-in-oil emulsion comprising microcapsules according to the invention. The concentration of the microbeads can be adjusted such that a single microbead, on average, appears in each microcapsule.

The term "compound" is used herein in accordance with the meaning normally assigned thereto in the art. The term compound is used in its broadest sense i.e. a substance comprising two or more elements in fixed proportions, including molecules and supramolecular complexes. This definition includes small molecules (typically <500 Daltons) which make up the majority of pharmaceuticals. However, the definition also includes larger molecules, including polymers, for example polypeptides, nucleic acids and carbohydrates, and supramolecular complexes thereof.

A "repertoire" of compounds is a group of diverse compounds, which may also be referred to as a library of compounds. Repertoires of compounds may be generated by any means known in the art, including combinatorial chemistry, compound evolution, such as by the method of our copending UK patent application entitled "Compartmentalised Combinatorial Chemistry" filed on even date herewith, or purchased from commercial sources such as Sigma Aldrich, Discovery Partners International, Maybridge and Tripos. A repertoire advantageously comprises at least 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} or more different compounds, which may be related or unrelated in structure or function.

A "subset" of a repertoire is a part thereof, which may be a single compound or a group of compounds having related or unrelated structures. Advantageously, the subset is a single compound. Preferably, multiple copies of each compound are encapsulated in a

microcapsule. Subsets of the repertoire, which may be attached to microbeads, are advantageously attached in multiple copies of each compound; for example, where each microbead has attached thereto only one compound, multiple molecules of that compound are attached to said microbead. The amount of compound attached to the microbead will determine the concentration of the compound in the microcapsule.

Compounds can be "released" from a microbead by cleavage of a linker which effects the attachment of the compound to the microbead. Release of the compounds from the microbead allows the compounds to interact more freely with other contents of the microcapsule, and to be involved in reactions therein and optionally to become combined with other reagents to form new compounds, complexes, molecules or supramolecular complexes. Cleavage of linkers can be performed by any means, with means such as photochemical cleavage which can be effected from without the microcapsule being preferred. Photochemically cleavable linkers are known in the art (see for example (Gordon and Balasubramanian, 1999)) and further described below.

As used herein, the "target" is any compound, molecule, or supramolecular complex. Typical targets include targets of medical significance, including drug targets such as receptors, for example G protein coupled receptors and hormone receptors; transcription factors, protein kinases and phosphatases involved in signalling pathways; gene products specific to microorganisms, such as components of cell walls, replicases and other enzymes; industrially relevant targets, such as enzymes used in the food industry, reagents intended for research or production purposes, and the like.

A "desired activity", as referred to herein, is the modulation of any activity of a target, or an activity of a molecule which is influenced by the target, which is modulatable directly or indirectly by a compound or compounds as assayed herein. The activity of the target may be any measurable biological or chemical activity, including binding activity, an enzymatic activity, an activating or inhibitory activity on a third enzyme or other molecule, the ability to cause disease or influence metabolism or other functions, and the like. Activation and inhibition, as referred to herein, denote the increase or decrease of a desired activity 1.5 fold, 2 fold, 3 fold, 4 fold, 5 fold, 10 fold, 100 fold or more. Where the modulation is inactivation, the inactivation can be substantially complete inactivation.

The desired activity may moreover be purely a binding activity, which may or may not involve the modulation of the activity of the target bound to.

A compound defined herein as "low molecular weight" or a "small molecule" is a molecule commonly referred to in the pharmaceutical arts as a "small molecule". Such compounds are smaller than polypeptides and other, large molecular complexes and can be easily administered to and assimilated by patients and other subjects. Small molecule drugs can advantageously be formulated for oral administration or intramuscular injection. For example, a small molecule may have a molecular weight of up to 2000 Dalton; preferably up to 1000 Dalton; advantageously between 250 and 750 Dalton; and more preferably less than 500 Dalton.

A "selectable change" is any change which can be measured and acted upon to identify or isolate the compound which causes it. The selection may take place at the level of the microcapsule, the microbead, or the compound itself, optionally when complexed with another reagent. A particularly advantageous embodiment is optical detection, in which the selectable change is a change in optical properties, which can be detected and acted upon for instance in a FACS device to separate microcapsules or microbeads displaying the desired change.

As used herein, a change in optical properties refers to any change in absorption or emission of electromagnetic radiation, including changes in absorbance, luminescence, phosphorescence or fluorescence. All such properties are included in the term "optical". Microcapsules or microbeads can be identified and, optionally, sorted, for example, by luminescence, fluorescence or phosphorescence activated sorting. In a preferred embodiment, flow cytometry is employed to identify and, optionally, sort microcapsules or microbeads. A variety of optical properties can be used for analysis and to trigger sorting, including light scattering (Kerker, 1983) and fluorescence polarisation (Rolland et al., 1985). In a highly preferred embodiment microcapsules or microbeads are analysed and, optionally, sorted using a fluorescence activated cell sorter (FACS) (Norman, 1980; Mackenzie and Pinder, 1986).

The compounds in microcapsules or on beads can be identified using a variety of techniques familiar to those skilled in the art, including mass spectroscopy, chemical tagging or optical tagging.

General Techniques

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods. In addition Harlow & Lane, A Laboratory Manual Cold Spring Harbor, N.Y, is referred to for standard Immunological Techniques.

(A) GENERAL DESCRIPTION

The microcapsules of the present invention require appropriate physical properties to allow the working of the invention.

First, to ensure that the compounds and the target may not diffuse between microcapsules, the contents of each microcapsule must be isolated from the contents of the surrounding microcapsules, so that there is no or little exchange of compounds and target between the microcapsules over the timescale of the experiment. However, the permeability of the microcapsules may be adjusted such that reagents may be allowed to diffuse into and/or out of the microcapsules if desired.

Second, the method of the present invention requires that there are only a limited number of different compounds per microcapsule. In the case that compounds are attached to beads, the method of the present invention requires that there are only a limited number of beads per microcapsule.

Third, the formation and the composition of the microcapsules advantageously does not abolish the activity of the target.

Consequently, any microencapsulation system used preferably fulfils these three requirements. The appropriate system(s) may vary depending on the precise nature of the requirements in each application of the invention, as will be apparent to the skilled person.

A wide variety of microencapsulation procedures are available (see Benita, 1996) and may be used to create the microcapsules used in accordance with the present invention. Indeed, more than 200 microencapsulation methods have been identified in the literature (Finch, 1993).

These include membrane enveloped aqueous vesicles such as lipid vesicles (liposomes) (New, 1990) and non-ionic surfactant vesicles (van Hal et al., 1996). These are closed-membranous capsules of single or multiple bilayers of non-covalently assembled molecules, with each bilayer separated from its neighbour by an aqueous compartment. In the case of liposomes the membrane is composed of lipid molecules; these are usually phospholipids but sterols such as cholesterol may also be incorporated into the membranes (New, 1990). A variety of enzyme-catalysed biochemical reactions, including RNA and DNA polymerisation, can be performed within liposomes (Chakrabarti et al., 1994; Oberholzer et al., 1995a; Oberholzer et al., 1995b; Walde et al., 1994; Wick & Luisi, 1996).

With a membrane-enveloped vesicle system much of the aqueous phase is outside the vesicles and is therefore non-compartmentalised. This continuous, aqueous phase should be removed or the biological systems in it inhibited or destroyed in order that the reactions are limited to the microcapsules (Luisi et al., 1987).

Enzyme-catalysed biochemical reactions have also been demonstrated in microcapsules generated by a variety of other methods. Many enzymes are active in reverse micellar solutions (Bru & Walde, 1991; Bru & Walde, 1993; Creagh et al., 1993; Haber et al., 1993; Kumar et al., 1989; Luisi & B., 1987; Mao & Walde, 1991; Mao et al., 1992; Perez

et al., 1992; Walde et al., 1994; Walde et al., 1993; Walde et al., 1988) such as the AOT-isooctane-water system (Menger & Yamada, 1979).

Microcapsules can also be generated by interfacial polymerisation and interfacial complexation (Whateley, 1996). Microcapsules of this sort can have rigid, nonpermeable membranes, or semipermeable membranes. Semipermeable microcapsules bordered by cellulose nitrate membranes, polyamide membranes and lipid-polyamide membranes can all support biochemical reactions, including multienzyme systems (Chang, 1987; Chang, 1992; Lim, 1984). Alginate/polylysine microcapsules (Lim & Sun, 1980), which can be formed under very mild conditions, have also proven to be very biocompatible, providing, for example, an effective method of encapsulating living cells and tissues (Chang, 1992; Sun et al., 1992).

Non-membranous microencapsulation systems based on phase partitioning of an aqueous environment in a colloidal system, such as an emulsion, may also be used.

Preferably, the microcapsules of the present invention are formed from emulsions; heterogeneous systems of two immiscible liquid phases with one of the phases dispersed in the other as droplets of microscopic or colloidal size (Becher, 1957; Sherman, 1968; Lissant, 1974; Lissant, 1984).

Emulsions may be produced from any suitable combination of immiscible liquids. Preferably the emulsion of the present invention has water (containing the biochemical components) as the phase present in the form of finely divided droplets (the disperse, internal or discontinuous phase) and a hydrophobic, immiscible liquid (an 'oil') as the matrix in which these droplets are suspended (the nondisperse, continuous or external phase). Such emulsions are termed 'water-in-oil' (W/O). This has the advantage that the entire aqueous phase containing the biochemical components is compartmentalised in discreet droplets (the internal phase). The external phase, being a hydrophobic oil, generally contains none of the biochemical components and hence is inert.

The emulsion may be stabilised by addition of one or more surface-active agents (surfactants). These surfactants are termed emulsifying agents and act at the water/oil

interface to prevent (or at least delay) separation of the phases. Many oils and many emulsifiers can be used for the generation of water-in-oil emulsions; a recent compilation listed over 16,000 surfactants, many of which are used as emulsifying agents (Ash and Ash, 1993). Suitable oils include light white mineral oil and decane. Suitable surfactants include: non-ionic surfactants (Schick, 1966) such as sorbitan monooleate (Span™80; ICI), sorbitan monostearate (Span™60; ICI), polyoxyethylenesorbitan monooleate (Tween™ 80; ICI), and octylphenoxyethoxyethanol (Triton X-100); ionic surfactants such as sodium cholate and sodium taurocholate and sodium deoxycholate; chemically inert silicone-based surfactants such as polysiloxane-polycetyl-polyethylene glycol copolymer (Cetyl Dimethicone Copolyol) (e.g. Abil™EM90; Goldschmidt); and cholesterol.

Emulsions with a fluorocarbon (or perfluorocarbon) continuous phase (Krafft et al., 2003; Riess, 2002) may be particularly advantageous. For example, stable water-in-perfluorooctyl bromide and water-in-perfluorooctylethane emulsions can be formed using F-alkyl dimorpholinophosphates as surfactants (Sadtler et al., 1996). Non-fluorinated compounds are essentially insoluble in fluorocarbons and perfluorocarbons (Curran, 1998; Hildebrand and Cochran, 1949; Hudlicky, 1992; Scott, 1948; Studer et al., 1997) and small drug-like molecules (typically <500 Da and Log P<5) (Lipinski et al., 2001) are compartmentalised very effectively in the aqueous microcapsules of water-in-fluorocarbon and water-in-perfluorocarbon emulsions – with little or no exchange between microcapsules.

