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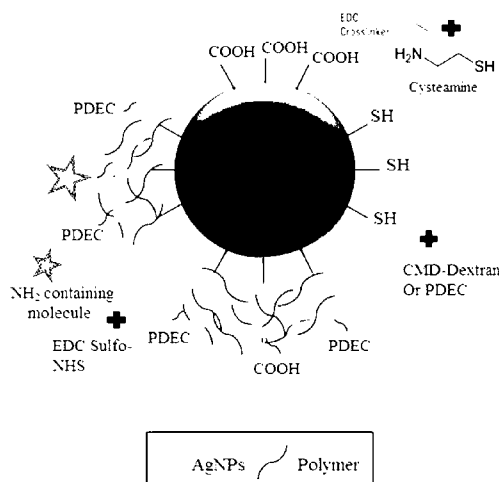
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FIG 8



(57) Abstract: A polymer which has a functionality capable of reacting with metallic or metallic alloy particles is used to form a scaffold for a sensor by binding the polymer to a substrate surface and allowing the polymer to crosslink with a plurality of metallic or metallic alloy particles to generate a three dimensional multi-layer polymeric network with the particles bonded in place therein. The polymer will accommodate subsequent binding of a detection agent, such as an antibody or antibody fragment, to form the sensor.

SENSOR SCAFFOLD

FIELD OF THE INVENTION

[0001] The present invention relates to sensors and in particular to a scaffold and
5 a method for constructing a scaffold for a sensor for detecting a target molecule in a sample.

BACKGROUND OF THE INVENTION

[0002] It is to be understood that, if any prior art information is referred to herein;
such reference does not constitute an admission that the information forms part of
10 the common general knowledge in the art, in Australia or any other country.

[0003] Detection of target molecules or analytes in biological samples is central to
diagnostic monitoring of health and diseases. Key requirements of analyte detection
are specificity and sensitivity, particularly when the target molecule or analyte is in a
limiting amount or concentration in a biological sample.

15 [0004] Typically, specificity is provided by detection agents such as antibodies
which specifically interact with (i.e. bind or recognize) the analyte. Sensitivity is
typically provided by a label bound to the specific antibody, or to a secondary
antibody which assists detection of relatively low levels of analyte. Current methods
to detect specific target molecules or analytes for diagnostic purposes include
20 biosensors, which are analytical devices that combine a solid support sensor surface
upon which the detection agents are immobilised, with a physicochemical detector.
One of the most well known and widely used sensors utilizing the diagnostic
approach above involves the enzyme-linked immunosorbent sandwich assay (ELISA)
format, in which the detection agent is immobilised to the surface of a solid support
25 such as a microplate and the fluorescence signal arising from the successful
recognition of the analyte in a biological sample by the detection agent is detected
using a microplate reader.

[0005] However, the methods via which the detection agents are immobilised
typically involve physical or covalent immobilisation of the detection agents to the
30 sensor surface in a specific manner while minimising non-specific binding of
undesirable materials (i.e. unwanted binding of proteins, cells, bacteria, and the like)
through the use of biocompatible coatings. In particular, such methods often suffer
from a number of limitations which significantly restrict their widespread application.

Such limitations most notably include poor sensitivity due to an insufficient number of capture probes being immobilised on the sensor surface or poor access to the immobilised capture probes.

5 [0006] Indeed, the accuracies of traditional ELISAs vary considerably when the sample concentrations are lower than 1 µg/L.

10 [0007] With the advent of nanotechnology, it has been possible to produce materials having dimensions of the same order as those of the biological molecules that are specifically targeted for detection in biological samples using such detection methods as ELISA. Among these particles are quantum dots which are fluorescent particles typically composed of semiconductor materials that bridge the gap between individual atoms and bulk semiconductor solids. Owing to this intermediate size, quantum dots possess unique properties unavailable in either individual atoms or bulk materials. Most notably, the small size of quantum dots means that the electronic characteristics (that is, the bandgap and emission wavelength of a quantum dot) may be tuned by simply adjusting its size, with smaller quantum dots emitting at shorter wavelengths. Thus, by adjusting their size and composition, it is possible to produce quantum dots that emit fluorescent light from the ultraviolet (UV) region, through the visible region, and into the infrared (IR) region.

20 [0008] Importantly, from a biological perspective, quantum dots can absorb and emit light very efficiently, allowing highly sensitive detection relative to conventionally used organic dyes and fluorescent proteins. Indeed, individual quantum dots are significantly brighter than organic dyes, thus enabling highly sensitive detection of analytes in low concentration. As well as having excellent fluorescence intensity, quantum dots also possess a photobleaching resistance ability making them ideally suited as labels for capture probes to detect specific target molecules or analytes in low concentration.

25 [0009] There remains a need to improve one or more of the brightness, sensitivity, and stability of scaffolds for use as probes in immunoassays.

30

SUMMARY OF THE INVENTION

[0010] According to a first aspect of the present invention, there is provided a method of constructing a scaffold for a sensor, the method comprising the steps of:

- 5 (a) contacting a scaffold surface with a polymer, the polymer having a reactive functionality suitable for binding to each of (i) the scaffold surface; (ii) a metal or metal alloy; and (iii) a detection agent;
- (b) contacting the polymer with a plurality of metallic or metallic alloy particles; and
- (c) allowing the polymer to bind to the scaffold surface and the metallic or metallic alloy particles to form a multi-layer polymeric network, cross-linked with the metallic or metallic alloy particles, which network is bound to the scaffold surface,
- 10 to thereby construct the scaffold.

[0011] In one embodiment, the scaffold surface may be a metallic or metal alloy surface, a ceramic surface, a silica surface or a polymeric surface.

[0012] The metallic surface may be a gold or silver plate or magnetic nanoparticle.

15 [0013] The silica surface may be colloidal silica or a surface of a silica-containing particle.

[0014] The polymeric surface may be a surface of a polymeric matrix, preferably of a polymer bead.

20 [0015] The contacting of the polymer with the plurality of metallic or metallic alloy particles may occur at the same time as or subsequent to the contacting of the scaffold surface with the polymer.

[0016] Preferably, the contacting of the polymer with the plurality of metallic or metallic alloy particles occurs at the same time as the contacting of the scaffold surface with the polymer.

25 [0017] The reactive functionality suitable for binding to each of the scaffold surface, the metal or metal alloy and the detection agent may be the same or different.

30 [0018] In one embodiment, the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy and the detection agent are the same functionality.

[0019] In an alternative embodiment, the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy and the detection agent may be a different functionality for one or more of the same.

5 [0020] The reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy and the detection agent may be a chalcogen-containing functionality.

[0021] Preferably, the reactive functionality comprises two bonded chalcogen atoms each of which may be independently selected from S, O, Se and Te, for example S-S.

10 [0022] In one embodiment, the chalcogen atoms may have one or two oxygen atoms bonded thereto.

[0023] Preferably, the terminal chalcogen atom is attached to an R group to form a leaving group. In one embodiment, the R group is selected such that the conjugate acid formed by the R group and attached chalcogen atom leaving the polymer has a
15 pKa of less than 8, preferably less than 6 and more preferably less than 4. For example HS-R could be the conjugate acid formed by the leaving group of an –S-S-R moiety.

[0024] In one embodiment, the reactive functionality suitable for binding to each of the scaffold surface, the metal or metal alloy and the detection agent may be
20 independently selected from a disulfide-containing functionality, a maleimide containing functionality and a functionality reactive with a thiol group.

[0025] In one embodiment, the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy and the detection agent comprises -S-S-R wherein R is selected from an electron withdrawing group, aryl, heteroaryl and C₁-C₁₂
25 alkyl, each of which may be substituted.

[0026] Preferably the substitution is with an electron withdrawing group such as halide, nitro, nitrile and carbonyl.

[0027] When R is heteroaryl it may be selected from pyrrole, imidazole, pyrazole, pyridine, furan, thiophene, oxazole, thiazole, diazine, oxazine and thiazine.

30 [0028] In one embodiment, R comprises a 2-pyridyl group.

[0029] The polymer may be a branched hydrophilic polymer.

[0030] The polymer may be a dendrimer or a polysaccharide, each of which may be optionally derivatised.

[0031] In one embodiment, the polymer is selected from the group consisting of dextran, cellulose, agarose, sepharose, hyaluronic acid, agarose, nitrocellulose, polyvinylalcohol, partially hydrolysed polyvinylacetate and polymethylmethacrylate, carboxymethyl cellulose, carboxymethyl dextran and the like, any one of which may optionally be derivatised.

[0032] In one preferred embodiment, the polymer is 2-(pyridinyldithio)ethyl carbomoyl dextran (PDEC-dextran), PDEA-dextran or CMD-dextran. Polymers such as CMD-dextran may be appropriately modified to display the required functionality.

[0033] In one embodiment, the metallic or metallic alloy particles are selected from the group consisting of gold particles, silver particles, platinum particles, iron particles and other magnetic particles, copper particles and metal-containing quantum dots.

[0034] In one embodiment, the particles may be nanoparticles.

[0035] Preferably, the metal-containing quantum dots are selected from the group consisting of a III-V group compound, a II-VI compound, and a IV group element.

[0036] Preferably, the metal-containing quantum dots are selected from the group consisting of CdSe, CdS, CdTe, ZnSe, ZnS, ZnTe, CdSe/ZnS, ZnCdSe/ZnS, InP, InN, InAs, InGaP, GaP and GaAs.

[0037] In one embodiment, the metal-containing quantum dots are organic metal-containing quantum dots.

[0038] In one embodiment, the metallic or metallic alloy particles have an average particle size of 0.5 to 100 nm.

[0039] The method may further comprise the step of choosing the amount of the metallic or metallic alloy particles which are made available to the polymer to thereby control the thickness and/or density of the multi-layer polymer network which forms.

[0040] In one embodiment, the method further comprises the step of attaching a fluorescent probe to the polymer.

[0041] The fluorescent probe may be attached to the reactive functionality, preferably via a thiol group.

[0042] In a further embodiment, the method further comprises the step of attaching a reporter agent, for example HRP enzyme, to the polymer

[0043] In one preferred embodiment wherein the scaffold surface is the surface of a polymer matrix then the method may further comprise, before step (a), the step of
5 incorporating a plurality of metallic or metallic alloy particles within the polymer matrix.

[0044] The metallic or metallic alloy particles incorporated within the polymer matrix may be the same or different from the metallic or metallic alloy particles cross-linked within the multi-layer polymeric network. They may be selected from the same
10 groups already described.

[0045] In one embodiment, the metallic or metallic alloy particles incorporated within the polymer matrix may be magnetic particles, including iron particles.

[0046] In one embodiment, the polymer matrix comprises both magnetic particles and a second population of particles selected from gold nanoparticles, silver
15 nanoparticles and quantum dots.

[0047] In one embodiment, the method further comprises, before the step of incorporating the metallic or metallic alloy particles within the polymer matrix, the step of swelling the polymer matrix in an organic solvent. This assists with incorporation of the metallic or metallic alloy particles therein.

[0048] In this embodiment, wherein the scaffold surface is the surface of a polymer matrix, then the method may further comprise, prior to contacting the scaffold surface with the polymer, the step of adding a polymerisable reagent to the swollen polymer matrix with incorporated metallic or metallic alloy particles.

[0049] In this embodiment, the method may further comprise the step of initiating
25 polymerisation of the polymerisable reagent to form polymer strands located within the polymer matrix and forming part of the scaffold surface to thereby entrap the plurality of metallic or metallic alloy particles incorporated into the polymer matrix.

[0050] Preferably, the polymerisable reagent has an anchoring group which, after the polymerisation of the polymerisable reagent, presents as part of the scaffold
30 surface as a group reactive with the reactive functionality of the polymer.

[0051] In one embodiment, the anchoring group of the polymerisable reagent is a thiol group.