Creation of an emulsion generally requires the application of mechanical energy to force the phases together. There are a variety of ways of doing this which utilise a variety of mechanical devices, including stirrers (such as magnetic stir-bars, propeller and turbine stirrers, paddle devices and whisks), homogenisers (including rotor-stator homogenisers, high-pressure valve homogenisers and jet homogenisers), colloid mills, ultrasound and 'membrane emulsification' devices (Becher, 1957; Dickinson, 1994).

Complicated biochemical processes, notably gene transcription and translation are also active in aqueous microcapsules formed in water-in-oil emulsions. This has enabled compartmentalisation in water-in-oil emulsions to be used for the selection of genes,

which are transcribed and translated in emulsion microcapsules and selected by the binding or catalytic activities of the proteins they encode (Doi and Yanagawa, 1999; Griffiths and Tawfik, 2003; Lee et al., 2002; Sepp et al., 2002; Tawfik and Griffiths, 1998). This was possible because the aqueous microcapsules formed in the emulsion were generally stable with little if any exchange of nucleic acids, proteins, or the products of enzyme catalysed reactions between microcapsules.

The technology exists to create emulsions with volumes all the way up to industrial scales of thousands of litres (Becher, 1957; Sherman, 1968; Lissant, 1974; Lissant, 1984).

The preferred microcapsule size will vary depending upon the precise requirements of any individual screening process that is to be performed according to the present invention. In all cases, there will be an optimal balance between the size of the compound library and the sensitivities of the assays to determine the identity of the compound and target activity.

The size of emulsion microcapsules may be varied simply by tailoring the emulsion conditions used to form the emulsion according to requirements of the screening system. The larger the microcapsule size, the larger is the volume that will be required to encapsulate a given compound library, since the ultimately limiting factor will be the size of the microcapsule and thus the number of microcapsules possible per unit volume.

Water-in-oil emulsions can be re-emulsified to create water-in-oil-in water double emulsions with an external (continuous) aqueous phase. These double emulsions can be analysed and , optionally, sorted using a flow cytometer (Bernath et al., 2004).

Highly monodisperse microcapsules can be produced using microfluidic techniques. For example, water-in-oil emulsions with less than 3% polydispersity can be generated by droplet break off in a co-flowing steam of oil (Umbanhowar et al., 2000). Microfluidic systems can also be used for laminar-flow of aqueous microdroplets dispersed in a stream of oil in microfluidic channels (Thorsen et al., 2001). This allows the construction of microfluidic devices for flow analysis and, optionally, flow sorting of microdroplets (Fu et al., 2002).

Microcapsules can, advantageously, be fused or split. For example, aqueous microdroplets can be merged and split using microfluidics systems (Link et al., 2004; Song et al., 2003). Microcapsule fusion allows the mixing of reagents. Fusion, for example, of a microcapsule containing the target with a microcapsule containing the compound could initiate the reaction between target and compound. Microcapsule splitting allows single microcapsules to be split into two or more smaller microcapsules. For example a single microcapsule containing a compound can be split into multiple microcapsules which can then each be fused with a different microcapsule containing a different target. A single microcapsule containing a target can also be split into multiple microcapsules which can then each be fused with a different microcapsule containing a different compound, or compounds at different concentrations.

Microcapsules can be optically tagged by, for example, incorporating fluorochromes. In a preferred configuration, the microcapsules are optically tagged by incorporating quantum dots: quantum dots of 6 colours at 10 concentrations would allow the encoding of 10^6 microcapsules (Han et al., 2001). Microcapsules flowing in an ordered sequence in a microfluidic channel can be encoded (wholly or partially) by their sequence in the stream of microcapsules (positional encoding).

Microbeads, also known by those skilled in the art as microspheres, latex particles, beads, or minibeads, are available in diameters from 20 nm to 1 mm and can be made from a variety of materials including silica and a variety of polymers, copolymers and terpolymers including polystyrene (PS), polymethylmethacrylate (PMMA), polyvinyltoluene (PVT), styrene/butadiene (S/B) copolymer, and styrene/vinyltoluene (S/VT) copolymer (www.bangslabs.com). They are available with a variety of surface chemistries from hydrophobic surfaces (e.g. plain polystyrene), to very hydrophilic surfaces imparted by a wide variety of functional surface groups: aldehyde, aliphatic amine, amide, aromatic amine, carboxylic acid, chloromethyl, epoxy, hydrazide, hydroxyl, sulfonate and tosyl. The functional groups permit a wide range of covalent coupling reactions for stable or reversible attachment of compounds to the microbead surface.

Microbeads can be optically tagged by, for example, incorporating fluorochromes. For example, one hundred different bead sets have been created, each with a unique spectral address due to labelling with precise ratios of red (>650 nm) and orange (585 nm) fluorochromes (Fulton et al., 1997) (www.luminex.com) and sets of up to 10^6 beads can be encoded by incorporating quantum dots of 10 intensities and 6 colours (Han et al., 2001).

The compounds can be connected to the microbeads either covalently or non-covalently by a variety of means that will be familiar to those skilled in the art (see, for example, (Hermanson, 1996)). Advantageously, the compounds are attached *via* a cleavable linker. A variety of such linkers are familiar to those skilled in the art (see for example (Gordon and Balasubramanian, 1999)), including for example, linkers which can be cleaved photochemically and reversible covalent bonds which can be controlled by changing the pH (e.g. imines and acylhydrazones), by adjusting the oxido-reductive properties (e.g. disulphides), or using an external catalyst (e.g. cross-metathesis and transamidation).

The method of the present invention permits the identification of compounds which modulate the activity of the target in a desired way in pools (libraries or repertoires) of compounds.

The method of the present invention is useful for screening repertoires or libraries of compounds. The invention accordingly provides a method according to preceding aspects of the invention, wherein the compounds are identified from a library of compounds.

The compounds identified according to the invention are advantageously of pharmacological or industrial interest, including activators or inhibitors of biological systems, such as cellular signal transduction mechanisms suitable for diagnostic and therapeutic applications. In a preferred aspect, therefore, the invention permits the identification of clinically or industrially useful products. In a further aspect of the invention, there is provided a product when isolated by the method of the invention.

The selection of suitable encapsulation conditions is desirable. Depending on the complexity and size of the compound library to be screened, it may be beneficial to set up

the encapsulation procedure such that one compound (or one or less than one microbead) is encapsulated per microcapsule. This will provide the greatest power of resolution. Where the library is larger and/or more complex, however, this may be impracticable; it may be preferable to encapsulate several compounds (or several microbeads) together and rely on repeated application of the method of the invention to identify the desired compound. A combination of encapsulation procedures may be used to identify the desired compound.

Theoretical studies indicate that the larger the number of compounds created the more likely it is that a compound will be created with the properties desired (see (Perelson and Oster, 1979) for a description of how this applies to repertoires of antibodies). It has also been confirmed practically that larger phage-antibody repertoires do indeed give rise to more antibodies with better binding affinities than smaller repertoires (Griffiths et al., 1994). To ensure that rare variants are generated and thus are capable of being identified, a large library size is desirable. Thus, the use of optimally small microcapsules is beneficial.

The largest repertoires of compounds that can be screened in a single experiment to date, using two dimensional microarrays of 1 nl volume spots, is ~10^3 (Hergenrother et al., 2000). Using the present invention, at a microcapsule diameter of 2.6 mm (Tawfik and Griffiths, 1998), by forming a three-dimensional dispersion, a repertoire size of at least 10^{11} can be screened using 1ml aqueous phase in a 20 ml emulsion.

In addition to the compounds, or microbeads coated with compounds, described above, the microcapsules according to the invention will comprise further components required for the screening process to take place. They will comprise the target and a suitable buffer. A suitable buffer will be one in which all of the desired components of the biological system are active and will therefore depend upon the requirements of each specific reaction system. Buffers suitable for biological and/or chemical reactions are known in the art and recipes provided in various laboratory texts, such as (Sambrook and Russell, 2001).

Other components of the system will comprise those necessary for assaying the activity of the target. These may for example comprise substrate(s) and cofactor(s) for a reaction catalysed by the target, and ligand(s) bound by the target. They may also comprise other catalysts (including enzymes), substrates and cofactors for reactions coupled to the activity of the target which allow for the activity of the target to be detected.

(B) SCREENING PROCEDURES

To screen compounds which bind to or modulate the activity of a target, the target is compartmentalised in microcapsules together with one or more compounds or compound-coated microbeads. Advantageously each microcapsule contains only a single sort of compound, but many copies thereof. Advantageously each microbead is coated with only a single sort of compound, but many copies thereof. Advantageously the compounds are connected to the microbeads *via* a cleavable linker, allowing them to be released from the microbeads in the compartments. Advantageously, each microcapsule or microbead is optically tagged to allow identification of the compounds contained within the microcapsule of attached to the microbead.

(i) SCREENING FOR BINDING

Compounds can be screened directly for binding to a target. In this embodiment, if the compound is attached to a microbead and has affinity for the target it will be bound by the target. At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds exhibiting the desired binding can be selected by affinity purification using a molecule that specifically binds to, or reacts specifically with, the target.

In an alternative embodiment, the target can be attached to microbeads by a variety of means familiar to those skilled in the art (see for example (Hermanson, 1996)). The compounds to be screened contain a common feature - a tag. The compounds are released from the microbeads and if the compound has affinity for the target, it will bind to it. At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds exhibiting the desired binding can be selected by affinity purification using a molecule that specifically binds to, or reacts specifically with, the "tag".

In an alternative embodiment, microbeads may be screened on the basis that the compound, which binds to the target, merely hides the ligand from, for example, further binding partners. In this eventuality, the microbead, rather than being retained during an affinity purification step, may be selectively eluted whilst other microbeads are bound.

Sorting by affinity is dependent on the presence of two members of a binding pair in such conditions that binding may occur. Any binding pair may be used for this purpose. As used herein, the term binding pair refers to any pair of molecules capable of binding to one another. Examples of binding pairs that may be used in the present invention include an antigen and an antibody or fragment thereof capable of binding the antigen, the biotin-avidin/streptavidin pair (Savage et al., 1994), a calcium-dependent binding polypeptide and ligand thereof (e.g. calmodulin and a calmodulin-binding peptide (Montigiani et al., 1996; Stofko et al., 1992), pairs of polypeptides which assemble to form a leucine zipper (Tripet et al., 1996), histidines (typically hexahistidine peptides) and chelated Cu^{2+}, Zn^{2+} and Ni^{2+}, (e.g. Ni-NTA; (Hochuli et al., 1987)), RNA-binding and DNA-binding proteins (Klug, 1995) including those containing zinc-finger motifs (Klug and Schwabe, 1995) and DNA methyltransferases (Anderson, 1993), and their nucleic acid binding sites.

In an alternative embodiment, compounds can be screened for binding to a target using a change in the optical properties of the microcapsule or the microbead.

The change in optical properties of the microcapsule or the microbead after binding of the compound to the target may be induced in a variety of ways, including:

(1) the compound itself may have distinctive optical properties, for example, it is fluorescent

(2) the optical properties of the compound may be modified on binding to the target, for example, the fluorescence of the compound is quenched or enhanced on binding (Voss, 1993; Masui and Kuramitsu, 1998).

(3) the optical properties of the target may be modified on binding of the compound, for example, the fluorescence of the target is quenched or enhanced on binding (Guixe et al., 1998; Qi and Grabowski, 1998)

(4) the optical properties of both target and compound are modified on binding, for example, there can be a fluorescence resonance energy transfer (FRET) from target to compound (or *vice versa*) resulting in emission at the "acceptor" emission wavelength when excitation is at the "donor" absorption wavelength (Heim & Tsien, 1996; Mahajan *et al.*, 1998; Miyawaki *et al.*, 1997).

The invention provides a method wherein a compound with the desired activity induces a change in the optical properties of the microcapsule, which enables the microcapsule containing the compound and the microbeads contained therein to be identified, and optionally, sorted.

In an alternative embodiment, the invention provides a method wherein microbeads are analysed following pooling of the microcapsules into one or more common compartments. In this embodiment, a compound having the desired activity modifies the optical properties of the microbead which carried it (and which resides in the same microcapsule) to allow it to be identified, and optionally, sorted.

In this embodiment, it is not necessary for binding of the compound to the target to directly induce a change in optical properties.

In this embodiment, if the compound attached to the microbead has affinity for the target it will be bound by the target. At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds exhibiting the desired binding can be identified by adding reagents that specifically bind to, or react specifically with, the target and thereby induce a change in the optical properties of the microbeads allowing their identification. For example, a fluorescently-labelled anti-target antibody can be used, or an anti-target antibody followed by a second fluorescently labelled antibody which binds the first.

In an alternative embodiment, the target can be attached to the microbeads by a variety of means familiar to those skilled in the art (see for example (Hermanson, 1996)). The compounds to be screened contain a common feature - a tag. The compounds are released from the microbeads and if the compound has affinity for the target, it will bind to it. At

the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds exhibiting the desired binding can be identified by adding reagents that specifically bind to, or react specifically with, the "tag" and thereby induce a change in the optical properties of the microbeads allowing their identification. For example, a fluorescently-labelled anti-"tag" antibody can be used, or an anti-"tag" antibody followed by a second fluorescently labelled antibody which binds the first.

In an alternative embodiment, microbeads may be identified on the basis that the gene product, which binds to the ligand, merely hides the ligand from, for example, further binding partners which would otherwise modify the optical properties of the microbeads. In this case microbeads with unmodified optical properties would be selected.

Fluorescence may be enhanced by the use of Tyramide Signal Amplification (TSA™) amplification to make the microbeads fluorescent (Sepp et al., 2002). This involves peroxidase (linked to another compound) binding to the microbeads and catalysing the conversion of fluorescein-tyramine in to a free radical form which then reacts (locally) with the microbeads. Methods for performing TSA are known in the art, and kits are available commercially from NEN.

TSA may be configured such that it results in a direct increase in the fluorescence of the microbeads, or such that a ligand is attached to the microbeads which is bound by a second fluorescent molecule, or a sequence of molecules, one or more of which is fluorescent.

(ii) SCREENING FOR REGULATION OF BINDING

In an alternative embodiment, the invention can be used to screen compounds which act to regulate a biochemical process. If the compound activates a binding activity of a target, a ligand for the target which is activated can be attached to microbeads by a variety of means familiar to those skilled in the art (see for example (Hermanson, 1996)). At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds exhibiting the desired binding can

be selected by affinity purification using a molecule that specifically binds to, or reacts specifically with, the target.

In an alternative embodiment, microbeads may be screened on the basis that the compound inhibits the binding activity of a target. In this eventuality, the microbead, rather than being retained during an affinity purification step, may be selectively eluted whilst other microbeads are bound.

In an alternative embodiment, compounds can be screened for the ability to modulates a binding activity of a target using a change in the optical properties of the microcapsule or the microbead.

The change in optical properties of the microcapsule or the microbead after binding of the target to its ligand may be induced in a variety of ways, including:

(1) the ligand itself may have distinctive optical properties, for example, it is fluorescent

(2) the optical properties of the ligand may be modified on binding to the target, for example, the fluorescence of the ligand is quenched or enhanced on binding (Voss, 1993; Masui and Kuramitsu, 1998).

(3) the optical properties of the target may be modified on binding of the ligand, for example, the fluorescence of the target is quenched or enhanced on binding (Guixe et al., 1998; Qi and Grabowski, 1998)

(4) the optical properties of both target and ligand are modified on binding, for example, there can be a fluorescence resonance energy transfer (FRET) from target to ligand (or *vice versa*) resulting in emission at the "acceptor" emission wavelength when excitation is at the "donor" absorption wavelength (Heim & Tsien, 1996; Mahajan *et al.*, 1998; Miyawaki *et al.*, 1997).

The invention provides a method wherein a compound with the desired activity induces a change in the optical properties of the microcapsule, which enables the microcapsule containing the compound and the microbeads contained therein to be identified, and optionally, sorted.

In an alternative embodiment, the invention provides a method wherein microbeads are analysed following pooling of the microcapsules into one or more common compartments. In this embodiment, a compound having the desired activity modifies the optical properties of the microbead which carried it (and which resides in the same microcapsule) to allow it to be identified, and optionally, sorted.

In this embodiment, it is not necessary for binding of the target to the ligand to directly induce a change in optical properties.

In this embodiment, if a ligand attached to the microbead has affinity for the target it will be bound by the target. At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds which modulate the binding activity can be identified by adding reagents that specifically bind to, or react specifically with, the target and thereby induce a change in the optical properties of the microbeads allowing their identification. For example, a fluorescently-labelled anti-target antibody can be used, or an anti-target antibody followed by a second fluorescently labelled antibody which binds the first.

In an alternative embodiment, the target can be attached to the microbeads by a variety of means familiar to those skilled in the art (see for example (Hermanson, 1996)). The ligand to be screened contains a feature - a tag. At the end of the reaction, all of the microcapsules are combined, and all microbeads pooled together in one environment. Microbeads carrying compounds which modulate binding can be identified by adding reagents that specifically bind to, or react specifically with, the "tag" and thereby induce a change in the optical properties of the microbeads allowing their identification. For example, a fluorescently-labelled anti-"tag" antibody can be used, or an anti-"tag" antibody followed by a second fluorescently labelled antibody which binds the first.

Fluorescence may be enhanced by the use of Tyramide Signal Amplification (TSA™) amplification to make the microbeads fluorescent (Sepp et al., 2002), as above.

(iii) SCREENING FOR REGULATION OF CATALYSIS

In an alternative embodiment, the invention provides a method wherein a compound with the desired activity induces a change in the optical properties of the microcapsule, which enables the microcapsule containing the compound and, optionally, the microbeads contained therein to be identified, and optionally, sorted. The optical properties of microcapsules can be modified by either:

(1) the substrate and product of the regulated reaction having different optical properties (many fluorogenic enzyme substrates are available commercially, see for example (Haugland, 1996 and www.probes.com) including substrates for glycosidases, phosphatases, peptidases and proteases, or

(2) the presence of reagents which specifically bind to, or react with, the product (or substrate) of the regulated reaction in the microcapsule and which thereby induce a change in the optical properties of the microcapsules allowing their identification.

A wide range of assays for screening libraries of compounds for those which modulate the activity of a target are based on detecting changes in optical properties and can be used to screen compounds according to this invention. Such assays are well known to those skilled in the art (see for example Haugland, 1996 and www.probes.com).

Alternatively, selection may be performed indirectly by coupling a first reaction to subsequent reactions that takes place in the same microcapsule. There are two general ways in which this may be performed. First, the product of the first reaction could be reacted with, or bound by, a molecule which does not react with the substrate(s) of the first reaction. A second, coupled reaction will only proceed in the presence of the product of the first reaction. A regulatory compound can then be identified by the properties of the product or substrate of the second reaction.

Alternatively, the product of the reaction being selected may be the substrate or cofactor for a second enzyme-catalysed reaction. The enzyme to catalyse the second reaction can be incorporated in the reaction mixture prior to microencapsulation. Only when the first reaction proceeds will the coupled enzyme generate an identifiable product.

This concept of coupling can be elaborated to incorporate multiple enzymes, each using as a substrate the product of the previous reaction. This allows for selection of regulators of enzymes that will not react with an immobilised substrate. It can also be designed to give increased sensitivity by signal amplification if a product of one reaction is a catalyst or a cofactor for a second reaction or series of reactions leading to a selectable product (for example, see (Johannsson, 1991; Johannsson and Bates, 1988). Furthermore an enzyme cascade system can be based on the production of an activator for an enzyme or the destruction of an enzyme inhibitor (see (Mize et al., 1989)). Coupling also has the advantage that a common screening system can be used for a whole group of enzymes which generate the same product and allows for the selection of regulation of complicated multi-step chemical transformations and pathways.

In an alternative embodiment, if the target is itself an enzyme, or regulates a biochemical process which is enzymatic, the microbead in each microcapsule may be coated with the substrate for the enzymatic reaction. The regulatory compound will determine the extent to which the substrate is converted into the product. At the end of the reaction the microbead is physically linked to the product of the catalysed reaction. When the microcapsules are combined and the reactants pooled, microbeads which were coated with activator compounds can be identified by any property specific to the product. If an inhibitor is desired, selection can be for a chemical property specific to the substrate of the regulated reaction.

It may also be desirable, in some cases, for the substrate not to be attached to the microbead. In this case the substrate would contain an inactive "tag" that requires a further step to activate it such as photoactivation (e.g. of a "caged" biotin analogue, (Pirrung and Huang, 1996; Sundberg et al., 1995)). After convertion of the substrate to product the "tag" is activated and the "tagged" substrate and/or product bound by a tag-binding molecule (e.g. avidin or streptavidin) attached to the microbead. The ratio of substrate to product attached to the nucleic acid *via* the "tag" will therefore reflect the ratio of the substrate and product in solution. A substrate tagged with caged biotin has been used to select for genes encoding enzymes with phosphotriesterase activity using a procedure based on compartmentalisation in microcapsules (Griffiths and Tawfik, 2003). The phosphotriesterase substrate was hydrolysed in solution in microcapsules containing

active enzyme molecules, and after the reaction was completed, the caging group was released by irradiation to allow the product to bind, *via* the biotin moiety, to microbeads to which the gene encoding the enzyme was attached.

After the microbeads and the contents of the microcapsules are combined, those microbeads coated with regulators can be selected by affinity purification using a molecule (e.g. an antibody) that binds specifically to the product or substrate as appropriate.

In an alternative embodiment, the invention provides a method wherein the microbeads are analysed following pooling of the microcapsules into one or more common compartments. Microbeads coated with regulator compounds can be identified using changes in optical properties of the microbeads. The optical properties of microbeads with product (or substrate) attached can be modified by either:

(1) the product-microbead complex having characteristic optical properties not found in the substrate-microbead complex, due to, for example;

(a) the substrate and product having different optical properties (many fluorogenic enzyme substrates are available commercially (see for example Haugland, 1996 and www.probes.com) including substrates for glycosidases, phosphatases, peptidases and proteases, or

(b) the substrate and product having similar optical properties, but only the product, and not the substrate binds to, or reacts with, the microbead;

(2) adding reagents which specifically bind to, or react with, the product (or substrate) and which thereby induce a change in the optical properties of the microbeads allowing their identification (these reagents can be added before or after breaking the microcapsules and pooling the microbeads). The reagents

bind specifically to, or react specifically with, the product, and not the substrate, (or *vice versa*) if both substrate and product are attached to the microbeads, or optionally bind both substrate and product if only the product, and not the substrate binds to, or reacts with, the microbeads (or *vice versa*).