[0052] In one embodiment, the method is a method of constructing a scaffold for a sensor comprising the steps of:

- (ai) swelling a polymer matrix in an organic solvent and incorporating a plurality of metallic or metallic alloy particles within the polymer matrix;
 - 5 (a) contacting the polymer matrix with a polymer, the polymer having a reactive functionality suitable for binding to each of (i) the polymer matrix; (ii) a metal or metal alloy; and (iii) a detection agent;
 - (b) contacting the polymer with a plurality of additional metallic or metallic alloy particles; and
 - 10 (c) allowing the polymer to bind to both the polymer matrix and the additional metallic or metallic alloy particles to form a multi-layer polymeric network, cross-linked with the metallic or metallic alloy particles, the network thereby bound to the polymer matrix,
- to thereby construct the scaffold.

15 [0053] According to a second aspect of the present invention, there is provided a scaffold for a sensor, comprising:

- (a) a scaffold surface;
- (b) a multi-layer polymeric network bound to the scaffold surface; and
- (c) a plurality of metallic or metallic alloy particles bound within and
- 20 crosslinking the multi-layer polymeric network.

[0054] The scaffold surface, multi-layer polymeric network and metallic or metallic alloy particles are as described for the first aspect.

[0055] In one embodiment, wherein the scaffold surface is the surface of a polymer matrix then the scaffold may further comprise a plurality of metallic or

25 metallic alloy particles within the polymer matrix.

[0056] The metallic or metallic alloy particles incorporated within the polymer matrix may be the same or different from the metallic or metallic alloy particles cross-linked within the multi-layer polymeric network.

[0057] The metallic or metallic alloy particles incorporated within the polymer matrix may be substantially physically entrapped therein by the presence of polymer strands formed from polymerisation of a polymerisable reagent.

[0058] The polymer strands formed by the polymerisable agent are preferably
5 different from the polymer forming the multi-layer polymeric network.

[0059] In one embodiment, the metallic or metallic alloy particles incorporated within the polymer matrix may comprise magnetic particles.

[0060] The multi-layer polymeric network may be bound to the scaffold surface, at least in part, via an anchoring group presented by the polymer strand formed from
10 the polymerisable reagent, at least a portion of the polymer strand forming part of the scaffold surface.

[0061] According to a third aspect of the present invention, there is provided a sensor for use in detecting a target molecule in a sample comprising:

- (a) a scaffold according to the second aspect; and
- 15 (b) at least one detection agent bound to the multi-layer polymeric network of the scaffold which is capable of detecting the target molecule in the sample.

[0062] According to a fourth aspect of the present invention, there is provided a method of detecting a target molecule in a sample comprising the steps of:

- 20 (a) contacting the sensor of the third aspect with a sample to expose the at least one detection agent to the target molecule in the sample; and
- (b) detecting a signal resulting from the formation of a complex or conjugate between the at least one detection agent and the target molecule.

[0063] According to the aforementioned aspects, the target molecule may be any
25 molecule that is detectable by the detection agent. Such target molecules may be any molecule that is capable of forming a complex or conjugate with the detection agent. Non-limiting examples of target molecules include proteins inclusive of antibodies or antibody fragments, peptides, enzymes, epitopes and other antigens, antigen receptors, hormone receptors, growth factor receptors, cytokine receptors,
30 hormones, growth factors or cytokines, small organic molecules inclusive of drugs and metabolites, carbohydrates, lipids, cells and cellular fractions, organelles or other components thereof, biological markers such as cell surface markers, nucleic

acids such as single- or double stranded DNA and RNA, and derivatives, cooligomers and any fragments or combinations thereof. Small molecules, ions, carbohydrates, whole cells, pathogens, bacteria (inclusive of Gram positive and Gram negative bacteria), viruses, protozoa, fungi and the like and/or molecular components thereof may also be, or comprise, a target molecule.

[0064] In certain embodiments of the aforementioned aspects, the detection agent may be any molecule that can be bound to the multi-layer polymeric network which is capable of forming a complex or conjugate with the target molecule. Non-limiting examples of the at least one detection agent include small molecules, ions, carbohydrates, whole cells, bacteria, viruses or molecular components thereof, lectins including WGA and ConA, fungi as well as proteins inclusive of antibodies or antibody fragments, peptides, enzymes, epitopes and other antigens, antigen receptors, hormone receptors, growth factor receptors, cytokine receptors, hormones, growth factors or cytokines, , cells and cellular fractions, organelles or other components thereof, biological markers such as cell surface markers, nucleic acids such as single- or double stranded DNA and RNA, and derivatives, cooligomers and any fragments or combinations thereof.

[0065] The detection agent may present a functional group for binding to the polymer of the multi-layer polymeric network. The functional group may be naturally present on the detection agent or the detection agent may be modified to present the required group. In one preferred embodiment, the detection agent presents a free thiol group to enable binding to the multi-layer polymeric network.

[0066] It will therefore be appreciated that in some embodiments, the detection agent and the target molecule may be "complementary binding partners". By this is meant, for example, an antibody and antigen (e.g. an epitope), whereby the antigen or epitope may be the target molecule and the antibody or antibody fragment may be the detection agent, or *vice versa*. It will also be apparent to a skilled person that complementary binding partners could include complementary DNA strands, growth factors and growth factor receptors and enzymes and substrates, although without limitation thereto.

[0067] Accordingly, the sensor and method of detecting a target molecule may be useful for detecting target molecules in samples such as biological samples, diagnostic samples, food samples, air samples, water samples and the like. Non-

limiting examples of biological and/or diagnostic samples include tissue, organ and tumour biopsies, body fluids such as urine, blood, serum, cerebrospinal fluid, semen, tears and sputum, although without limitation thereto.

[0068] In one particular embodiment, the detection agent is an antibody or antibody fragment. The antibody or antibody fragment may be polyclonal, monoclonal or recombinant synthetic inclusive of single chain fragments such as scFvs, F(ab) and F(ab')₂ fragments, diabodies and triabodies, although without limitation thereto.

[0069] Accordingly, a preferred sensor may be suitable for use in an immunoassay.

10 [0070] One particular embodiment of the method of the fourth aspect includes:

(a) contacting the sensor of the third aspect with a sample to expose the at least one antibody or antibody fragment of the sensor to a target molecule in the sample; and

15 (b) detecting a signal resulting from the formation of a complex or conjugate between the at least one antibody or antibody fragment and the target molecule.

[0071] In a preferred form of this embodiment, the target molecule is bound to another antibody or antibody fragment prior to step (a). By way of example, said another antibody or antibody fragment may be coupled or immobilized to a surface of a vessel, such as a well of a microwell plate.

[0072] In one embodiment of the fourth aspect the method may include the step of exposing the sensor comprising metal nanoparticles to a second population of particles comprising incorporated quantum dots.

25 [0073] In this embodiment, the metal nanoparticles are preferably gold or silver nanoparticles within the multi-layer polymeric network.

[0074] In this embodiment, the two populations of particles are in a sample with the target molecule and the reduction in fluorescence of the quantum dots is measured as an output.

30 [0075] Throughout this specification, unless otherwise indicated, "*comprise*", "*comprises*" and "*comprising*" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

[0076] It will also be appreciated that the indefinite articles “a” and “an” are not to be read as singular indefinite articles or as otherwise excluding more than one or more than a single subject to which the indefinite article refers. For example, “a” protein includes one protein, one or more proteins or a plurality of proteins.

5

BRIEF DESCRIPTION OF THE DRAWINGS

[0077] Notwithstanding any other forms which may fall within the scope of the present invention, preferred embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings in which:

[0078] Fig. 1 shows (i) a standard curve of fluorescence intensity (a.u.) versus
10 quantum dot concentration (mol) for a supernatant of a first washing step (in 5:95 toluene-butanol) following washing of a suspension of swollen polymer microspheres loaded with quantum dots according to one preferred embodiment of the present invention, and (ii) a corresponding chart to estimate the incorporation efficiency of quantum dots within the loaded microspheres;

15 [0079] Fig. 2 shows (i) a standard curve of absorbance (at 412 nm) versus sulfhydryl group concentration (mol), and (ii) a corresponding chart to quantify the density of sulfhydryl groups on the surface of five different batches of polymer bead loaded with quantum dots of Fig. 1;

[0080] Fig. 3 shows (i) a standard curve of fluorescence intensity (a.u.) versus
20 quantum dot concentration (pmol) for a supernatant of a first washing step (in 5:95 toluene-butanol) following washing of a suspension of polymer beads after incubation in 2-(pyridinyldithio)ethyl carbomoyl dextran (PDEC-dextran) in the presence of a plurality of additional quantum dots to produce a multi-layer polymeric network with cross-linked additional quantum dots, and (ii) a corresponding chart to estimate the
25 incorporation efficiency of additional quantum dots within the multi-layer polymeric network;

[0081] Fig. 4 shows (i) a standard curve of absorbance (at 562 nm) versus
immunoglobulin (IgG) concentration ($\mu\text{g/mL}$), and (ii) a corresponding chart to
30 estimate the density of antibody fragments (mFabs) immobilized on the surfaces of scaffolds prepared according to one preferred embodiment of the present invention; and (iii) a chart of fluorescence intensity (a.u.) versus $\log_{10}$ quantum dot concentration (ng/mL) showing a comparison of a scaffold of the present invention

with functionalised commercial particles (Life Tech. F8811) with the same antibody pair used in each experiment;

[0082] Fig. 5 shows a chart of fluorescence intensity (a.u.) versus storage time (days) which provides a comparison of the stability of a batch of scaffolds constructed according to the present invention against batches of two reference scaffolds;

[0083] Fig. 6 a) – c) is a series of graphs showing the progressive increase in the fluorescence intensity due to a) increase in HRP activity with an increase in number of polymer layers in the multi-layer polymer network b) increase in fluorescence intensity with increase in number of polymer layers in the multi-layer polymer network c) increase in fluorescence intensity with increase in QDs incorporated due to increase in number of polymer layers in the multi-layer polymer network;

[0084] Fig. 7 a) – c) is a series of graphs showing dose response curves for the detection of Myoglobin using a) HY_QD, b) HY_FP and c) HY_HRP particles;

[0085] Fig. 8 is a diagrammatic representation of the polymer bioconjugation chemistry for experiments on the attachment of lectins to substrate particles;

[0086] Fig. 9 is a graphical representation of the BCA protein quantification standard curve with comparison of protein content achieved on scaffolds using direct (triangle) and multi-layer polymeric network (square) coupling of WGA;

[0087] Fig. 10 is a graphical demonstration of the bacterial capture from undiluted platelets;

[0088] Fig. 11 is a graphical representation of the results of non-specific binding experiments, with negative controls, in platelets;

[0089] Fig. 12 is a graphical representation of the detection limit for *S.Aureus* in platelets using MCC174 blue beads functionalized with a CMD-dextran multilayer polymeric network;

[0090] Fig. 13 is a graphical representation of the decrease in fluorescence of quantum dots with the use of either gold or silver nanoparticles after (a) 1 hour and (b) 2 hours; and

[0091] Fig. 14 is a graphical representation of the decrease in fluorescence of quantum dots with the use of varying amounts of silver nanoparticles.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

[0092] The present invention is predicated, at least in part, on the finding that a polymer which has a functionality capable of reacting with metallic or metallic alloy particles can be used to form the basis of a scaffold for a sensor by binding the polymer to a substrate surface of the proposed scaffold and allowing the polymer to crosslink with a plurality of the metallic or metallic alloy particles to generate a three dimensional multi-layer polymeric network with the particles bonded in place therein. The polymer also has a functionality which allows the subsequent binding of a detection agent, such as an antibody or antibody fragment, to form the sensor.