In this scenario, the substrate (or one of the substrates) can be present in each microcapsule unlinked to the microbead, but has a molecular "tag" (for example biotin,

DIG or DNP or a fluorescent group). When the regulated enzyme converts the substrate to product, the product retains the "tag" and is then captured in the microcapsule by the product-specific antibody. When all reactions are stopped and the microcapsules are combined, these microbeads will be "tagged" and may already have changed optical properties, for example, if the "tag" was a fluorescent group. Alternatively, a change in optical properties of "tagged" microbeads can be induced by adding a fluorescently labelled ligand which binds the "tag" (for example fluorescently-labelled avidin/streptavidin, an anti-"tag" antibody which is fluorescent, or a non-fluorescent anti-"tag" antibody which can be detected by a second fluorescently-labelled antibody).

(iv) SCREENING FOR COMPOUND SPECIFICITY/SELECTIVITY

Compounds with specificity or selectivity for certain targets and not others can be specifically identified by carrying out a positive screen for regulation of a reaction using one substrate and a negative screen for regulation of a reaction with another substrate. For example, two substrates, specific for two different target enzymes, are each labelled with different fluorogenic moieties. Each target enzymes catalyse the generation of a product with with a different fluorescence spectrum resulting in different optical properties of the microcapsules depending on the specificity of the compound for two targets.

(v) SCREENING USING CELLS

In the current drug discovery paradigm, validated recombinant targets form the basis of *in vitro* high-throughput screening (HTS) assays. Isolated proteins cannot, however, be regarded as representative of complex biological systems; hence, cell-based systems can provide greater confidence in compound activity in an intact biological system. A wide range of cell-based assays for drug leads are known to those skilled in the art. Cells can be compartmentalised in microcapsules, such as the aqeous microdroplets of a water-in-oil emulsion (Ghadessy, 2001). The effect of a compound(s) on a target can be determined by compartmentalising a cell (or cells) in a microcapsule together with a compound(s) and using an appropriate cell-based assay to identify those compartments containing compounds with the desired effect on the cell(s). The use of water-in-fluorocarbon emulsions may be particularly advantageous: the high gas dissolving

capacity of fluorocarbons can support the exchange of respiratory gases and has been reported to be beneficial to cell culture systems (Lowe, 2002) .

(vi) FLOW CYTOMETRY

In a preferred embodiment of the invention the microcapsules or microbeads will be analysed and, optionally, sorted by flow cytometry. Many formats of microcapsule can be analysed and, optionally, sorted directly using flow cytometry. Some formats of microcapsule may require that the microcapsules be further processed before analysis or sorting. For example, water-in-oil emulsions can be converted into water-in-oil-in-water double emulsions to facilitate analysis by flow cytometry (Bernath et al., 2004). Multiple emulsions are prepared by the re-emulsification of a simple primary water-in-oil (or oil-in-water) emulsion to provide water-in-oil-in-water (or oil-in-water-in-oil) emulsions (Davis and Walker, 1987).

Highly monodisperse microcapsules can be produced using microfluidic techniques. For example, water-in-oil emulsions with less than 3% polydispersity can be generated by droplet break off in a co-flowing steam of oil (Umbanhowar, 2000). Microfluidic systems can also be used for laminar-flow of aqueous microdroplets dispersed in a stream of oil in microfluidic channels (Thorsen, 2001) . This allows the construction of microfluidic devices for flow analysis and, optionally, flow sorting of microdroplets (Fu, 2002).

A variety of optical properties can be used for analysis and to trigger sorting, including light scattering (Kerker, 1983) and fluorescence polarisation (Rolland et al., 1985). In a highly preferred embodiment the difference in optical properties of the microcapsules or microbeads will be a difference in fluorescence and, if required, the microcapsules or microbeads will be sorted using a fluorescence activated cell sorter (Norman, 1980; Mackenzie and Pinder, 1986), or similar device. Flow cytometry has a series of advantages:

(1) commercially available fluorescence activated cell sorting equipment from established manufacturers (e.g. Becton-Dickinson, Coulter, Cytomation) allows the analysis and sorting at up to 100,000 microcapsules or microbeads s^{-1}.

(2) the fluorescence signal from each microcapsule or microbead corresponds tightly to the number of fluorescent molecules present. As little as few hundred fluorescent molecules per microcapsules or microbeads can be quantitatively detected;

(3) the wide dynamic range of the fluorescence detectors (typically 4 log units) allows easy setting of the stringency of the sorting procedure, thus allowing the recovery of the optimal number microcapsules or microbeads from the starting pool (the gates can be set to separate microcapsules or microbeads with small differences in fluorescence or to only separate out microcapsules or microbeads with large differences in fluorescence, dependant on the selection being performed);

(4) commercially available fluorescence-activated cell sorting equipment can perform simultaneous excitation and detection at multiple wavelengths (Shapiro, 1995). allowing positive and negative selections to be performed simultaneously by monitoring the labelling of the microcapsules or microbeads with two to thirteen (or more) fluorescent markers, for example, if substrates for two alternative targets are labelled with different fluorescent tags the microcapsules or microbeads can labelled with different fluorophores dependent on the target regulated.

If the microcapsules or microbeads are optically tagged, flow cytometry can also be used to identify the compound or compounds in the microcapsule or coated on the microbeads (see below). Optical tagging can also be used to identify the concentration of the compound in the microcapsule (if more than one concentration is used in a single experiment) or the number of compound molecules coated on a microbead (if more than one coating density is used in a single experiment). Furthermore, optical tagging can be used to identify the target in a microcapsule (if more than one target is used in a single experiment). This analysis can be performed simultaneously with measuring activity, after sorting of microcapsules containing microbeads, or after sorting of the microbeads.

(vii) MICROCAPSULE IDENTIFICATION AND SORTING

The invention provides for the identification and, optionally, the sorting of intact microcapsules where this is enabled by the sorting techniques being employed. Microcapsules may be identified and, optionally, sorted as such when the change induced by the desired compound either occurs or manifests itself at the surface of the

microcapsule or is detectable from outside the microcapsule. The change may be caused by the direct action of the compound, or indirect, in which a series of reactions, one or more of which involve the compound having the desired activity leads to the change. For example, where the microcapsule is a membranous microcapsule, the microcapsule may be so configured that a component or components of the biochemical system comprising the target are displayed at its surface and thus accessible to reagents which can detect changes in the biochemical system regulated by the compound on the microbead within the microcapsule.

In a preferred aspect of the invention, however, microcapsule identification and, optionally, sorting relies on a change in the optical properties of the microcapsule, for example absorption or emission characteristics thereof, for example alteration in the optical properties of the microcapsule resulting from a reaction leading to changes in absorbance, luminescence, phosphorescence or fluorescence associated with the microcapsule. All such properties are included in the term "optical". In such a case, microcapsules can be identified and, optionally, sorted by luminescence, fluorescence or phosphorescence activated sorting. In a highly preferred embodiment, flow cytometry is employed to analyse and, optionally, sort microcapsules containing compounds having a desired activity which result in the production of a fluorescent molecule in the microcapsule.

In an alternative embodiment, a change in microcapsule fluorescence, when identified, is used to trigger the modification of the microbead within the compartment. In a preferred aspect of the invention, microcapsule identification relies on a change in the optical properties of the microcapsule resulting from a reaction leading to luminescence, phosphorescence or fluorescence within the microcapsule. Modification of the microbead within the microcapsules would be triggered by identification of luminescence, phosphorescence or fluorescence. For example, identification of luminescence, phosphorescence or fluorescence can trigger bombardment of the compartment with photons (or other particles or waves) which leads to modification of the microbead or molecules attached to it. A similar procedure has been described previously for the rapid sorting of cells (Keij et al., 1994). Modification of the microbead may result, for example, from coupling a molecular "tag", caged by a photolabile protecting group to the

microbeads: bombardment with photons of an appropriate wavelength leads to the removal of the cage. Afterwards, all microcapsules are combined and the microbeads pooled together in one environment. Microbeads coated with compounds exhibiting the desired activity can be selected by affinity purification using a molecule that specifically binds to, or reacts specifically with, the "tag".

(C) COMPOUND LIBRARIES

Libraries of compounds can be obtained from a variety of commercial sources. The compounds in the library can be made by a variety of means well known to those skilled in the art. Optionally, compound libraries can be made by combinatorial synthesis using spatially resolved parallel synthesis or using split synthesis, optionally to generate one-bead-one-compound libraries. The compounds can, optionally, be synthesised on beads. These beads can be compartmentalised in microcapsules directly or the compounds released before compartmentalisation.

Advantageously, only a single type of compound, but multiple copies thereof is present in each microcapsule.

The compounds can, optionally, be connected to microbeads either covalently or non-covalently by a variety of means that will be familiar to those skilled in the art (see, for example, (Hermanson, 1996)).

Microbeads are available with a variety of surface chemistries from hydrophobic surfaces (e.g. plain polystyrene), to very hydrophilic surfaces imparted by a wide variety of functional surface groups: aldehyde, aliphatic amine, amide, aromatic amine, carboxylic acid, chloromethyl, epoxy, hydrazide, hydroxyl, sulfonate and tosyl. The functional groups permit a wide range of covalent coupling reactions, well known to those skilled in the art, for stable or reversible attachment of compounds to the microbead surface.

Advantageously, the compounds are attached to the microbeads *via* a cleavable linker. A variety of such linkers are familiar to those skilled in the art (see for example (Gordon and Balasubramanian, 1999)), including for example, linkers which can be cleaved

photochemically and reversible covalent bonds which can be controlled by changing the pH (e.g. imines and acylhydrazones), by adjusting the oxido-reductive properties (e.g. disulphides), or using an external catalyst (e.g. cross-metathesis and transamidation).

Advantageously, only a single type of compound, but multiple copies thereof is attached to each bead.

(D) IDENTIFICATION OF COMPOUNDS

The compounds in microcapsules or on microbeads can be identified in a variety of ways. If the identified microcapsules are sorted (e.g. by using a fluorescence activated cell sorter - FACS) the compounds can be identified by by direct analysis, for example by mass-spectroscopy. If the compounds remain attached to beads isolated as a result of selection (for example by affinity purification) or sorting (for example using a FACS) they can also be identified by direct analysis, for example by mass-spectroscopy. The microcapsules or beads can also be tagged by a variety of means well known to those skilled in the art and the tag used to identify the compound attached to the beads (Czarnik, 1997). Chemical, spectrometric, electronic, and physical methods to encode the compounds may all be used. In a preferred embodiment microcapsules or beads have different optical properties and are thereby optically encoded. In a preferred embodiment encoding is based on microcapsules or beads having different fluorescence properties. In a highly preferred embodiment the microcapsules or beads are encoded using fluorescent quantum dots present at different concentrations in the microcapsule or bead (Han, 2001). Microcapsules flowing in an ordered sequence in a microfluidic channel can also be encoded (wholly or partially) by their sequence in the stream of microcapsules (positional encoding).

Advantageously, each compounds is present in different microcapsules at different concentrations (typically at concentrations varying from mM to nM) allowing the generation of a dose-response curve. This would, for example, allow the determination of the inhibition constant (K_i) of an inhibitory compound. The concentration of the compounds in the microcapsules can be determined by, for example, optical encoding or positional encoding of the microcapsules or microbeads as above.

(E) IDENTIFICATION OF TARGETS

Advantageously, multiple different targets can be compartmentalised in microcapsules such that each microcapsule contains multiple copies of the same target. For example, multiple protein kinases, or multiple polymorphic variants of a single target, can be compartmentalised to allow the specificity of compounds to be determined. The identity of the target in a microcapsule can be determined by, for example, optical encoding or positional encoding of the microcapsules or microbeads as above.

EXAMPLES

Example 1

Screening for inhibitors of the enzyme protein tyrosine phosphatase 1B (PTP1B)

PTP1B is a negative regulator of insulin and leptin signal transduction. Resistance to insulin and leptin are hallmarks of type 2 diabetes mellitus and obesity and hence PTP1B is an attractive drug target for diabetes and obesity therapy (Johnson et al., 2002). Two water-in-oil emulsions are made as follows.

A solution of 1% (w/v) Span 60 and 1% (w/v) cholesterol in decane (all from Sigma Aldrich) is prepared by dissolving 80 mg of Span 60 and 80 mg of cholesterol into 7.84 ml of decane. The decane is heated to 45°C to allow complete solubilization of the surfactant and cholesterol. The surfactant/decane solution is divided over batches of 200 µl and placed in a block-heater at 37°C.

A hand-extruding device (Mini extruder, Avanti Polar Lipids Inc, Alabaster, AL, USA) is assembled according to the manufacturer's instructions. For extrusion, a single 19 mm Track-Etch polycarbonate filter with average pore size of 14 µm (Whatman Nuclepore, Whatman, Maidstone, UK) is fitted inside the mini extruder. Two gas-tight 1 ml Hamilton syringes (Gastight #1001, Hamilton Co, Reno, Nevada, USA) are used for extrusion. The extruder was pre-rinsed with 3 x 1 ml of decane by loading one of the Hamilton syringes with 1 ml of decane, placing the syringe at one and of the mini extruder and extruding it through the filters into the empty Hamilton syringe on the other side of the extruder.

The first emulsion is made by loading 50 µl of 100 µM compound **2** (Fig. 1), which has a *bis*-difluoromethylene phosphonate and is a known PTP1B inhibitor (Johnson et al., 2002), the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at 5 mU/ml, the fluorogenic PTP1B substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Molecular Probes), and 100 µM Texas Red (Sigma; excitation/emmission maxima 595/615 nm; red fluorescence) in a buffer compatible with PTP1B activity (25 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM EDTA) (Doman et al., 2002) into one of the Hamilton syringes, and 200 µl of the pre-heated decane/surfactant mix into the other Hamilton syringe. The syringes are fitted into the openings on both sides of the filter holder of the extruder. The compound mix is forced through the filter holder into the alternate syringe containing the decane/surfactant mix and directly forced back into the original syringe to complete one round of extrusion. In total, 7.5 rounds of extrusion are completed. The filled syringe is removed from the extruder and emptied into a 1.7 ml Axygen tube (# MCT-175-C, Axygen Scientific, Inc., Union City, CA, USA).

A second water-in-oil emulsion is made identical to the emulsion above but containing 100 µM hydrocinnamic acid (Aldrich), a compound that is not a PTP1B inhibitor, in place of compound **2**, and 100 µM calcein (Sigma; excitation/emmission maxima 470/509 nm; green fluorescence) in place of Texas Red.

The two emulsions are mixed by vortexing in ratios varying from 1:1000 to 1:1 (compound 2 emulsion: hydrocinnamic acid emulsion) and incubated at 37°C for 30 min. Inhibitors reduce the amount of non-fluorescent substrate (DiFMUP) converted to the dephosphorylated product (DiFMU; excitation/emmission maxima 358/452 nm; blue fluorescence).

The water-in-oil emulsions are then converted into water-in-oil-in water double emulsions as follows. The extruder (see above) is disassembled, cleaned extensively with soap and reversed-osmosis water, and re-assembled. A single 19 mm Track-Etch polycarbonate filter with an average pore size of 8 µm is fitted. The extruder is pre-rinsed with 3 x 1 ml phosphate-buffered saline solution (PBS). 750 µl of PBS containing 0.5% (w/v) Tween 80 (Sigma Aldrich) is loaded into a 1ml gas-tight Hamilton syringe and fitted into the

extruder. 250 µl of the water-in-oil emulsion is loaded into the alternate 1 ml Hamilton syringe and fitted into the extruder. The emulsion is forced through the filter into the alternate syringe containing the PBS/0.5% Tween 80 and immediately forced back into the original syringe to complete one cycle of extrusion. In total, 4.5 cycles of extrusion are performed. The filled syringe is removed from the extruder and emptied into a 1.7 ml Axygen tube. The water-in-oil-in-water double emulsions formed are placed on ice.

The double emulsions are then analysed by multi-colour flow cytometery using a MoFlo (Cytomation) flow cytometer. Predominantly, microcapsules exhibiting green fluorescence (and containing hydrocinnamic acid) also show blue fluorescence due to dephosphorylation of DiFMUP by PTP1B. Predominantly, microcapsules exhibiting red fluorescence (and containing containing compound **2**) also show little or no blue fluorescence due to inhibition of PTP1B.

Example 2

Two aqueous mixtures are made on ice (to prevent reaction). The first mixture contains 100 µM compound **2** (Fi.g. 1), which has a *bis*-difluoromethylene phosphonate and is a known PTP1B inhibitor (Johnson et al., 2002), the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at 5 mU/ml, the fluorogenic PTP1B substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Molecular Probes), and 100 µM Texas Red (Sigma; excitation/emmission maxima 595/615 nm; red fluorescence) in a buffer compatible with PTP1B activity (25 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM EDTA) (Doman et al., 2002). A second mixture is created identical to the above but containing 100 µM hydrocinnamic acid (Aldrich), a compound that is not a PTP1B inhibitor, in place of compound **2**, and 100 µM calcein (Sigma; excitation/emmission maxima 470/509 nm; green fluorescence) in place of Texas Red.

50 µl of each of the compound mixtures is added sequentially to a solution of 1% (w/v) Span 60 and 1% (w/v) cholesterol in decane, made and held at 37°C as example 1, whilst homogenising at 25,000 r.p.m. using an Ultra-Turrax T8 Homogenizer (IKA) with a 5 mm dispersing tool. Homogenisation is continued for 3 minutes after the addition of the second aliquot. The coarse emulsion produced is then extruded as in example 1 to create a

fine water-in-oil emulsion and incubated at 37°C for 30 min. Inhibitors reduce the amount of non-fluorescent substrate (DiFMUP) converted to the dephosphorylated product (DiFMU; excitation/emmission maxima 358/452 nm; blue fluorescence). The water-in-oil emulsion is then converted into a water-in-oil-in water double emulsion and analysed by multi-colour flow cytometery as in example 1. Predominantly, microcapsules exhibiting green fluorescence (and containing hydrocinnamic acid) also show blue fluorescence due to dephosphorylation of DiFMUP by PTP1B. Predominantly, microcapsules exhibiting red fluorescence (and containing containing compound **2**) also show little or no blue fluorescence due to inhibition of PTP1B.

Example 3

Screening of PTP1B inhibitors from a compound library

100 water-in-oil emulsions are made on ice (to prevent reaction) as in example 1. The first emulsion is made by dispersing a mixture of 100 μM compound **2** (Fi.g. 1), which has a *bis*-difluoromethylene phosphonate and is a known PTP1B inhibitor (Johnson et al., 2002), the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at 5 mU/ml, the fluorogenic PTP1B substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Molecular Probes), and a pre-defined ratio of Qdot™ Streptavidin Conjugates with emmission maxima at 585 nm, 655 nm and 705 nm (Quantum Dot Corporation, Hayward CA) in a buffer compatible with PTP1B activity (25 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM EDTA) (Doman et al., 2002). The 99 other water-in-oil emulsions are identical to the above but each contain one of 99 carboxylic acids from the Carboxylic Acid Organic Building Block Library (Aldrich) in place of compound **2**, and different ratios of Qdot™ Streptavidin Conjugates with emmission maxima at 585 nm, 655 nm and 705 nm. In all emulsions the concentration of the 705 nm Qdot™ Streptavidin Conjugates is 100 nM, and the concentrations of the 585 nm and 655 nm Qdot™ Streptavidin Conjugates is either 0, 11, 22, 33, 44, 55, 66, 77, 88 or 100 nM. Hence, there are 100 (10 x 10) permutations of Qdot™ Streptavidin Conjugate concentrations which allows the microcapsules containing each compound to have a unique fluorescence signature which is read by determining the fluorescence ratios of fluorescence at at 705 nm, 585 nm and 655 nm.

The 100 emulsions are mixed in equal ratios by vortexing and the temperature raised to 25°C for 30 min. Inhibitors reduce the amount of non-fluorescent substrate (DiFMUP) converted to the dephosphorylated product (DiFMU; excitation/emmission maxima 358/452 nm; blue fluorescence). The water-in-oil emulsion is then converted into a water-in-oil-in water double emulsion and analysed by multi-colour flow cytometery as in example 1n. Predominantly, all microcapsules exhibited blue fluorescence due to dephosphorylation of DiFMUP by PTP1B except those with the Qdot fluorescence signature of the microcapsules containing compound **2**.

Example 4

Screening of PTP1B inhibitors from a compound library

100 aqueous mixtures are made on ice (to prevent reaction). The first mixture contains 100 μM compound **2** (Fi.g. 1), which has a *bis*-difluoromethylene phosphonate and is a known PTP1B inhibitor (Johnson et al., 2002), the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at 5 mU/ml, the fluorogenic PTP1B substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Molecular Probes), and a pre-defined ratio of Qdot™ Streptavidin Conjugates with emmission maxima at 585 nm, 655 nm and 705 nm (Quantum Dot Corporation, Hayward CA) in a buffer compatible with PTP1B activity (25 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM EDTA) (Doman et al., 2002). The 99 other aqueous mixtures are identical to the above but each contain one of 99 carboxylic acids from the Carboxylic Acid Organic Building Block Library (Aldrich) in place of compound **2**, and different ratios of Qdot™ Streptavidin Conjugates with emission maxima at 585 nm, 655 nm and 705 nm. In all mixtures the concentration of the 705 nm Qdot™ Streptavidin Conjugates is 100 nM, and the concentrations of the 585 nm and 655 nm Qdot™ Streptavidin Conjugates is either 0, 11, 22, 33, 44, 55, 66, 77, 88 or 100 nM. Hence, there are 100 (10 x 10) permutations of Qdot™ Streptavidin Conjugate concentrations which allows the microcapsules containing each compound to have a unique fluorescence signature which is read by determining the fluorescence ratio of fluorescence at at 705 nm, 585 nm and 655 nm.

0.5 μl of each of the compound mixtures is added sequentially to a solution of 1% (w/v) Span 60 and 1% (w/v) cholesterol in decane, made and held at 37°C as example 1, whilst

homogenising at 25,000 r.p.m. using an Ultra-Turrax T8 Homogenizer (IKA) with a 5 mm dispersing tool. Homogenisation is continued for 3 minutes after the addition of the second aliquot. The coarse emulsion produced is then extruded as in example 1 to create a fine water-in-oil emulsion and incubated at 37°C for 30 min. Inhibitors reduce the amount of non-fluorescent substrate (DiFMUP) converted to the dephosphorylated product (DiFMU; excitation/emmission maxima 358/452 nm; blue fluorescence). The water-in-oil emulsion is then converted into a water-in-oil-in water double emulsion and analysed by multi-colour flow cytometery as in example 1. Predominantly, all microcapsules exhibited blue fluorescence due to dephosphorylation of DiFMUP by PTP1B except those with the Qdot fluorescence signature of the microcapsules containing compound **2**.