[0093] The presence of this multi-layer polymeric network provides great advantages in operation compared with the formation of a simple monolayer of polymer or other species displaying a detection agent, including that it substantially enhances the reactive polymer surface area available for anchoring of detection agents. This has the beneficial knock on effect that the greater density of detection agent, for example an antibody, on the scaffold will greatly improve capture kinetics thereby providing for improved sensitivity and increased speed during detection. The multi-layer polymeric network also has the beneficial effect of greatly reducing non-specific binding to the substrate.

[0094] Further, the fixing of the metallic or metallic alloy particles within the multi-layer polymeric network reduces the level of quenching that is observed compared to the situation when metallic or metallic alloy particles are only present within the body of the scaffold substrate, such as within the matrix of a polymer bead or microsphere. The polymer matrix of the polymer bead can reduce the excitation signal observed during use of the sensor. Surprisingly, this problem is substantially reduced or avoided when the metallic or metallic alloy particles are incorporated within the multi-layer polymeric network and therefore distanced from the polymeric matrix of the bead resulting in superior brightness being observed.

[0095] In one preferred embodiment, the brightness of the emitted signal can be greatly increased by entrapping metallic or metallic alloy particles both within the polymer matrix of the polymer bead substrate and also within the cross-linked multi-layer polymeric network thereby greatly amplifying the response from the biosensor.

[0096] The invention has been developed primarily for use in sensing and detecting analytes of interest in biological samples and will be described hereinafter

with reference to this application. However, it will be appreciated that the invention is not limited to this particular field of use.

[0097] The term “multi-layer polymeric network”, as used herein, signifies that the polymer structure referred to extends beyond a single layer (monolayer) on the scaffold surface. That is, the polymer structure referred to will have a complex 3-dimensional structure due to crosslinking with a plurality of metallic or metallic alloy particles comprising a plurality of layers of the polymer extending away from the scaffold surface to which only the lowermost polymers are actually directly physically anchored or bound to. Therefore, the multi-layer polymeric network comprises multiple interconnected polymers in extending between the substrate surface and the outer extent of the formed polymeric scaffold.

[0098] The scaffold surface may be a surface of a substrate such as a polymer bead, ceramic particle, silica-containing particle or colloidal silica, metal, such as a gold or silver electrode plate, or other metal or metal alloy structure. It will be appreciated that the nature of the substrate, and hence the scaffold surface, is not particularly limited and potentially any surface which can bind, or be coated, functionalised or modified in any manner to bind, the polymer forming the multi-layer polymeric network may be suitable. A wide range of such substrates and scaffold surfaces are already in use commercially and would be known to the person skilled in the art. Hence, any functionalised surface presenting, for example -NH_2 , -SH , -OH , -COOH and similar reactive moieties may be suitable. Of course the functionality presented by the scaffold surface may dictate the conjugation strategy with the polymer but such adjustments would be within the skill of the skilled artisan and modifications could be tested in a standard manner. In one further non-limiting example, an APTES or MPTS modified ELISA plate may be a suitable option as a substrate and hence scaffold surface.

[0099] It will be appreciated that the shape or form which the substrate takes is not particularly limited. The only requirement is that the polymer to form the multi-layer polymeric network can bind itself to the scaffold surface of that substrate. This may be achieved by a natural reactivity with the surface, for example the reactivity of a disulfide moiety attached to a good leaving group with a metallic or metallic alloy surface, or by modification of the surface, for example thiol modification of a polymer bead functionality or introduction into such a bead of a thiol-containing polymer to

thereby react with the same disulfide moiety. Such a modification to introduce the required functionality is within the present use of the term "scaffold surface". That is, the term "scaffold surface" as used herein includes situations wherein a further polymer presenting the functionality is added to the polymeric matrix and the polymer to form the multi-layer polymeric matrix can access that functionality whether or not it physically sits at the surface of the substrate or not. Such a situation is explicitly considered to present the functionality on or as forming a component of the "scaffold surface".

[00100] When the substrate, and hence scaffold surface, is polymeric then the nature of that substrate may be selected, in certain embodiments, on the basis of its being capable of swelling to a reasonable size when suspended in a suitable solvent or solvent system to then receive a plurality of metallic or metallic alloy particles, such as quantum dots, within the pores of the swollen polymer matrix. Such polymer substrates can be purchased commercially in various sizes and with various functional groups on their surface. For example, polyethylene-based microspheres, polystyrene-based microspheres and latex microspheres are common, but the present scaffold is not limited thereto.

[00101] In one embodiment of the present invention it is preferred to swell the polymer matrix of the scaffold substrate to allow incorporation of metallic or metallic alloy particles therewithin. It has been found through a study of various solvents that a preferred solvent or solvent system comprises an organic component to not only induce the swelling of the polymer matrix, for example latex microspheres, but also assists in preventing aggregation of the latex microspheres.

[00102] Of the solvents tested, those organic solvents that are immiscible in water were found to induce a degree of aggregation and so, while still allowing construction of the scaffold, are less preferred. Those solvents that were found to exhibit a pronounced polarity but have a minimal hydrophobic component were found to induce the least amount of aggregation of latex microspheres.

[00103] In the case of latex microspheres, it was found that protic solvents were useful. Examples of such solvents may include methanol, ethanol, isopropanol and butanol, which all successfully swelled the microspheres to a sufficient size, with the latter solvent demonstrating the best results. The solvent preference may change with changing nature of the substrate polymer. Information on the swelling

characteristics of various solid-phase particles is readily available in commercial catalogues and the like.

[00104] Good results were observed when a plurality of latex microspheres were suspended in a mixed solvent system of 5% toluene in butanol or 5% THF in butanol to induce swelling of the latex microspheres. Good results were also observed for the following solvent systems: 50% propylene glycol in methanol, 5% toluene in butanol and 5% THF in butanol.

[00105] Once swollen, this embodiment comprises the step of incorporating a plurality of metallic or metallic alloy particles, such as quantum dots, within the pores of the polymer matrix of the swollen substrate, for example latex microspheres, to produce a plurality of substrate particles loaded with quantum dots, referred to herein as the loaded substrate.

[00106] The inventors have found that the organic component within the solvent or solvent system maintains the plurality of quantum dots colloidally stable. It will be appreciated that this latter property of the solvent will be particularly relevant in the case of hydrophobic quantum dots.

[00107] In this embodiment, a polymerisable reagent is optionally added to the solution of loaded substrates. The polymerisable reagent may, in one embodiment, be mercaptopropyltrimethoxysilane (MPTS) which in the presence of a trace amount of water causes the MPTS to polymerise inside the pores of the polymeric matrix of the loaded substrates to form polymer strands and complete the entrapment of the quantum dots within the loaded substrates. This step not only completes the entrapment process, it also presents a plurality of sulfhydryl groups, from the polymerised MPTS, to form part of the scaffold surface of the loaded substrate.

[00108] It will be appreciated that, if metallic or metallic alloy particles are to be incorporated into a polymer matrix and this subsequently used as the substrate for the scaffold then it is not essential that a polymerisable agent such as MPTS be used to entrap the incorporated metallic or metallic alloy particles but it is nonetheless preferred to prevent leakage of the particles.

[00109] A person of skill in the art will also appreciate that the role of the polymerised MPTS is essentially a physical one whereby the formed polymer strands are physically entangled or engaged with the polymer matrix of the substrate, such as the latex microsphere. This effect could be achieved in a number of alternative

ways. The inclusion of the MPTS also provides a thiol anchoring group which effectively forms part of the scaffold surface and so provides a functionality to react with a complimentary functionality of the polymer used to form the multi-layer polymeric network, but this may not be necessary if such a functionality is inherently provided by the scaffold surface or the surface is otherwise modified to present such reactive functionality.

[00110] The nature of the polymerisable reagent will therefore depend on the anchoring group required, if any, to be located at the scaffold surface by that reagent once polymerisation is complete. In a preferred embodiment of the present invention the polymerisable reagent will present at least two alkoxy or aryloxy groups to allow polymerisation upon the addition of small amounts of water or aqueous acid.

[00111] Preferably the polymerisable reagent comprises two or more C₁ to C₁₂, preferably C₁ to C₆ alkoxy groups.

[00112] Preferably, the polymerisable reagent further comprises at least one group capable of reacting with a disulphide functionality. In one embodiment, the polymerisable reagent, in addition to the alkoxy or aryloxy groups described above, further comprises a thiol anchoring group.

[00113] It will be appreciated that the scaffold substrate and the metallic or metallic alloy particles used in the preferred embodiments of the present invention will depend largely on the specific requirements of the applications for which the scaffold is intended. This is particularly relevant in the case of quantum dots as the electronic characteristics of a quantum dot are closely related to its size and shape. For example, the bandgap in a quantum dot, which determines the frequency range of emitted light, is inversely related to its size. In fluorescent dye applications, the frequency of emitted light increases as the size of the quantum dot decreases. Consequently, the color of emitted light shifts from red to blue when the size of the quantum dot is made smaller. This allows the excitation and emission of the quantum dots to be highly tunable.

[00114] The metallic or metallic alloy particles may be in the nature of gold, silver, platinum, copper or iron particles or may be metal-containing quantum dots. In the case of quantum dots, these can be selected from the group consisting of an III-V group compound, an II-VI compound, or an IV group element (which are referred hereon in as an III-V group quantum dot, an II-VI quantum dot, or an IV group

quantum dot, respectively). Specific examples thereof include, but are not limited to, and may be selected from the following: CdSe, CdS, CdTe, ZnSe, ZnS, ZnTe, CdSe/ZnS, ZnCdSe/ZnS, InP, InN, InAs, InGaP, GaP and GaAs.

[00115] Good results have been obtained using CdSe/ZnS hydrophobic quantum dots (PL-QD-O-590, as commercially supplied by PlasmaChem Tech GmbH). The average size of the CdSe core of these quantum dots may vary between about 2 nm and about 10 nm, depending on the desired emission maximum required for the scaffold. For example, PlasmaChem Tech indicate on their website that a CdSe core diameter of 2.5 nm has an emission maximum of 530 nm, while a CdSe core diameter of 6.7 nm has an emission maximum of 650 nm. The website also indicates that the ZnS outer shell surrounding the core has a thickness of between about 0.5 nm and 0.7 nm.

[00116] Depending on the application, for example sorting or isolation of bound target analyte from a sample, it may be desirable to have magnetic particles incorporated into the substrate or multi-layer polymeric network. It may further be beneficial to have at least a second population of metallic or metallic alloy particles which provide alternative properties including better formation of the multi-layer polymeric network or fluorescence. For example, in one non-limiting embodiment, it may be desirable to have magnetic particles incorporated within the substrate and to optionally have quantum dots also incorporated. The multi-layer polymeric network generated on the surface of the substrate may then employ either quantum dots or metal particles, such as gold or silver particles, to form the scaffold.

[00117] Once loaded into the scaffold substrate, such as the latex microsphere, comes the step of contacting the scaffold surface of the loaded substrates with a polymer having a reactive functionality suitable for binding with the scaffold surface and attaching the polymer thereto. One non-limiting example of such a reaction is reaction of a disulfide functionality on the polymer with sulfhydryl groups on the scaffold surface of the loaded substrate.

[00118] The polymer can be any type of polymer that comprises a plurality of functional groups that can react with the plurality of complimentary groups on the scaffold surface. Preferably, the polymer is a branched hydrophilic polymer.

[00119] Preferably, the polymer is capable of binding to a functional group present on the surface of the substrate. This functional group may be part of the substrate

structure such as, for example, a pre-functionalised commercially available resin or may be added to the substrate by subsequent functionalisation or functional group transformation or via the polymerisable reagent which bears the functional group and either reacts with or simply physically interacts with the particle. If the substrate is a metal or metal alloy then it may simply be that the reactive functionality of the polymer reacts with and binds to the metal or metal alloy surface.

[00120] The polymer will also have a reactive functionality, being the same or different to that which binds to the substrate surface, allowing it to crosslink with a plurality of metallic or metallic alloy particles, such as quantum dots, to thereby fix them within the multi-layer polymeric network. Finally, the polymer will have, or may be modified to have, a further reactive functionality, including a suitable leaving group, which allows attachment of a desired detection agent.

[00121] The polymer is not limited to any particular molecular weight range as this may be dependent on the specific application. For example, if the polymer is immobilised in surface sensitive sensors a small molecular weight polymer may be preferred whereas, for particle substrates, a longer molecular weight polymer might be beneficial to extend over the surface roughness.

[00122] In one preferred embodiment, the polymer is PDEA-dextran functionalised with a reactive group, for example 2-(pyridinyldithio)ethyl carbomoyl dextran (PDEC-dextran). Sulfhydryl groups on the scaffold surface of the loaded substrate, with entrained polymerised MPTS, react with the available (2-pyridinyl)disulfanyl group of PDEC-dextran to form a corresponding disulphide bond with the subsequent formation of a 2-mercaptopyridine leaving group.

[00123] It will be appreciated that, in the embodiment wherein the reactive functionality of the polymer suitable for binding with the scaffold surface, the metal or metal alloy surface and the detection agent comprises a disulfide linkage then the leaving group forming part of this functionality is not limited to a 2-pyridinyl moiety. A person skilled in the art will appreciate that this may be any suitably reactive leaving group. In one embodiment, the polymer reactive functionality comprises $---S-S-R$ wherein R is selected from aryl, heteroaryl and C_1-C_{12} alkyl, each of which may be substituted preferably with electron withdrawing groups. When R is heteroaryl and aryl is may be selected from a C5-C7 ring, preferably a C6 ring.

[00124] In one embodiment, when R is heteroaryl it may be selected from pyrrole, imidazole, pyrazole, pyridine, furan, thiophene, oxazole, thiazole, diazine, oxazine and thiazine.

[00125] In embodiments, the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy and the detection agent may be a chalcogen-containing functionality.

[00126] Preferably, the reactive functionality comprises two bonded chalcogen atoms each independently selected from S, O, Se and Te. In one embodiment S-S may be preferred.

[00127] In one embodiment, the chalcogen atoms may further have one or two oxygen atoms bonded thereto. Preferably, the terminal chalcogen atom is attached to an R group such that a good leaving group is formed, for example the reactive functionality may comprise $-S-SO_2-CF_3$.

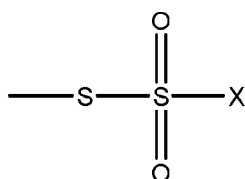
[00128] Therefore, in one embodiment, the reactive functionality may comprise:



wherein each dotted line may represent a bond, each W is independently selected from S, O, Se and Te, O is an oxygen atom which is either doubly bonded to the W or has a further atom attached directly to it and X is an electron-withdrawing atom or group and each W may have further atoms bonded thereto which are not shown.

20

[00129] In one specific embodiment the reactive functionality may comprise:



wherein X is an electron-withdrawing atom or group.

[00130] The electron-withdrawing atom or group may be selected from halogen, C_1 - C_6 alkylhalide, nitro, nitrile and carbonyl.

25

[00131] As mentioned above, the binding of the polymer to the scaffold surface preferably happens concurrently with the contacting of the polymer with an additional plurality of metallic or metallic alloy particles, such as a plurality of additional quantum dots, to enable the cross linking reaction to form the three dimensional multi-layer polymeric network rather than a simple monolayer. In the embodiment described thus far, not only will there be quantum dots entrapped within the loaded substrate, such as the latex microspheres, but there will also be additional quantum dots crosslinking the multi-layer polymeric network of the resulting scaffold.

[00132] The plurality of additional metallic or metallic alloy particles may also be selected from the same group as those discussed for incorporation into the polymer matrix of the substrate for example gold, silver, platinum, copper and iron particles as well as quantum dots selected from the group consisting of a III-V group compound, a II-VI compound, or a IV group element (which are referred hereon in as a III-V group quantum dot, a II-VI quantum dot, or a IV group quantum dot, respectively).

[00133] Preferably, the additional metallic or metallic alloy particles are organic quantum dots. Alternatively, if the metallic or metallic alloy particles are gold, silver, platinum, copper or iron particles then hydrophilic embodiments are preferred.

[00134] Good results have been obtained in practice using Qdot® ITK™ organic quantum dots (Q21711MP, as commercially supplied by Life Technologies). As indicated on the Life Technologies website, the Q21711MP quantum dots comprise a CdSe or CdTe core, a ZnS outer shell to stabilize the core, and an amphiphilic polymer coating around the layer to confer water solubility and to provide surface functional groups for use in physical or chemical interaction with the functional groups of other species.

[00135] When the metallic or metallic alloy particles are quantum dots then they preferably have an average particle size of 0.5 nm to 30 nm, more preferably 1 to 10 nm, and optimally 1.2 to 8 nm.

[00136] It will be appreciated that the plurality of additional metallic or metallic alloy particles within the cross-linked multi-layer polymeric network may have the same functional characteristics as the metallic or metallic alloy particles already entrapped within the loaded substrates, or different functional characteristics depending largely on the specific requirements of the application for which the scaffold is intended. In one non-limiting example, the metallic or metallic alloy particles within the loaded

substrate may be magnetic particles and the metallic or metallic alloy particles within the multi-layer polymeric network may be quantum dots. Hybrid fluoro/magnetic particles are already used to advantage in multi-modal imaging, for example MRI combined with fluorescence signalling. It also allows for the option of performing a magnetic enrichment step from a large volume (e.g. 10mL) into a small volume such as would be suitable for the typical ELISA 96-well plate format. The embedded fluorescence due to the quantum dots in the multi-layer polymeric network could be used for generating a signal as in fluorescent ELISA. This flexibility in approach and choice of emission signal can provide advantages in operation.

[00137] It will be appreciated that when the metallic or metallic alloy particles are all quantum dots and those entrapped within the loaded substrates have the same electronic characteristics as the additional quantum dots entrapped within the multi-layer polymeric network surrounding the loaded substrates, then the intensity of fluorescence emitted by all of the quantum dots combined will be significantly brighter than in the case where quantum dots are entrapped within the loaded substrates alone.

[00138] The discussion of the preferred embodiment above has focused on the use of a polymeric matrix containing substrate, such as latex microspheres. However, as has already been indicated, the invention is not so limited. If the substrate is a gold electrode, or other simple metallic or metallic alloy structure, then the steps involving swelling in organic solvent, incorporating metallic or metallic alloy particles within the substrate itself and entrapping them therein by use of a polymerisable reagent, such as MPTS, are not performed. Rather, in this embodiment of the present method, the metal or metal alloy substrate scaffold surface is simply contacted with the polymer, such as PDEC-dextran, and the metallic or metallic alloy particles which are to be incorporated within the forming multi-layer polymeric network. Due to the disulfide moiety and 2-pyridinyl leaving group on the PDEC-dextran the polymer reacts with the metal or metal alloy substrate surface to anchor itself there while at the same time a similar reaction with the surface of the metallic or metallic alloy particle effects the crosslinking reaction to form a scaffold comprising a multi-layer polymeric network comprising metallic or metallic alloy particles, such as gold, silver, platinum, copper or iron particles or quantum dots, bound to the metal or metal alloy substrate surface.

[00139] Such an embodiment will provide all of the advantages of increased surface area for detection agent binding and thereby increased sensitivity and reaction time for target molecule detection as well as reduced non-specific binding. Only the advantages provided by having metallic or metallic alloy particles incorporated within the substrate, and the possibility of increased brightness and dual functionality that this offers, are absent from this embodiment but these may not be required when a simple metal or metal alloy substrate is employed.

[00140] As is known in the art it may, in some embodiments, be beneficial to modify the surface of the metal or metal alloy substrate prior to contacting it with the polymer to either better prepare the surface for binding with the polymer or reduce non-specific binding thereto.

SENSOR

[00141] To assess the capability of the scaffolds of the preferred embodiments of the present invention for application in biosensors, the scaffolds were biofunctionalized with antibody fragments (mFABs) according to the protocol described in the Materials and Methods section below and employed in a sandwich-type immunoassay format as described. The results strongly emphasise the benefits provided by the present scaffolds which are derived from the increased surface area obtained by multi-layering the PDEC-dextran polymer. The effect of the multi-layer polymeric network is made clear by the observed greater density of detection agents compared with commercial sensors and the greater sensitivity thereby provided. Such levels of incorporation of detection agent onto a scaffold are not obtainable by use of sensor scaffolds described in the art.

[00142] By way of general comparison the present scaffold allows for immobilisation of a detection agent in greater than 5 times, preferably greater than 10, 20, 30, 40, 50, 60, 70 80 or 90 times the amount which can be incorporated into a scaffold employing the same polymer and detection agent but in a monolayer arrangement. In one embodiment, the present scaffold allows for incorporation of detection agent in about 100 times the amount as can be incorporated onto a functionally comparable theoretical monolayer. Put another way, the present scaffold allows for an at least 5, at least 10, at least 20, at least 30 at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 fold increase in the number of available reactive functionalities suitable for reaction with a detection

agent on the multi-layer polymeric network compared with the same polymer in the form of a monolayer covering the same area of scaffold surface. This comparative amount could be measured in a number of ways which would be clear to a person of skill in the art. For example, one useful methodology is described in Saha et al
5 Analytical Chemistry, July 2014. If antibodies or proteins are employed in the biofunctionalization then a BCA assay could provide a quantitative estimation of the number of, for example, IgG molecules present. Further, a functional assay where a fluorescent target is bound to the particles could offer a quantitative estimate of the number of (active) detection agents on the scaffolds.

10 [00143] Saha et al., discusses the functionalization of particles with antibodies. This paper shows that when the particle is loaded with antibody to a density close to that for a theoretical monolayer then functionality is significantly impaired due to crowding of the antibody impacting on the ability to capture targets. The crowding may result in steric hindrance and/or adoption of unfavourable conformations. They
15 conclude, from their experiments, that only a small fraction (about 4 %) of the total immobilized antibodies were able to capture antigen from solution, which number decreases even more as the antibody coverage increases. They attributed this result to the random orientation and possible unfolding of the immobilized antibodies at low surface coverage, and to additional crowding effects at higher coverage and state
20 that these mechanisms can impair the availability of Fab fragments to capture antigen molecules. The results presented herein demonstrate that biofunctionalization of the present scaffold can result in an approximate 100 fold greater number of antibodies being incorporated when compared to that achieved in the experiments of Saha et al.

25 [00144] It is exactly this kind of limitation in capacity and sensitivity which the present scaffold solves. The generation of a 3 dimensional 'shell' in the form of the multi-layer polymeric network with cross-linked metallic or metallic alloy particles allows these overcrowding and unfavourable orientation issues to be avoided and enables the number of detection agents which can be incorporated into the scaffold
30 without encountering these issues to be greatly increased by comparison to such a monolayer approach. Even without the added benefit of the increased brightness provided by the incorporation of metallic or metallic alloy particles, such as quantum dots, into the multi-layer polymeric network the present scaffold provides surprising levels of benefits purely in greatly increased sensitivity due to the greater density of

detection agent which can be accommodated without significant impairment of binding ability.

[00145] The sensor resulting from binding of the detection agent to the scaffold of the second aspect may, in one embodiment, be provided in the form of an ELISA kit for performing immunoassays, and comprises a plurality of scaffolds prepared according to the embodiments described above in, for example, a Nunc Maxisorp polystyrene multi-well plate 210 (44-2404-21, as supplied by Nunc).

[00146] The scaffolds may be modified with a plurality of detection agents using suitable coupling protocols according to the functional groups on the scaffold. In the case of the preferred scaffolds described above employing PDEC-dextran to form the multi-layer polymeric network, the immobilised PDEC-dextran presents a plurality of available (2-pyridinyl)disulfanyl groups at the surface. Therefore, for the purpose of binding the detection agent to form the sensor of the third aspect, a detection agent, such as an antibody fragment (mFAB), comprising a sulfhydryl group at the free cysteine at the hinge region of the antibody that is capable of reacting with the available (2-pyridinyl)disulfanyl group may be used to form a disulphide linkage and the subsequent formation of a 2-mercaptopyridine leaving group to result in biofunctionalized scaffolds.