Example 5

Screening for PTP1B inhibitors using microcapsules in microfluidic systems

Microchannels are fabricated with rectangular cross-sections using rapid prototyping in poly(dimethylsiloxane) (PDMS) (McDonald and Whitesides, 2002) and rendered hydrophobic as (Song and Ismagilov, 2003). Syringe pumps were used to drive flows (Harvard Apparatus PHD 2000 Infusion pumps). For aqueous solutions, 50 µl Hamilton Gastight syringes (1700 series, TLL) with removeable needles of 27-gaugeare used with 30-gauge Teflon tubing (Weico Wire and Cable). For the carrier fluid, 1 ml Hamilton Gastight syringes (1700 series, TLL) are used with 30-gauge Teflon needles with one hub from Hamilton (Song and Ismagilov, 2003). The carrier fluid is 9% (v/v) $C_6F_{11}C_2H_4OH$ in perfluorodecaline (PFD) (Song et al., 2003). All water-soluble reagents were dissolved in (25 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA), a buffer compatible with PTP1B activity.

A solution of the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at 50 mU/ml and a solution of either a) 100 µM compound **2** (Fi.g. 1), which has a *bis*-difluoromethylene phosphonate and is a known PTP1B inhibitor (Johnson et al., 2002), or b) 100 µM hydrocinnamic acid (Aldrich), a compound that is not a PTP1B inhibitor are flowed in a microchannel as two laminar streams, with an inert centre stream (of 25 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA) to separate them and prevent the enzyme and compound coming into contact prior to droplet microcapsule formation (Song et al., 2003). These three steams are continuously injected into a flow of

water immiscible fluorocarbon carrier fluid (9% (v/v) $C_6F_{11}C_2H_4OH$ in PFD). Inlet channels for the aqeous solutions are 50 μm^2 wide and channel for PFD is 28 μm wide. A variety of PFD/water volumetric flow rates (in $\mu l\ min^{-1}$) can be used including 0.6:0.3, 1.0:0.6, 12.3:3.7, 10:6 and 20:6, resulting in flow rates of 10, 19, 190, 190 and 300 $mm\ s^{-1}$ respectively. Aqueous microcapsules which occupy the entire width of the channel are formed by drop-breakoff in the PFD stream (Song et al., 2003). Microcapsules containing either compound **2** or hydrocinnamic acid can be formed by switching between injection with syringes containing compound **2** and hydrocinnamic acid.

The channel immediately downstream of the point of droplet formation is winding with a peak to peak distance of 50 μm for a distance of 1 mm. This results in rapid mixing of the contents of the microcapsule by chaotic advection (Song et al., 2003). After this point the microcapsules are run for up to 1 min through a 60 cm long microchannel (to allow inhibitor binding). This microchannel is then merged with a 60 x 50 μm^2 microchannel containing aqueous microcapsules in (9% (v/v) $C_6F_{11}C_2H_4OH$ in PFD) formed as above. These larger microcapsules contain the fluorogenic PTP1B substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) (Molecular Probes) in 25 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM EDTA. After the junction between the microchannels the expanded main channel is 100 x 50 μm^2 and the microcapsules do not block the channel and can move at different speeds until a large microcapsule (containing DiFMUP) coalesces with a small microcapsule (containing PTP1B and the compound) (Song et al., 2003). The frequency of production of large and small microcapsules is equal such that each large microcapsule has a small microcapsule with which to fuse. The fused microcapsules are then run for up to 2 min through a 60 cm long microchannel. Fluorescence of the microcapsules due to production of DiFMU (excitation/emmission maxima 358/452 nm; blue fluorescence) is measured using an epifluorescence microscope. Predominantly, microcapsules exhibiting blue fluorescence are those containing hydrocinnamic acid whereas microcapsules containing compound **2** exhibit low fluorescence due to inhibition of PTP1B.

Example 6

Attachment of a compound library to microbeads

5.5 μm diameter polystyrene microbeads that bear carboxylate functional groups on the surface are commercially available (www.luminexcorp.com) in an optically tagged form, as a result of incorporation of precise ratios of orange (585 nm), and red (>650 nm) fluorochromes (Fulton et al., 1997). A set of 100 such beads, each with a unique optical signature (www.luminexcorp.com) are modified with an excess of ethylenediamine and EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce) as (Hermanson, 1996) to create primary amino groups on the surface. The photocleavable linker 4-(4-hydroxymethyl-2-methoxy-5-nitrophenoxy)butanoic acid (NovaBiochem) (Holmes and Jones, 1995) is then attached to the beads by forming an amide bond using EDC as above. 100 different carboxylic acids from the Carboxylic Acid Organic Building Block Library (Aldrich) are then coupled to the beads, by reacting with the linker alcohol to form a carboxylate ester, each of the 100 different optically tagged beads being coupled to a different carboxylic acid, and each bead being derivatised with ~10^6 molecules of carboxylic acid. Irradiation for 4 min on ice using a B100 AP 354 nm UV lamp (UVP) from a distance of ~5 cm results in release of the compounds from the beads as carboxylic acids.

Example 7

Screening for inhibitors of the enzyme protein tyrosine phosphatase 1B (PTP1B) using compounds attached to microbeads

PTP1B is a negative regulator of insulin and leptin signal transduction. Resistance to insulin and leptin are hallmarks of type 2 diabetes mellitus and obesity and hence PTP1B is an attractive drug target for diabetes and obesity therapy (Johnson et al., 2002). 5.5 μm diameter polystyrene microbeads that bear carboxylate functional groups on the surface are commercially available (www.luminexcorp.com) in an optically tagged form, as a result of incorporation of precise ratios of orange (585 nm), and red (>650 nm) fluorochromes (Fulton et al., 1997). First, the carboxylate functional groups on the microbeads are converted to primary amines using ethylenediamine and EDC as in example 6. A phosphopeptide substrate for PTP1B, the undecapaptide $EGFR_{988-998}$ (DADEpYLIPQQG) (Zhang et al., 1993), is then coupled to both sets of microbeads via the surface amino groups using EDC. This peptide is made by solid phase synthesis on

Sieber Amide resin (9-Fmoc-amino-xanthen-3-yloxy-Merrifield resin) (Novabiochem) with orthogonal protection on the side chain carboxylate groups using carboxylate-*O*-allyl esters. A linker comprised of tetradecanedioic acid is coupled to the N-terminus and the peptide cleaved from the beads using 1% TFA to yield a peptide with a C-terminal amide The peptide is coupled to the beads (using EDC) via the linker to give ~10^5 peptides per bead. The remaining surface amino groups are then modified by attaching the photochemically cleavable linker 4-(4-hydroxymethyl-2-methoxy-5-nitrophenoxy)butanoic acid as in example 6. The protecting groups on the side chain carboxylates of the peptide are then removed using $Pd(Ph_3)_4/CHCl_3$/HOAc/N-methyl morpholine. A first set of microbeads is derivatised with 3-(4-difluorophosphonomethylphenyl)propanoic acid (compound 1, Fig. 1), a compound that is a known PTP1B inhibitor (Johnson et al., 2002). A second set of beads, with a distinct optical tag from the first set of beads, is derivatised with hydrocinnamic acid (Aldrich), a compound that is not a PTP1B inhibitor. In each case the compound is coupled by reacting with the linker alcohol to form a carboxylate ester as in example 6. Each microbead is derivatised with ~10^6 molecules (Fulton et al., 1997).

The microbeads are then screened using the method outlined in Fig 2. The two sets of microbeads are mixed in ratios varying from 1:1000 to 1:1 (compound 1 beads: hydrocinnamic acid beads) and 10^8 total microbeads are mixed with the target enzyme (human recombinant PTP1B, residues 1-322; Biomol Research Laboratories, Inc.) at a concentration of 10 nM, on ice (to prevent reaction) in a buffer compatible with PTP1B activity (25 mM HEPES, pH 7.4, 125 mM NaCl, 10% glycerol, 1 mM EDTA) (Doman et al., 2002). Single beads and target enzyme (PTP1B) are then colocalised in microcapsules by forming a water-in-oil emulsion (also on ice). The concentration of beads is such that most microcapsules contain one or no beads. The compound is released photochemically (as in example 6) and the temperature raised to 25°C. Inhibitors reduce the amount of substrate converted to product (dephosphorylated peptide). The emulsion is cooled to 4°C and broken as (Griffiths and Tawfik, 2003) into 100 μM vanadate to stop the reaction (Harder et al., 1994). After labelling with an anti-substrate (anti-phosphotyrosine) antibody labelled with the green (530 nm) fluorochrome fluorescein isothiocyanate (mouse monoclonal IgG_{2b} PY20 (Santa Cruz) according to the manufacturer's instructions, beads are analysed by 3-colour flow cytometry using a

FACScan (Becton-Dickinson), FACScalibur (Becton-Dickinson) or MoFlo (Cytomation) flow cytometers to simultaneously determine the extent of inhibition and the compound on the beads. Predominantly, dephosphorylation of the peptide is only observed on those microbeads which were coated with PTPIB inhibitors, and not on other microbeads.

Example 8

Screening of PTP1B inhibitors from a compound library attached to microbeads

A set of 100 5.5 μm diameter polystyrene microbeads, bearing carboxylate functional groups on the surface and each with a unique optical signature (www.luminexcorp.com) as a result of incorporation of precise ratios of orange (585 nm), and red (>650 nm) fluorochromes (Fulton et al., 1997) are derivatised with a phosphopeptide substrate for PTP1B, the undecapaptide $EGFR_{988-998}$ (DADEpYLIPQQG) (Zhang et al., 1993), and 100 different carboxylic acids, each attached *via* a photochemically cleavable linker, as in example 7. One of these carboxylic acids is 3-(4-difluorophosphonomethylphenyl) propanoic acid (compound 1, Fig. 1), a compound that is a known PTP1B inhibitor (Johnson et al., 2002). The other 99 carboxylic acids are from the Carboxylic Acid Organic Building Block Library (Aldrich) as example 6. Equal numbers of each of the 100 bead sets are then mixed and screened as for example 7. Predominantly, dephosphorylation of the peptide is only observed on those microbeads which were coated with the PTPIB inhibitor 3-(4-difluorophosphonomethylphenyl) propanoic acid (compound 1, Fig. 1), and not on microbeads coated with other compounds.

Example 9

Compartmentalisation of small molecules in a water-in-fluorocarbon emulsions.