[00147] The multi-well plate may be incubated overnight with 100 μ L/well of mFAB (5 μ g/mL in carbonate buffer 50 mM, pH 8.5), cross-reactive against all four dengue serotypes. The wells of the resulting biofunctionalized multi-well plate may then be washed with PBSP buffer and subsequently blocked with KPL buffer (KPL50-82-01).

[00148] The biofunctionalized multi-well plate may then be incubated with a test sample comprising a target molecule in the form of NS1 protein, serially diluted in human serum (or PBSP buffer). The NS1 protein is capable of forming a conjugate with the immobilized mFABs on the surface of the biofunctionalized multi-well plate such that the NS1 protein becomes immobilized on the surface of the biofunctionalized multi-well plate (denoted herein as the bioconjugated multi-well plate).

[00149] After a brief washing step, the biofunctionalized scaffolds may then be added to the wells of the bioconjugated multi-well plate and incubated for 1 hour to allow for the immobilized mFABs on the surface of the biofunctionalized scaffolds to

recognise and ligate with the NS1 proteins immobilized on the surface of the bioconjugated multi-well plate.

- [00150] The unbound biofunctionalized scaffolds may be removed via a washing step with PBSP buffer and the fluorescence intensity of the remaining
5 biofunctionalized scaffolds bound to the surface of the bioconjugated multi-well plate can be read with a Perkin Elmer Envision 2104-0010 plate reader to determine the presence and/or concentration of the NS1 protein in the test sample.

EXAMPLES

- 10 [00151] Polybead Amino Microspheres (15699-5 and 07763-5) were purchased from PolySciences Inc. PDEA-Thiol Coupling kit was purchased from GE Healthcare LifeSciences. Dextran T70 was purchased from Pharmacosmos. CdSe/ZnS quantum dots were purchased from PlasmaChem GmbH (PL-QD-O-590) and Invitrogen (Q21711MP). All other chemical reagents were purchased from Sigma Aldrich.

15 **Polymer**

Preparation of 4-nitrophenyl carbonated dextran.

- [00152] To a solution of dextran T70 (180 mg) in 1.8 mL of anhydrous pyridine and 2 mL of anhydrous DMSO, were added nitrophenylchloroformate (90mg) and a catalytic amount of 4-di(methylamino)pyridine. The solution was gently stirred for 5
20 hours at 0°C before adding a mixture of 1:1 methanol:diethyl ether. After overnight precipitation, the solid content was washed three times with 1:1 methanol:diethyl ether solution using a filter under vacuum. The precipitate was dried under high vacuum yielding 110mg of product.

Preparation 2-(pyridinyldithio)ethyl carbomoyl dextran (PDEC-dextran).

- 25 [00153] 4-Nitrophenyl carbonated dextran (110mg) and 2-(pyridinyldithio)ethaneamine (PDEA, 45 mg) were dissolved in 0.42 mL of anhydrous pyridine. 1.4 mL DMSO and 28 µL of methylmorpholine were added and the mixture was stirred O/N at RT. The mixture was precipitated by slowly adding methanol/diethyl ether (1:1) mixture while stirring. The precipitates were washed
30 three times with 1:1 methanol:diethyl ether solution using a filter under vacuum. The

compound was dried under high vacuum for ~ 3 hours, yielding 76 mg of 2-(pyrindinyldithio) ethyl carbamoyl dextran.

Scaffolds

Step 1: incorporation of QDs by swelling.

5 [00154] A 100 μ L solution of amino-functionalised latex particles (Polybead amino microspheres, 200 nm or 500 nm) was washed twice with butanol by centrifugation (2 minutes at 13k rcf). The washed particles were then suspended in a mixture of toluene-butanol 5:95 and placed in a sonic bath for 60s. A solution of 500 pmol of PL-QD-O-590 quantum dots in toluene-butanol 5:95 was added to the latex particle
10 suspension and then incubated for 30 minutes under gentle shaking in darkness.

[00155] The fluorescence intensity of a 100 μ L of supernatant collected at the first wash step is compared with the fluorescence intensity of the solution of the PL-QD-O-590 quantum dots provided at input and a solution of the loaded particles as reference samples to determine the number of PL-QD-O-590 quantum dots
15 incorporated within the loaded particles.

[00156] Mercaptopropyltrimethoxysilane (MPTS, 10 μ mol) was added to the solution of the loaded particles and then incubated for 20 minutes before adding trace amounts of deionized water to trigger polymerisation of the MPTS and to present sulfhydryl groups at the surface of the loaded particles. The resulting loaded
20 particles were washed three times in absolute ethanol.

[00157] As shown in Fig. 1(i), a standard curve is obtained by serially diluting the supernatant from the first washing step in 5:95 toluene-butanol. The concentration of quantum dots remaining within the supernatant from the first washing step can be determined using the linear fit inset of Fig. 1(i), and from this one can estimate the
25 incorporation efficiency of quantum dots within the loaded substrate. As is clear from the fluorescence intensity of the supernatant from the first washing step in Fig. 1(i), and from the corresponding chart shown in Fig. 1(ii), a highly efficient incorporation of quantum dots within the loaded substrate is achieved, with a yield higher than 99%.

Density of sulfhydryl groups on the surface of the loaded particles.

30 [00158] An Ellman's assay was used to quantify the density of the sulfhydryl groups on the surface of the loaded substrate and to ensure batch-to-batch reproducibility. 5 μ L of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), Thermo

Scientific Pierce 22582) 1mg/mL in PBS buffer was added to 10 μ L of the loaded substrate and 85 μ L of PBS buffer. After 15 minutes of incubation, the loaded substrates were removed by centrifugation and the absorbance at 412 nm was measured and correlated with a standard curve obtained by serially diluting cysteamine hydrochloride (Sigma Aldrich M6500-25G) in PBS buffer.

[00159] Fig. 2(i) and 2(ii) shows the raw data from the Ellman's assay obtained for five different batches of loaded substrate. By comparing with the reference curve, it was estimated that each loaded substrate comprised $(5.7 \pm 2.2) \times 10^7$ sulfhydryl groups at the surface of the loaded substrate.

10 **Step 2: Generation of polymer layer.**

[00160] PDEC-dextran (0.1 mg) and a 20 pmol solution of Q21711MP quantum dots were added to the loaded substrates obtained at step 1. After 4 hours of incubation, the resulting multi-layer polymeric network comprising the additional quantum dots (Q21711MP) was formed and the scaffold was washed in absolute ethanol and resuspended in PBSP buffer (PBS, pH 7.4, 0.1% Pluronic F127).

Incorporation of QDs in the polymer layer.

[00161] The quantification of the density of additional quantum dots (Q21711MP) entrapped within the multi-layer polymeric network of the scaffold was carried out following the same protocol as for the incorporation of quantum dots into the substrate, as described in step 1. The fluorescence intensity of 100 μ L of supernatant collected at the first wash step is compared with the fluorescence intensity of a solution of the loaded substrates (comprising PL-QD-O-590 quantum dots entrapped therewithin) provided at input and a solution of the scaffold to determine the number of additional quantum dots (Q21711MP) incorporated within the multi-layer polymeric network of the scaffold.

[00162] As shown in Fig. 3(i), a reference curve was obtained by serially diluting the supernatant from the first washing step in 5:95 toluene-butanol. The concentration of the supernatant from the first washing step was determined using the linear fit of Fig. 3(i), and from this one can estimate the number of additional quantum dots (Q21711MP) remaining in the supernatant after the first washing step.

[00163] As is clear from the fluorescence intensity of the supernatant from the first washing step in Fig. 3(i), and from the corresponding chart shown in Fig. 3(ii), when

performing the reaction with 40 pmol of additional quantum dots (Q21711MP), the amount of additional quantum dots remaining in the supernatant was 0.28 ± 0.08 pmol. This corresponds to a reaction efficiency higher than 99%, which is highly suggestive of a unitary coupling efficiency, and indicative of a highly efficient incorporation of additional quantum dots (Q21711MP) within the multi-layer polymeric network of the scaffold. As a result, it is possible to estimate a theoretical average of 800 additional quantum dots (Q21711MP) per scaffold. The measured fluorescence intensity correlates with 49 ± 9 pmol of quantum dots per batch, indicating that no quenching occurs during the cross-linking reaction.

Bio-functionalized Scaffolds

[00164] Scaffolds prepared according to the procedures outlined in Steps 1 and 2 above, were biofunctionalized with antibody fragments (mFABs) according to the following protocol and assessed against commercially available scaffolds as reference samples, also biofunctionalized with mFABs, to compare the density of mFABs immobilized on the surfaces of the scaffolds tested, as well as their corresponding fluorescence intensity data and stability data.

Bio-functionalization.

[00165] Antibody fragments against dengue non-structural protein (NS1) were added to the scaffolds (25 μ g every mg of scaffolds) and incubated overnight at 4°C.

The resulting bio-functionalized scaffolds were washed three times and stored in PBSP buffer at a concentration of 10mg/mL.

[00166] Reference scaffold: 0.1mL of Life-Technologies (F8811) FluoSpheres® carboxylate-modified latex particles were washed into 50mM MES buffer (pH 6 with 0.01% Tween20). 1.5 mg of EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, Sigma Aldrich 39391) and 1.5mg of sulfo-NHS (N-hydroxysulfosuccinimide, Thermo Fisher 24510) were dissolved in deionized water and added right away to the carboxylate-modified latex particles. After 10 minutes incubation, the modified latex particles were rapidly washed twice in PBS buffer, 0.1% Pluronic F127 and mFAB were added and incubated for 2h at room temperature. The reaction was quenched with large excess of ethanolamine. The resulting biofunctionalized latex particles were resuspended in PBSP buffer.

[00167] Two versions of the scaffold of the present invention were biofunctionalized with mFABs for comparison.

[00168] First scaffold the loaded substrates were prepared according to the procedure outlined in Step 1 above, incubated with PDEC-dextran in the absence of any additional quantum dots (Q21711MP).

5 [00169] Second scaffold the loaded substrates were prepared according to the procedure outlined in Step 1 above, were incubated with PDEC-dextran in the presence of the additional quantum dots (Q21711MP) within the multi-layer polymeric network according to the procedure outlined in Step 2 above.

[00170] All three biofunctionalized scaffolds were prepared using the same excess of mFAB: 25 µg of mFAB per mg of latex particles.

10 *Density of mFABs on the scaffold surface.*

[00171] Thermo Scientific Pierce BCA Protein Assay Kit. According to a general method, 5 µL of the biofunctionalized scaffolds with a diameter of 200 nm and at a concentration of 2% w/v were incubated with 200 µL BCA reagent (bicinchoninic acid) as detailed in the kit protocol. After 30 minutes incubation at 37°C, the
15 biofunctionalized scaffolds were removed by centrifugation and the absorbance at 562 nm was compared with a reference curve (denoted in Fig. 4(i) as "Ref. Curve") obtained by serially diluting an IgG standard (2mg/mL).

[00172] As shown in Fig. 4(i), the BCA assay estimates obtained for the reference scaffold (0.7ug), the monolayer scaffold (3ug) and the multilayer scaffold of the
20 invention (18ug) correspond respectively to a 1.5%, 6% and 36% coupling efficiency between the immobilized mFABs and IgG. The EDC/NHS step corresponds to a theoretical monolayer, assuming spherical particles with no surface roughness. Since the polymer in this experiment cannot crosslink with quantum dots to form a multi-layer polymeric network, only a monolayer is formed by reacting only with the thiols
25 on the surface.

[00173] As shown in Fig. 4(ii), the larger density of mFABs immobilized on the surface of the multilayer scaffold of the present invention as compared to the monolayer scaffold makes apparent the greater density of PDEA groups on the scaffold of the invention available for biofunctionalization due to the formation of a
30 multi-layer polymeric network enabled by crosslinking of the polymer with the quantum dots to form the scaffold of the present invention.

[00174] Fig. 4(iii) presents a dose-response curve obtained with the multi-layer polymeric network containing scaffolds of the invention (labelled 'our approach'). F8811 is a functionalised commercial particle. In both curves seen in FIG. 4(iii) the antibody pair used is the same and the curves clearly demonstrate the superior sensitivity and lower limit of detection provided for by the scaffolds of the invention compared to commercial approaches.

Stability in aqueous buffers.

[00175] A batch of multi-layer scaffolds of the present invention was prepared as outlined above and stored at 4°C in darkness. The fluorescence intensity of 10 fmol of the multilayer scaffolds was periodically recorded under optimized readout settings. Two reference samples were also prepared according to protocols adapted from literature methods (Wang, D.S., et al., *Superparamagnetic Fe₂O₃ Beads-CdSe/ZnS quantum dots core-shell nanocomposite particles for cell separation*. Nano Letters, 2004. **4**(3): p. 409-413; and Koktysh, D., V. Bright, and W. Pham, *Fluorescent magnetic hybrid nanoprobe for multimodal bioimaging*. Nanotechnology, 2011. **22**(27).).

[00176] Amide bond batch: 0.1mL of Polybead Amino Microspheres 07763-5 is buffer exchanged to 50mM MES pH 6, 0.1% Pluronic F127 and 0.1mL of Crystalplex –COOH quantum dots (Trilite™ Green, Emission 525 nm) were added. 1.5mg EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide, Sigma Aldrich 39391) dissolved in deionized water was added to the mixture and incubated for 2h at room temperature. Unbound quantum dots were removed with three washing steps.

[00177] Sulfhydryl/metal batch: Polybead 09836-15, functionalized with –COOH groups were surface modified to introduce sulfhydryl groups by means of standard carbodiimide chemistry. The successful modification was monitored by means of an Ellman's assay, following the same protocol as outlined above. A very high density of sulfhydryl groups of 0.5-1 SH/nm² was found. The modified particles were suspended in 1:1 mixture of toluene/ethanol, to ensure solubility of both the quantum dots and the latex particles. Organic quantum dots (Life Technologies, 545 nm) were added and incubated for 1.5h under gentle shaking. Unbound quantum dots were removed by several washing steps. The successful conjugation was verified by measuring the fluorescence intensity of the conjugates. All constructs were stored in 10mM PBS buffer (pH 7.4, 0.1% Pluronic F127).

[00178] As shown in Fig. 5, based on a comparison of the normalized fluorescence intensity data obtained for the multilayer scaffold of the invention, the amide bond batch and the sulfhydryl/metal batch tested, the multilayer scaffold demonstrates a markedly improved stability over time compared with the stabilities of the reference samples. The multilayer scaffold also demonstrates a superior brightness corresponding to a 100-fold higher density of detection agents immobilized on the surface of the multilayer scaffold as a result of the thickness of the multi-layer polymeric network formed around the loaded substrate. The combination of these advantages results in a 4-fold higher signal and superior sensitivity on a model dengue assay.

Magnetic Particles for Scaffold Formation

[00179] Background: In traditional sandwich assays approaches (e.g. ELISAs), recognition by the antibodies is diffusion-limited. These experiments show that magnetic nanoparticles can be incorporated into the scaffold of the present invention allowing for subsequent extraction, based on magnetic driven sorting, of the target molecule from a complex biological fluid and its transfer into a simpler/clear matrix to facilitate more accurate detection.

[00180] The magnetic labelling may be accompanied by fluorescent labelling of the scaffold, as described below. In the following experiment polystyrene particles are doped with nanometer-sized ferromagnetic grains and capped with a hydrophilic polymer that enables immobilization of reporter probes and the fluorescent agent. Composite particles were prepared and tested on a model assay to detect the exemplar cardiac biomarker myoglobin, clinically relevant for detection of myocardial infarction.

[00181] Methods: Three different types of hybrid constructs were prepared; HY_QD, HY_FP and HY_HRP all having a ferromagnetic core and gold nanoparticles within the particle substrate. The HY_QD has a multi-layer polymer network formed from quantum dots and PDEC-Dextran while the HY_FP scaffold has a multi-layer polymer network formed from gold nanoparticles and PDEC-Dextran and, finally, the HY_HRP has a multi-layer polymer network formed from gold nanoparticles and PDEC-Dextran with horse radish peroxidase (HRP) enzyme additionally anchored thereto.

Preparation of Constructs

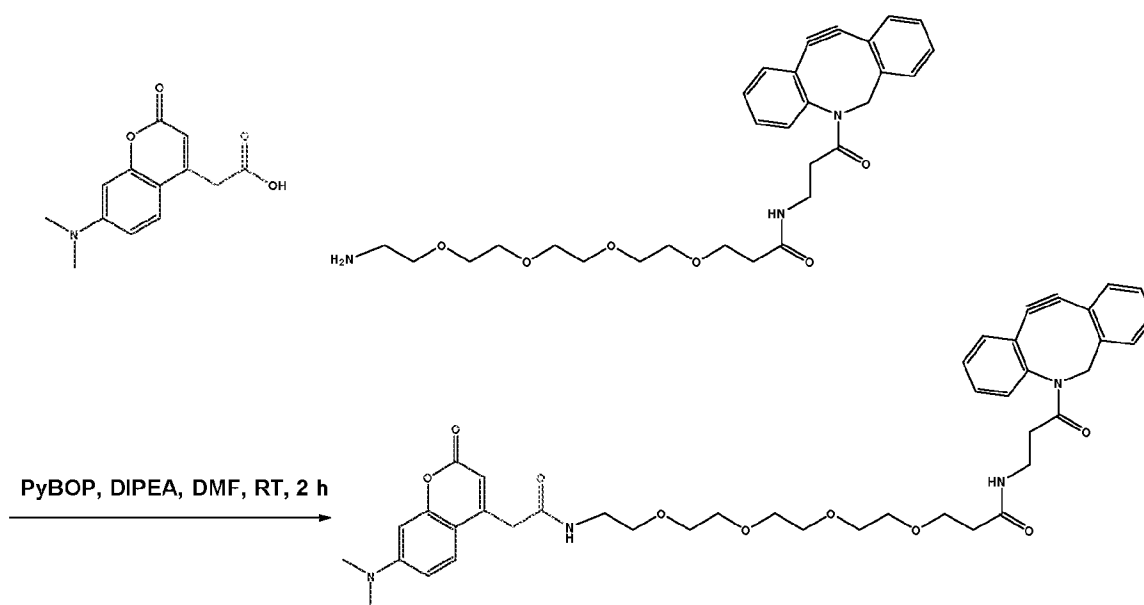
[00182] Doping of magnetic grains to produce magnetic substrate: 20 µg of Polybead® Amino Microspheres 0.20µm (Polybeads product# 15699-5) were washed with Tetrahydrofuran by centrifugation (2 mins at 17K rcf). The particles were then resuspended in a mixture of ethanol-isopropylglycol (1:1) by sonication. Various concentrations of magnetic grains in toluene (sigma product# 700320; Iron oxide(II,III), magnetic nanoparticles solution i.e. Fe₃O₄) were added to separate portions of the microsphere particles to achieve different amounts of incorporated magnetic material in separate batches, and hence a range of magnetic susceptibilities for the scaffolds, and incubated for 1 hour. A catalytic amount of gold nanoparticles (Sigma product# 741981; Gold nanoparticles; 40 nm diameter, OD 1, stabilized suspension in citrate buffer) were added to the microsphere particles. The mixture was incubated for 30 minutes. B-mercaptopropyltrimetoxysilane (MPTS) was added to the mixture and incubated for 20 minutes before adding traces of water. The microsphere particle substrates with incorporated magnetic and gold nanoparticles were finally washed three times with mixture of ethanol-isopropylglycol (1:1).

[00183] Production of HY_QD particles: The doped substrates, formed as just described, were divided into three different batches. For HY_QD, 10 pmol of quantum dots (product P1191580; semiconductor CdSe core and ZnS shell) were added together with polymer PDEC-Dextran. Complete encapsulation of the particles by the polymer to form a multi-layer polymer network was achieved with 4 hours of incubation. The substrate microsphere particles were washed three times with a mixture of ethanol-isopropylglycol (1:1) and finally resuspended in PBSP buffer (10mM PBS pH 7.4, 0.1% Pluronic F127).

[00184] Production of HY_FP particles: The same approach for the formation of HY_QD particles was followed here, however the QDs were replaced with 10 pmol of nanogold particles to anchor the polymer and form the multi-layer polymer network. Fluorescent probes were then attached to these substrate microsphere particles using a bio-orthogonal copper free click chemistry approach. 5 µl Thiol-PEG-Azido linkers (100 mg/ml) were added to the substrate batches of 20 µl and incubated for 1 hour at ambient temperature. The particles were washed three times with PBSP and resuspended with 20 µl of PBSP. The amount of the azide linker was estimated as follows. 5 µl of particles was added to 10 µl of the Alkyne dye at 100 mg/ml (Click chemistry tools, TA106). 10 µl of 10mg/ml freshly prepared sodium ascorbate (Sigma

Aldrich, 268550) was added to the reaction together with 0.5 μ l of 1mM CuSO₄ (Sigma Aldrich, 209198) and 5mM THPTA (Sigma Aldrich, 762342). PBSP was added to make the final volume to 100 μ l. After the reaction was performed at 37°C for 2.5 hours the particles were washed three times with PBSP. Fluorescent intensity was read at excitation at 490 nm and emission at 510 nm. The amount of N₃ was calculated against a standard curve.

[00185] In-house developed fluorescent probes (synthesis protocol below) with a cyclooctane functional group were then added to the particles for the click reaction. The reaction was continued for 3 hours at 37°C. The particles were washed three times with PBSP and finally resuspended with 20 μ l of PBSP. A fluorescent scan of the scaffolds thus formed were performed to ensure incorporation of the fluorescent probe onto the scaffold.



Synthetic route for preparing fluorescent probe with dibenzylcyclooctane functional group for bio-orthogonal click chemistry

[00186] Fluorescent probe synthesis protocol: DBCO-PEG4-Amine (15 mg, 2.86 x 10⁻⁵ mol), PyBOP (16.4 mg, 3.15 x 10⁻⁵ mol) and DMACA acid (7.8 mg, 3.15 x 10⁻⁵ mol) in DMF (5 mL) were stirred until dissolve. DIPEA (40 μ L, 2.29 x 10⁻⁴ mol) was added slowly to the reaction mixture. The reaction was stirred at room temperature 2 h, followed by removal of solvent under reduced pressure. Purification by column chromatography (Grace MPLC; C18 reverse phase with eluents 0.1% TFA in CAN and 0.1% TFA in H₂O; gradient timetable: 0 to 100 % B for 10 min, wash) gave

DBCO-PEG4-DMACA (4.5 mg, 21 % yield) as a green solid. LCMS analysis was conducted using Agilent Technologies 1200 Series instrument with a G1316A UV-Vis detector ($\lambda = 210$ nm and 254 nm), 1200 Series ELSD and 6110 quadrupole ESI-MS, using Agilent Eclipse XDB-Phenyl, 3.5 μ m, 3x100mm: Flow: 1 mL/min: Column temperature RT: Scan mode, positive 100-1500: UV lamp 210 nm: Gradient timetable: 5% B for 1 min; 5% - 100 % B for 8 min, wash. The eluents are 0.05% formic acid in water and 0.05% formic acid in acetonitrile. LCMS: Rt = 8.298 min @ 210 nm, found MS = 753.9, 377.0.