Water-in-fluorocarbon emulsions containing 95% (v/v) perfluorooctyl bromide, 5% (v/v) phosphate buffered saline containing the molecule of interest in solution, and 2% (w/v) $C_8F_{17}C_{11}H_{22}OP(O)[N(CH_2CH_2)_2O]_2$ (F8H11DMP) as surfactant were formed essentially as (Sadtler et al., 1996) by extrusion (15times) through 14μm filters (Osmonics) or by homogenising for 5 min at 25,000 r.p.m. using an Ultra-Turrax T8 Homogenizer (IKA) with a 5 mm dispersing tool. Emulsions were made containing a series of small fluorescent molecules dissolved in the aqueous phase at concentrations from 100 μm to 2mM. These molecules, including calcein, texas red, fluorescein, coumarin 102, 7-

hydroxycoumarin-3-carboxylic acid and 7-diethylamino-4-methyl coumarin (coumarin 1), had molecular weights from 203 to 625 Da and LogP values - calculated using SRC's LogKow/KowWin Program (Meylan and Howard, 1995) - ranging from –0.49 to 4.09. Emulsions containing different coloured fluorochromes were mixed by vortexing. Compartmentalisation was observed by epifluorescence microscopy of the mixed emulsions. No exchange between compartments was observed 24 hours after mixing (see Fig. 3).

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All publications mentioned in the above specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method for identifying a compound or compounds in a repertoire of compounds, which compound or compound(s) possess(es) a desired activity, comprising the steps of:

a) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;

b) identifying the compounds which possess the desired activity.

2. A method according to claim 1, wherein step (a) comprises forming separate emulsion compartments comprising individual compounds and mixing the emulsion compartments to form an emulsified compound repertoire wherein a subset of the repertoire is represented in multiple copies in any one microcapsule.

3. A method according to claim 1, wherein step (a) comprises

c) attaching the repertoire of compounds onto microbeads, such that only a subset of the repertoire is represented on any one microbead;

d) compartmentalising the microbeads into microcapsules;

such that a subset of the repertoire is represented in multiple copies in any one microcapsule.

4. A method according to any preceding claim, wherein the desired activity is selected from the group consisting of a binding activity and the modulation of the activity of a target.

5. A method according to claim 4, wherein the binding activity is binding to a target.

6. A method according to claim 4, wherein the modulated activity is a binding activity.

7. A method according to claim 4, wherein the modulated activity is a catalytic activity.

8. A method according to any one of claims 1 to 3, wherein the compound reacts with a target to generate a reaction product.

9. A method according to claim 3 or any claim dependent thereon, wherein the compounds are coupled to the microbeads via a non-cleavable linker.

10. A method according to claim 3 or any claim dependent thereon, wherein the compounds are coupled to the microbeads via a cleavable linker.

11. A method according to claim 10, wherein the compounds are coupled to the microbeads via a photochemically cleavable linker.

12. A method according to claim 3 or any claim dependent thereon, wherein the compounds are coupled to the microbeads by a combinations of a cleavable linker and a non-cleavable linker.

13. A method according to any one preceding claim, wherein the target is compartmentalised into microcapsules together with the compound(s).

14. A method according to any preceding claim, wherein the compound having a desired activity causes a change in the microbead or microcapsule with which it is associated which allows the microbead or microcapsule to be identified, sorted or selected.

15. A method according to claim 14, wherein the compound is bound to a microbead and the desired activity within the microcapsule results, directly or indirectly, in the modification of the microbead to which the compound is attached to enable the isolation of the microbead.

16. A method according to claim 15, wherein the target is bound to the microbead and the compound having the desired activity within the microcapsule binds, directly or indirectly, to the target to enable the isolation of the microbead.

17. A method according to claim 15 or claim 16, wherein the microbeads are isolated by affinity purification.

18. A method according to claim 7, wherein a substrate is present in the microcapsules and the desired activity of the compound within the microcapsule results, directly or indirectly, in the regulation of conversion of said substrate into product.

19. A method according to claim 18, wherein the substrate is converted into the product by the catalytic action of the target.

20. A method according to claims 18 or 19, wherein the substrate and the product have different optical properties.

21. A method according to claim 20, wherein the the substrate and the product have different fluorescence properties.

22. A method according to claim 18, wherein the substrate is bound to the microbead, and the desired activity of the compound within the microcapsule results, directly or indirectly, in the regulation of conversion of said substrate into a product which remains part of the microbead and enables its identification and, optionally, isolation.

23. A method according to claim 18, wherein the product and, optionally, the unreacted substrate are subsequently complexed with the microbead in the microcapsule.

24. A method according to claim 18, wherein the desired activity enhances the conversion of substrate into product.

25. A method according to claim 18, wherein the desired activity inhibits the conversion of substrate into product.

26. A method according to claim 7, wherein the desired activity of the compound within the microcapsule results, directly or indirectly, in the generation of a product which is subsequently complexed with the microbead and enables its isolation.

27. A method according to any one of claims 1 to 14, wherein the compound having a desired activity causes a change in the microcapsule which allows the microcapsule to be identified, sorted or selected.

28. A method according to any preceding claim, wherein the desired activity of the compound within the microcapsule induces a change in the optical properties of the microcapsule.

29. A method according to claim 28, wherein the microcapsules are identified by detection of a change in their fluorescence.

30. A method according to claim 29, wherein the change in fluorescence is due to fluorescence resonance energy transfer (FRET).

31. A method according to claim 28, wherein the compound in a microcapsule is identified by a difference in the optical properties of the microcapsule.

32. A method according to claim 31, wherein the difference in the optical properties of the microcapsule is a difference in fluorescence.

33. A method according to claim 32, wherein the difference in the fluorescence of the microcapsule is due to the presence of quantum dots.

34. A method according to any preceding claim, wherein the compounds are present in different microcapsules at different concentrations.

35. A method according to claim 34, wherein the concentration of the compound in a microcapsule is identified by a difference in the optical properties of the microcapsule.

36. A method according to claim 35, wherein the difference in the optical properties of the microcapsule is a difference in fluorescence.

37. A method according to claim 36, wherein the difference in the fluorescence of the microcapsule is due to the presence of quantum dots.

38. A method according to claim 28, wherein the target in a microcapsule is identified by a difference in the optical properties of the microcapsule.

39. A method according to claim38, wherein the difference in the optical properties of the microcapsule is a difference in fluorescence.

40. A method according to claim 39, wherein the difference in the fluorescence of the microcapsule is due to the presence of quantum dots.

41. A method according to any one of claims 28 to 40, wherein the identification of microcapsules is by flow cytometry.

42. A method according to claim 41, wherein the sorting of microcapsules is performed using a fluorescence activated cell sorter (FACS).

43. A method according to claim 42, wherein the microcapsules are sorted using a fluorescence activated cell sorter (FACS) after conversion of the water-in-oil emulsion to a water-in-oil-in water emulsion.

44. A method according to claim 15, wherein the modification of the microbead comprises a change in its optical properties.

45. A method according to claim 44, wherein the difference in the optical properties of the microbead is a difference in fluorescence.

46. A method according to claim 44, wherein the compound on a microbead is identified by a difference in the optical properties of the microbead.

47. A method according to claim 46, wherein the difference in the optical properties of the microbead is a difference in fluorescence.

48. A method according to claim 47, wherein the difference in the fluorescence of the microbead is due to the presence of quantum dots.

49. A method according to claim 3, wherein the microbead is nonmagnetic, magnetic or paramagnetic.

50. A method according to claim 45, wherein the microbeads are sorted by detection of a change in their fluorescence.

51. A method according to claim 50, wherein the identification of microbeads is by flow cytometry.

52. A method according to claim 50, wherein the sorting of microbeads is performed using a fluorescence activated cell sorter (FACS).

53. A method according to claim 50, wherein the change in fluorescence is due to fluorescence resonance energy transfer (FRET).

54. A method according to claim 45, wherein the modification of the microbead enables it to be further modified outside the microcapsule so as to induce a change in its optical properties.

55. A method according to claim 45, wherein the change in optical properties of the microbead is due to binding of a compound with distinctive optical properties to the microbead.

56. A method according to claim 45 or claim 46, wherein the change in optical properties of the microbead is due to binding of a target with distinctive optical properties by the compound.

57. A method according to claim 45 or claim 46, wherein the change in optical properties of the microbead is due to a change in the optical properties of the compound when bound to target.

58. A method according to claim 45 or claim 46, wherein the change in optical properties of the microbead is due to a change in the optical properties of the target when bound by the compound.

59. A method according to claim 45 or claim 46, wherein the change in optical properties of the microbead is due to a change in the optical properties of both target and compound on binding.

60. A method according to claim 45 or claim 46, wherein the compound with the desired activity acts to cause a change in the target, and the change in optical properties of the microbead is due to the different optical properties of the target and the product of the action of the compound on the target.

61. A method according to claim 45 or claim 46, wherein the compound with the desired activity acts to cause a change in the target without altering its optical properties, but only the product of the action, and not the target, binds to, or reacts with, the microbead, thereby changing the optical properties of the microbead.

62. A method according to claim 45 or claim 46, wherein further reagents specifically bind to, or specifically react with, the product (and not the substrate) of the regulated reaction, which is attached to the microbead, thereby altering the optical properties of the microbead.

63. A method according to claim 45 or claim 46, wherein further reagents specifically bind to, or specifically react with, the substrate (and not the product) of the regulated reaction, which is attached to the microbead, thereby altering the optical properties of the microbead.

64. A method according to any preceding claim, wherein a non-desired activity of a compound results in a change in the microbead or the microcapsule which is distinct from that resulting from the desired activity.

65. A method according to claim 64, wherein the change resulting from the non-desired activity is used to negatively select the microcapsules or the microbeads.

66. A method according to claim 65, wherein negative selection is combined with positive selection to improve reaction specificity.

67. A method according to claim 66, wherein the improved reaction specificity is an improvement in binding specificity.

68. A method according to claim 66, wherein the improved reaction specificity is an improvement in regio- and/or stereo-selectivity for the target.

69. A method according to any one of claims 45 to 68, wherein microbeads modified directly or indirectly by the activity of the desired compound are further modified by Tyramide Signal Amplification (TSA™; NEN), resulting directly or indirectly in a change in the optical properties of said microbeads thereby enabling their identification and, optionally, isolation.

70. A method according to any preceding claim, wherein each microbead or microcapsule comprises two or more compounds and each compound must have a desired activity in order for the microbead or the microcapsule to be selected.

71. Method according to any preceding claim, wherein the compound or compounds are low molecular weight compounds.

72. Method according to any preceding claim, wherein the subset of the repertoire is a single compound.

73. Method according to claim 72, wherein each microcapsule or microbead has associated therewith multiple molecules of a single compound.

74. A method according to any preceding claim further comprising iteratively repeating one or more of steps (a) to (d).

75. A method according to any preceding claim, wherein microencapsulation is achieved by forming a water-in-oil emulsion.

76. A method according to claim 75, wherein the water-in-oil emulsion is made using a microfluidic system.

77. A method according to claim 76, wherein the emulsion is formed by aqueous droplet break off in a co-flowing steam of an immiscible liquid.

78. A method according to claim 76, wherein microcapsules are transported for analysis by laminar-flow of aqueous microdroplets dispersed in a stream of oil in microfluidic channels.

79. A method according to claim 76 wherein the microcapsules are aqueous microdroplets dispersed in a stream of oil in microfluidic channels.

80. A method according to claim 76 wherein the compound in a microcapsule is identified by the relative position of of the microcapsule compared to other microdroplets in a microfluidic channel.

81. A method according to claim 76 wherein the concentration of a compound in a microcapsule is identified by the relative position of of the microcapsule compared to other microdroplets in a microfluidic channel.

82. A method according to claim 76 wherein the target in a microcapsule is identified by the relative position of of the microcapsule compared to other microdroplets in a microfluidic channel.