[00187] Production of HY HRP particles: The same approach for preparing HY_FP particles was used here to get to the point of having the multi-layer polymer network formed on the substrate surface. Following this, to anchor the HRP, 100 μ l (10 mg/ml) of HRP (sigma # P8375) was added with 5 μ l of NHS-PEG-SH (click chemistry tools # PG2-NSTH-2k) linker and was allowed to react for 1 hour at room temperature. The excess of linker was removed using a Zeba spin column. Thiolated HRP thereby produced was reacted with the scaffold particles for 1 hour at room temperature. The particles were washed three times with PBSP and finally resuspended with 20 μ l of PBSP. HRP and antibodies were buffer exchanged to PBS from the original storage buffer using a Zeba spin desalting column (MWCO 7KDa, ThermoFisher, 89882) and following the manufacturer's instructions. Summarizing the protocol, the storage buffer of the column was removed by spinning the column at 1500 RPM for 1 min. 300 μ l of PBS was added and the column and buffer removed by spinning. Finally, protein (up to 130 μ l) was added carefully and the column was spun at 1500 RPM for 2 mins. The flow through was collected and the concentration of protein was determined using a ThermoScientific Nanodrop at 280 nm.

[00188] Bio-functionalization of the Scaffold Microspheres: Antibodies (MY1 and BNP1) were thiolated using NHS-PEG-SH linker. Twenty times molar excess of NHS-PEG4-SH (Nanocs, PG2-NSTH-5k) linker was added onto the antibodies. The molar excess was calculated using the below equation, where V is the volume, C is the concentration and MW is the molecular weight:

$$V[\mu\text{L}] = 10[\text{uL/mg}] \frac{C_{\text{IgG}}[\text{mg/mL}] V[\text{mL}]}{MW_{\text{IgG}}[\text{g/mol}]} MW_{\text{PEG}}[\text{g/mol}]$$

The unreacted linkers were removed using zeba spin column (MWCO 7KDa, ThermoFisher, 89882). The thiolated antibodies were reacted with the HY_QD,

HY_FP and HY_HRP scaffolds, to form the corresponding sensors, for 1 hour at room temperature. The sensors were washed three times with PBSP and finally resuspended with 20 μ l of PBSP.

[00189] Results: The number of layers or density of the multi-layer polymer network increases with an increase in the amount of Quantum Dots/gold nanoparticles (or other metallic or metallic alloy particles) which are made available when forming said layers thereby indicating a progressive layer formation. This is important as it provides for an unprecedented level of control over the thickness of the multi-layer polymer network formed on the surface of the particles, and hence the number of fluorescent particles, reporter agents, detection agent etc. Fig. 6 a) – c) demonstrates this increase in the number of layers or density of the multi-layer polymer network by indicating the observed increase in fluorescence of the particle for batches the HY_QDs and HY_FP scaffolds and an increase in HRP activity for the HY_HRP scaffold.

[00190] A progressive increase in the amount of antibodies attached to the various was also noted after addition of the thiolated antibodies to these scaffolds with increasing layers/density of the multi-layer polymer network. Having a high density of the antibodies facilitates more efficient target capture.

[00191] The scaffolds with the maximum amount of antibodies on the surface were then selected. These sensors were used to sequester target myoglobin from 50 μ l of PBSP and then detected using surface functionalized ELISA plates. Up to 320 pg/ml of myoglobin was detected using HY_QDs and HY_FP and 1 μ g/ml of myoglobin was detected using HY_HRP particles indicating a good level of detection. These results are demonstrated in Fig. 7 a) – c).

Use of Scaffolds for the Capture of Pathogens

[00192] Two lectins, wheat germ agglutinin (WGA) and concanavalin A (ConA) were chosen because of their sugar recognition to N-acetyl glucosamine and mannose, respectively, which are cell wall sugar moieties. WGA and ConA were conjugated to magnetic substrate particles of different sizes (170 and 500 nm) and used for the capture of bacterial cells. Lectin coupling was performed with different particle based chemistries, assessed and quantified to select the highest conjugation efficiency. Two chemistries had been tested for determination of lectin coupling efficiency to the surface of the substrate particles: direct coupling to the substrate

particle surface or anchoring onto a polymeric hydrogel. The polymeric hydrogel is a functional dextran polymer, synthesized in-house, in which dextran was modified to introduce other functional groups such PDEC (PDEC-dextran) or PDEC and carboxylic acid (CMD-dextran). In direct coupling, WGA was attached to carboxylated magnetic substrate particles using standard carbodiimide chemistry. However, the polymer approach chemistry involved the conjugation of PDEC-dextran polymer (synthesized according to the procedure of Li, X., C. Abell, and M.A. Cooper, *Single-step biocompatible coating for sulfhydryl coupling of receptors using 2-(pyridinyldithio)ethylcarbonyl dextran*. Colloids Surf B Biointerfaces, 2008. **61**(1): p. 113-7) to magnetic substrate particles and the formation of a multi-layer network of the polymer with the support of silver nanoparticles according to protocol 1 (below) (the two approaches are demonstrated graphically in Fig. 8). Successful coupling was assessed by bicinchoninic acid assay, substantially as described earlier) (BCA, Thermo Scientific) to measure total protein content. The former allowed delocalization of WGA molecules within the hydrogel, thereby achieving up to 6 times (6.5 µg/mL) higher density of bound lectins compared to carbodiimide direct coupling chemistry (0.9 µg/mL) (results shown in Fig. 9).

[00193] **Protocol 1 for substrate particle functionalization using CMD-dextran polymer:** Carboxylated magnetic particles (50 mg/mL) and 0.2 µm FluoSpheres carboxylate-modified Microspheres (20 mg/mL) (Life Technology) were modified and passivated with CMD-PDEC-dextran polymer (Synthesized according to Li and Cooper paper discussed above). Briefly, an equal volume of cysteamine (35 mg/mL) was added to 500 µL of carboxylated nanoparticles then 6.25 mg of carbodiimide EDC was added in cold MES buffer (pH 5.0) and left for incubation for 2 hours at room temperature. Then, washed particles were suspended in CHES buffer pH 9.0 and add 8.5 µL CMD-PDEC dextran (13 mg/mL) in CHES buffer pH 9.0 as well as 10 µL of Ag nanoparticles (10 nm) (Sigma Aldrich) where the Ag nanoparticles were added to build the multi-layer polymeric network.

[00194] These obtained CMD-dextran modified substrate particles were reacted with NH₂-PEG4-DBCO linker (Click Chemistry tools) via an EDC reaction. Briefly, 12.5 µL of NH₂-PEG4-DBCO linker (100 mg/mL) (Click Chemistry tools) dissolved in DMSO were added to 500 µL of CMD-dextran substrate particles washed in cold MES buffer (pH 5.0) and 6.25 mg of carbodiimide EDC dissolved in cold MES buffer (pH 5.0) and left for incubation for 2 hours at room temperature. Then, the washed

particles were suspended in PBSP and N₃-antibiotics derivative (colistin) (0.5 mg/mL) were added and left for incubation for 4 hours at 37°C.

Bacterial Capture

[00195] Selected Gram-negative and Gram-positive bacterial strains were used in this study (Table 1). All bacterial strains were cultured on LB broth at 37 °C and diluted as required when their corresponding O.D₆₀₀ was about 0.5, which corresponded to 10⁹ cfu/mL. All biological samples (platelets, plasma, and blood (obtained from Australian Red Cross Blood Service (ARCBS)), were checked for absence of previous bacterial contamination by culture. The sterile fluids were spiked with bacterial strains and processed for magnetic capturing. Bacterial cell capturing was done on 1 mL scale of spiked samples using magnetic particles (500 nm) according to protocol 2. Capturing efficiency was calculated based on the number of counted colonies of the output plates in comparison to the input plates (protocol 3). Bacteria capturing from undiluted platelets showed almost 100% efficiency with Gram-positive and Gram-negative strains (results seen in Fig. 10).

[00196] **Protocol 2 for capturing bacterial cells from biological samples:** All bacterial strains were cultured on LB broth at 37 °C and diluted to the required concentrations when their corresponding O.D₆₀₀ is about 0.5, which corresponds to 10⁹ cfu/mL. All biological samples (platelets, plasma, and blood (Australian Red Cross Blood Service (ARCBS)) were sterile and spiked with bacterial strains (Gram-negative and Gram-positive) at different concentrations and processed for magnetic capturing. Bacterial cell capturing was done on 1 mL scale of spiked samples where 3 µL of WGA-MPs (500 nm) or 1 µL of WGA-MPs (170 nm) were added and incubated for 1 hour at 37°C with rotation.

ATCC number	Bacteria strain	Resistance Profile
ATCC 25923 (GP_01)	<i>Staphylococcus aureus</i>	Sensitive (MSSA)
ATCC 25922 (GN_01)	<i>E. coli</i>	Sensitive

Table 1. Bacterial strains used.

[00197] **Protocol 3 for quantification of capturing efficiency using culture methods:** WGA-MPs were magnetically washed with sterile phosphate buffered

saline (0.1% pluronic) (PBSP) 4 times. Then, WGA-MPs were resuspended in 150 μ L and cultured on LB agar and incubated for 24 hr at 37 °C. Capturing efficiency was calculated based on the number of counted colonies of the output plates in comparison to the input plates.

5 *Fluorescent detection of bacterial cells*

[00198] Reducing non-specific binding using the polymer approach: The surface of magnetic substrate particles were passivated by two approaches: 1) protein (HSA) and 2) hydrophilic polymer (CMD-dextran), by virtue of the present method, passivation and used as negative controls in capture assays in platelets followed by
10 detection with fluorescent nanoparticles functionalized with vancomycin. Fig. 11 demonstrates that the polymer (PDEC-dextran) passivation on the magnetic substrate particles (MPs) decreased non-specific binding compared to that seen with standard protein (HSA) passivation.

Fluorescent bacterial detection in platelets.

15 [00199] Vancomycin MCC_174 was functionalized on 0.2 μ m blue fluorescent (365/415) particles. Fluorescent blue beads were chosen to allow assessment of vancomycin coupling using Fam-KAA, which has overlapping and interfering fluorescence emission with Y/G beads. The surface of the particles was passivated with the polymer (CMD-dextran) based passivation method, through carbodiimide
20 bond (EDC) with cysteamine, before coupling to the vancomycin derivative with another carbodiimide bond (EDC) as to reduce the non-specific binding. The results showed significant improvement of the detection approach with a detection limit of approximately 10 cfu/mL (confirmed by culture) of *S. aureus* in platelets (results shown in Fig. 12). The extent of the error bars were due to variability between the
25 count of bacteria and manual washing which can be significantly improved by the use of robotics and would therefore lower the calculated limit of detection (LoD).

[00200] Figure 12 therefore exemplifies the results of an assay format where magnetic and fluorescent sensors of the invention simultaneously bind a target analyte (bacteria). Magnetic (due to the inclusion of the magnetic particles in the
30 substrate) sensors are able to be spiked directly into undiluted biological fluids and selectively bind onto the bacterial membrane. The PDEC dextran assists in passivating the surface to reduce non-specific interactions while at the same time enabling immobilization of a very high density of capture probes, essential to achieve

rapid capture from large sample volumes. The magnetically sequestered bacteria are then brought into contact with a fluorescent nanoparticle to facilitate fluorescent detection.

Multiplex Bacteremia Biomarkers Detection

5 [00201] This series of experiments aims to show the development of a novel biomarker assay which is tunable for detection of multiple biomarkers.