83. A method according to any preceding claim wherein the identified microcapsules are sorted using a microfluidic device.

84. A method according to claim 75, wherein microencapsulation is achieved by forming a water-in-fluorocarbon or water-in-perfluorocarbon emulsion.

85. A method according to claim 84, wherein the fluorocarbon is perfluorooctyl bromide or perfluorooctylethane.

86. A method according to claim 84, wherein the emulsion is formed using F-alkyl dimorpholinophosphate(s).

87. A method according to claim 86, wherein the F-alkyl dimorpholinophosphate(s) have the general formula $C_nF_{2n+1}C_mH_{2m}OP(O)[N(CH_2CH_2)_2O]_2$.

88. A method according to claim 87 wherein the F-alkyl dimorpholinophosphate is $C_8F_{17}C_{11}H_{22}OP(O)[N(CH_2CH_2)_2O]_2$.

89. A method according to any preceding claim, wherein the internal environment of the microcapsules is modified by the addition of one or more reagents to the oil phase.

90. A method according to any preceding claim, wherein the compounds are coupled to beads with different optical properties.

91. A method according to claim 90, wherein the beads have different fluorescent properties.

92. A method according to claim 90, wherein the different optical properties of the beads are due to incorporation of different levels of two or more fluorochromes into the beads.

93. A method according to claim 90, wherein the different optical properties of the beads are due to incorporation of different numbers of quantum dots with different emission spectra.

94. A method according to claim 90, wherein the different optical properties of the beads are used to identify the compound bound to the beads.

95. A method according to any preceding claim, wherein the compounds on the isolated beads are identified by releasing the compounds from the beads and direct analysis.

96. A method according to claim 95, wherein the compounds on the isolated beads are identified by mass-spectroscopy.

97. A product when isolated according to the method of any preceding claim.

98. A method for preparing a compound, comprising the steps of:
(a) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;
(b) identifying the compounds which possess the desired activity.
(c) identifying and producing the compound having the desired activity.

99. A method for preparing a compound, comprising the steps of:
(a) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;
(b) identifying and, optionally, sorting the microcapsules which have contain the compound(s) having the desired activity using a change in their optical properties; and
(c) identifying and producing the compound having the desired activity.

100. A method for screening a compound or compounds capable of modulating the activity of a target, comprising the steps of:
(a) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;

(b) identifying and, optionally, sorting the microcapsules which contain the compound(s) having the desired activity using a change in their optical properties; and

(c) contacting a target having a desired activity with the compound or compounds and monitoring the modulation of an activity of the target by the compound or compounds.

101. A method for preparing a target comprising the steps of:

(a) providing a synthesis protocol for a target wherein at least one step is facilitated by a compound;

(b) preparing variants of the compound which facilitates this step;

(c) compartmentalising the compounds into microcapsules, such that only a subset of the repertoire is represented in multiple copies in any one microcapsule;

(d) identifying and, optionally, sorting the microcapsules which contain the compound(s) having the desired activity using a change in their optical properties; and

(e) preparing the target using the compound identified in (d) to facilitate the relevant step of the synthesis.

102. A method according to any one of claims 98 to 102, wherein the compunds are attached to microbeads, and the compounds are optionally releaased from the microbeads inside the microcapsules.

103. A method according to claim 13, wherein a compound and a target are initially located in different microcapsules which are subsequently fused.

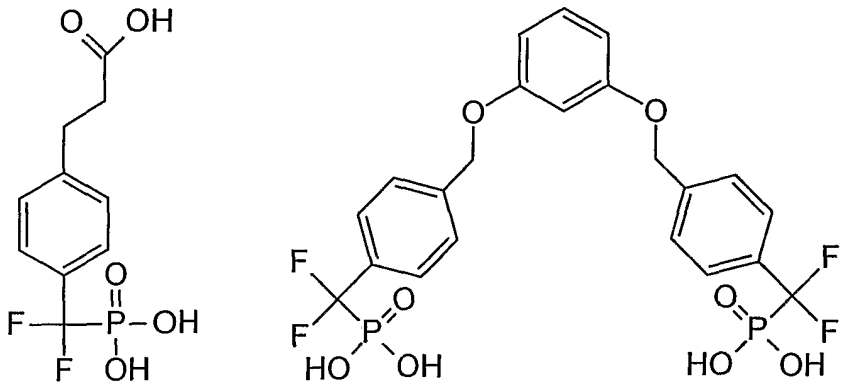
104. A method according to any preceding claim wherein a microcapsule containing a compound is split to generate two or more microcapsules.

105. A method according to claim 13 wherein a microcapsule containing a target is split to generate two or more microcapsules

106. A method according to any preceding claim wherein a cell or cells are located in a microcapsule.

107. A method according to claim 106 wherein the compound modulates the activity of a target in the cell(s).

108. A method according to claim 107 wherein the compound modulates an activity of the cell(s).



1 K_i = 420μM

2 K_i = 1.7μM

FIG. 1

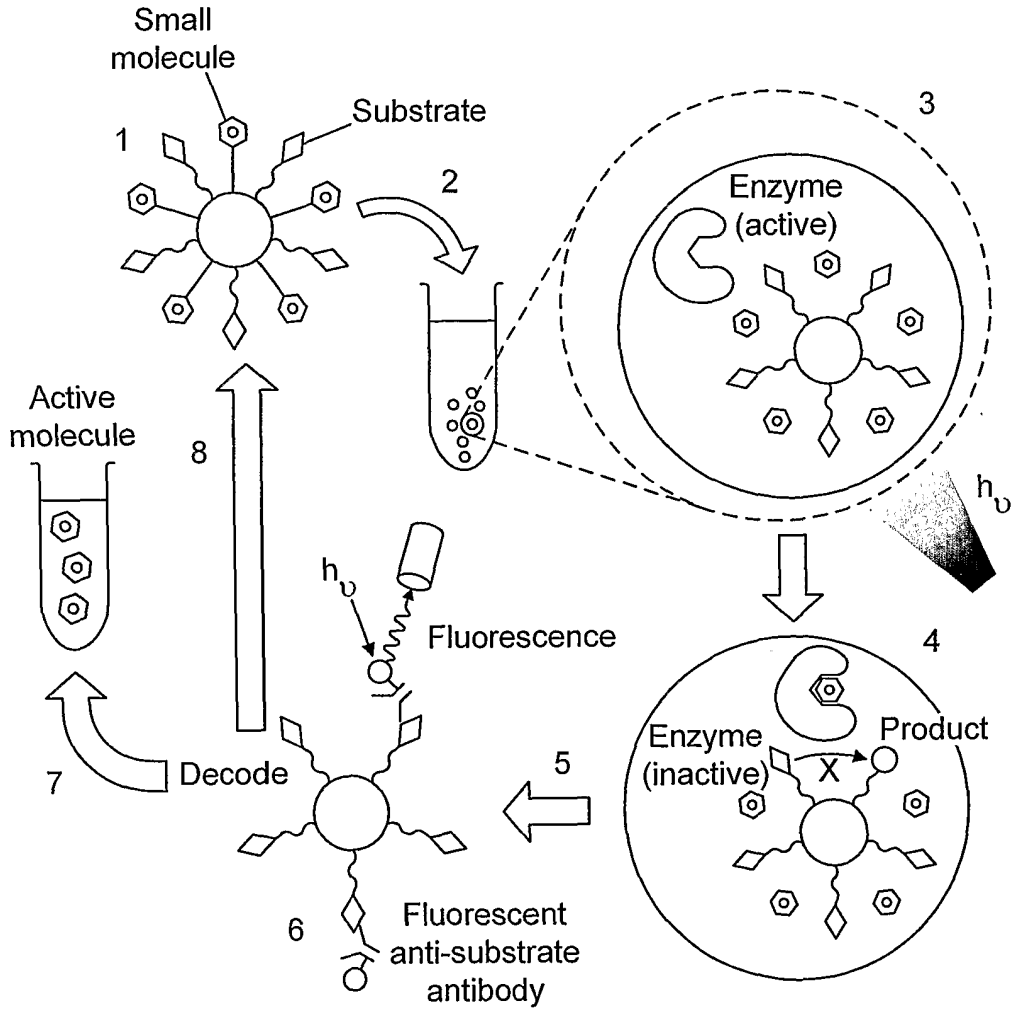


FIG. 2

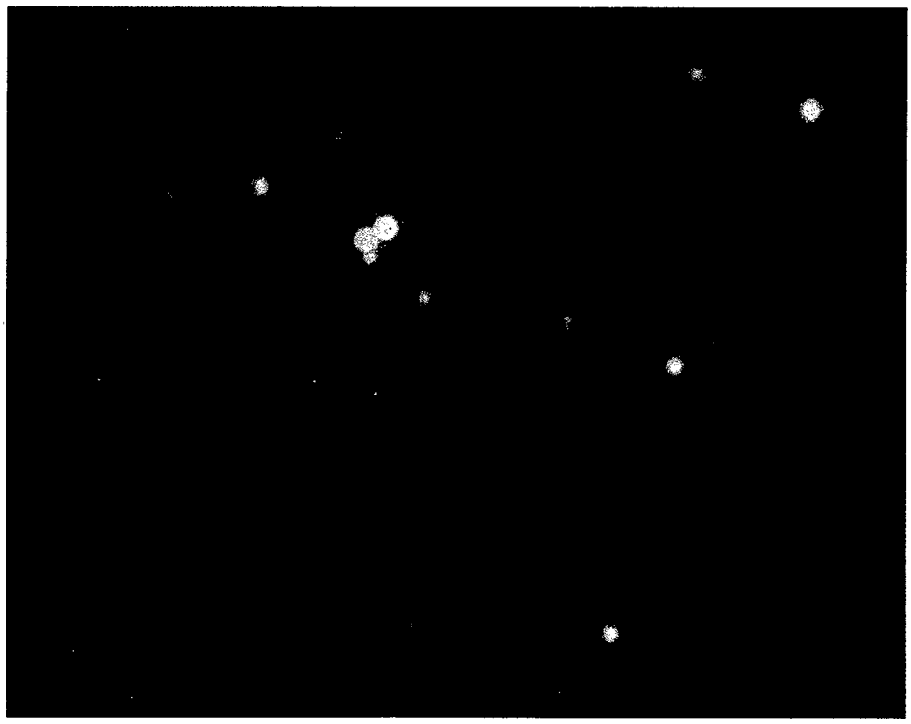


FIG. 3

INTERNATIONAL SEARCH REPORT

ıational Application No
'/GB2004/001362

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543

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 7 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)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 165 778 A (KEDAR HAIM) 26 December 2000 (2000-12-26) abstract column 38, line 35 - line 65 column 39, line 1 - line 12 column 54, line 41 - line 67 ---	1-96, 98-109
A	STRIZHKOV BORIS N ET AL: "PCR amplification on a microarray of gel-immobilized oligonucleotides: Detection of bacterial toxin- and drug-resistant genes and their mutations" BIOTECHNIQUES, EATON PUBLISHING, NATICK, US, vol. 29, no. 4, October 2000 (2000-10), pages 844-857, XP002167651 ISSN: 0736-6205 the whole document --- -/--	1-96, 98-108

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

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

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 June 2004	03/08/2004

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INTERNATIONAL SEARCH REPORT

national Application No
T/GB2004/001362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Form PCT/ISA/210 (continuation of second sheet) (January 2004)

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