[00202] Proof of concept, Approach, and Application: Enzyme linked immunosorbent assay (ELISA) has been extensively used to successfully detect antigens although it is time consuming and requires several washing steps. Here, a
10 proof of concept assay has been developed that is based on the ELISA sandwich approach. We selected Dengue NS1 antigen as a model to study the assay. In this assay no washing is required and a positive signal can be obtained in 1-2 hours. The assay kinetics are somewhat slow due to the K_{on} of the dengue antibodies ($10^4 \text{ M}^{-1} \text{ s}^{-1}$), but this will be enhanced by selecting other antibodies. Briefly, metal nanoparticles
15 can be used to quench the fluorescence signal of quantum dots because of their optical surface plasmon resonance, which leads to the use of that fluorescence energy transfer between quantum dots and metal nanoparticles in diagnostic assays. That energy is called Forster resonance energy transfer (FRET) which is distance dependent. In this assay, we are employing metal nanoparticles and quantum dots to
20 detect an antigen as a proof of concept. Since the sandwich formed is through Fabs or antibodies target recognition, the contribution of fluorescence absorption in the detection signal will be investigated.

[00203] Wash free biomarker assay: Amine modified polystyrene beads (200 nm) were doped with fluorescent quantum dots (QDs) as already described in the above
25 experimental, and passivated by PDEC-dextran to form the multi-layer polymeric network as well as reducing the non-specific signal. At the same time, another batch of polystyrene beads was loaded internally with silver nanoparticles (AgNPs) or gold nanoparticles (AuNPs), after being modified with PDEC-dextran to form the multi-layer polymeric network with the help of nanoparticles and passivate the substrate
30 particles at the same time (protocol 4 below).

[00204] This metal nanoparticle batch was used to absorb or quench the emitted fluorescence signal of the QDs. Both batches described above were functionalized with either Anti-Dengue Fabs or antibodies. In the presence of specific dengue

antigen, a sandwich of the two different batches of substrate polystyrene beads was formed and this resulted in a decrease in the fluorescence signal of the QDs upon excitation. The assay thus developed is wash free and the positive signal is recognised as a decrease in the fluorescence signal in comparison to the negative control (no antigen).

[00205] The same concentration of AuNPs (20 nm) and AgNPs (10 nm) were loaded, separately, on the polystyrene beads and used for detection in this model antigen dengue NS1 antigen (50 ng/mL) with 0.12 mg/mL of QDs-beads. The results showed AgNPs-beads had better fluorescence absorbance than AuNPs-beads although it should be noted the AgNPs were of smaller size compared to AuNPs (results shown in Fig. 13). It was observed that an increase in incubation time increased the fluorescence absorbance from about 25% for 1 hour to 50% for 2 hours, especially for low concentrations of beads due to slow binding kinetics.

[00206] Different amounts of the prepared AgNPs-beads were added for the detection of dengue NS1 antigen (50 ng/mL) with the same concentration of QDs_590 nm-beads and the signal was measured after 1 and 2 hours. It was observed that the increase of the fluorescence absorbance is directly proportional to the amount of AgNPs-beads in the assay (results shown in Fig. 14). One of the advantages of this assay is that only bound beads contributed to the drastic decrease of quenching signal; however, negative control (no antigen) fluorescence was decreased slightly because of some degree of absorption of QDs fluorescence in solution. Additionally, the concentration of the metal nanoparticles is directly proportional to the QDs fluorescence absorbed. The use of more metal nanoparticles beads changed the assay kinetics and allowed more binding events to occur resulting in a greater absorbed fluorescence signal for a given incubation time.

[00207] **Protocol 4 for preparation of fluorescence acceptor particles:** To prepare 200 μ L, 40 μ L Polybead amine microspheres (20 mg/mL), 0.20 μ m (Polysciences) were washed by 1:1 isopropyl glycol : methanol (0.1% pluronic F127) (S) and reacted with Sulfo-SMCC for 1 hour at room temperature. After which the particles were washed and suspended in CHES and sonicated for 5 min. Then, 1 μ L of PDEC-dextran (67 mg/mL) and 40 μ L AgNPs (10 nm) (Sigma Aldrich) and incubate for 2 hours at room temperature. Particles were washed by centrifugation at

maximum speed for 5 min at RT and suspended in CHES. Then, 25 µg/mL of species specific dengue Fabs were added for 20 mg/mL beads.

[00208] From the results shown it will be appreciated that the scaffolds as defined according to the embodiments of the present invention provide a number of advantages in use. They allow, in certain embodiment, the entrapment of highly fluorescent quantum dots or magnetic particles both within the swollen polymeric substrate and within the multi-layer polymeric network attached to the substrate, and immobilize a greater number of detection agents, fluorescent probes, report agents and the like on the surface of the scaffolds due to the thickness/density of the multi-layer polymeric network. This provides for a sensor exhibiting superior brightness, sensitivity and stability as compared to conventional sensors.

CLAIMS

1. A method of constructing a scaffold for a sensor comprising the steps of:
 - (a) contacting a scaffold surface with a polymer, the polymer having a reactive functionality suitable for binding to each of (i) the scaffold surface; (ii) a metal or metal alloy; and (iii) a detection agent;
 - (b) contacting the polymer with a plurality of metallic or metallic alloy particles; and
 - (c) allowing the polymer to bind to the scaffold surface and the metallic or metallic alloy particles to form a multi-layer polymeric network, cross-linked with the metallic or metallic alloy particles, which network is bound to the scaffold surface,to thereby construct the scaffold.
2. The method of claim 1 wherein the scaffold surface is selected from a metallic or metallic alloy surface, a ceramic surface, a silica surface or a polymeric surface.
3. The method of claim 1 or claim 2 wherein the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy surface and the detection agent is the same functionality.
4. The method of any one of the preceding claims wherein the reactive functionality suitable for binding to the scaffold surface, the metal or metal alloy surface and the detection agent is independently selected from the group consisting of carboxyl-containing groups, hydroxy-containing groups, carbonyl-containing groups and groups comprising at least two chalcogen atoms bonded to one another.
5. The method of claim 4 wherein the group comprising at least two bonded chalcogen atoms is selected from groups comprising two bonded S, Se or Te atoms, each of which may have one or two oxygen atoms attached, with an R group attached to the terminal chalcogen atom.
6. The method of claim 5 wherein the R group is selected such that the conjugate acid formed by the R group and attached chalcogen atom leaving the polymer has a pKa of less than 8.

7. The method of claim 6 wherein the R group is selected from aryl, heteroaryl and C₁-C₁₂ alkyl, each of which may be substituted.
8. The method of any one of the preceding claims wherein the metallic or metallic alloy particles are selected from the group consisting of gold, silver, platinum, copper, iron, palladium, nickel, chromium, titanium, and metal- or metal alloy-containing quantum dots.
9. The method of any one of the preceding claims wherein, when the scaffold surface is the surface of a polymer matrix, the method may further comprise, before step (a), the step of incorporating a plurality of metallic or metallic alloy particles within the polymer matrix.
10. The method of claim 9 further comprising, before the step of incorporating the metallic or metallic alloy particles, the step of swelling the polymer matrix in an organic solvent.
11. The method of claim 10 further comprising, prior to contacting the scaffold surface with the polymer, the step of adding a polymerisable reagent to the swollen polymer matrix with incorporated metallic or metallic alloy particles.
12. The method of claim 11 further comprising the step of initiating polymerisation of the polymerisable reagent to form polymer strands within the polymer matrix.
13. The method of claim 12 wherein the polymerisable reagent has an anchoring group which, after the polymerisation of the polymerisable reagent, presents as part of the scaffold surface as a group reactive with the reactive functionality of the polymer.
14. The method of any one of the preceding claims wherein the polymer having the reactive functionality comprises a polymer selected from the group consisting of dextran, cellulose, agarose, sepharose, hyaluronic acid, nitrocellulose, polyvinylalcohol, partially hydrolysed polyvinylacetate and polymethylmethacrylate, carboxymethyl cellulose and carboxymethyl dextran, any one of which may optionally be derivatised.
15. The method of any one of the preceding claims wherein the polymer comprises a 2-(pyridinyldithio)ethyl carbomoyl group.

16. The method of any one of the preceding claims wherein the multi-layer polymeric network provides for an at least 5 fold increase in the number of available reactive functionalities suitable for reaction with a detection agent compared with the same polymer in the form of a monolayer on the same area of scaffold surface.
17. A scaffold for a sensor, comprising:
- (a) a scaffold surface;
 - (b) a multi-layer polymeric network bound to the scaffold surface; and
 - (c) a plurality of metallic or metallic alloy particles bound within and crosslinking the multi-layer polymeric network.
18. The scaffold of claim 17 wherein the scaffold surface, multi-layer polymeric network and metallic or metallic alloy particles are as described in any one of claim 1 to claim 16.
19. The scaffold of claim 17 or claim 18, wherein, when the scaffold surface is the surface of a polymer matrix, then the scaffold may further comprise a plurality of metallic or metallic alloy particles within the polymer matrix.
20. The scaffold of claim 19 further comprising polymer strands entrapping the metallic or metallic alloy particles within the polymer matrix, the polymer strands formed from polymerisation of a polymerisable reagent.
21. The scaffold of claim 20 wherein the multi-layer polymeric network is bound to the scaffold surface, at least in part, via an anchoring group presented by the polymer strand formed from the polymerisable reagent, at least a portion of the polymer strand forming part of the scaffold surface.
22. The scaffold of any one of claim 17 to claim 21 wherein the multi-layer polymeric network provides for an at least 5 fold increase in the number of available reactive functionalities suitable for reaction with a detection agent compared with the same polymer in the form of a monolayer on the same area of scaffold surface.
23. A sensor for use in detecting a target molecule in a sample, comprising:
- (a) a scaffold as claimed in any one of claim 17 to claim 22; and

- (b) at least one detection agent bound to the multi-layer polymeric network of the scaffold which is capable of detecting the target molecule in the sample.
24. A method of detecting a target molecule in a sample comprising the steps of:
- 5 (a) contacting the sensor of claim 23 with a sample to expose the at least one detection agent to the target molecule in the sample; and
- (b) detecting a signal resulting from the formation of a complex or conjugate between the at least one detection agent and the target molecule.
- 10 25. The sensor of claim 23 or method of claim 24 wherein the target molecule is any molecule that is capable of forming a complex or conjugate with the detection agent.
26. The sensor or method of claim 25 wherein the target molecule is selected from small molecules, ions, carbohydrates, whole cells, bacteria, viruses, fungi as
- 15 well as proteins inclusive of antibodies or antibody fragments, peptides, enzymes, epitopes and other antigens, antigen receptors, hormone receptors, growth factor receptors, cytokine receptors, hormones, growth factors or cytokines, small molecules, cells and cellular fractions, organelles or other components thereof, biological markers such as cell surface markers, nucleic acids such as single- or double stranded DNA and RNA, and derivatives,
- 20 cooligomers and any fragments or combinations thereof.
27. The sensor of claim 23 or method of claim 24 wherein the detection agent is selected from small molecules, ions, carbohydrates, whole cells, bacteria, viruses, fungi as well as proteins inclusive of antibodies or antibody fragments,
- 25 peptides, enzymes, epitopes and other antigens, antigen receptors, hormone receptors, growth factor receptors, cytokine receptors, hormones, growth factors or cytokines, small molecules, cells and cellular fractions, organelles or other components thereof, biological markers such as cell surface markers, nucleic acids such as single- or double stranded DNA and RNA, and
- 30 derivatives, cooligomers and any fragments or combinations thereof.
28. The sensor or method of claim 27 wherein the detection agent is an antibody or antibody fragment.

29. The sensor or method of claim 28 wherein the antibody or antibody fragment is polyclonal, monoclonal or recombinant synthetic inclusive of single chain fragments such as scFvs, F(ab) and F(ab')₂ fragments, diabodies and triabodies.
- 5 30. The sensor or method of any one of claim 23 to claim 29 wherein the multi-layer polymeric network provides for an at least 5 fold increase in the number of detection agents compared with those incorporated on same polymer in the form of a monolayer on the same area of scaffold surface.

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1/ 13

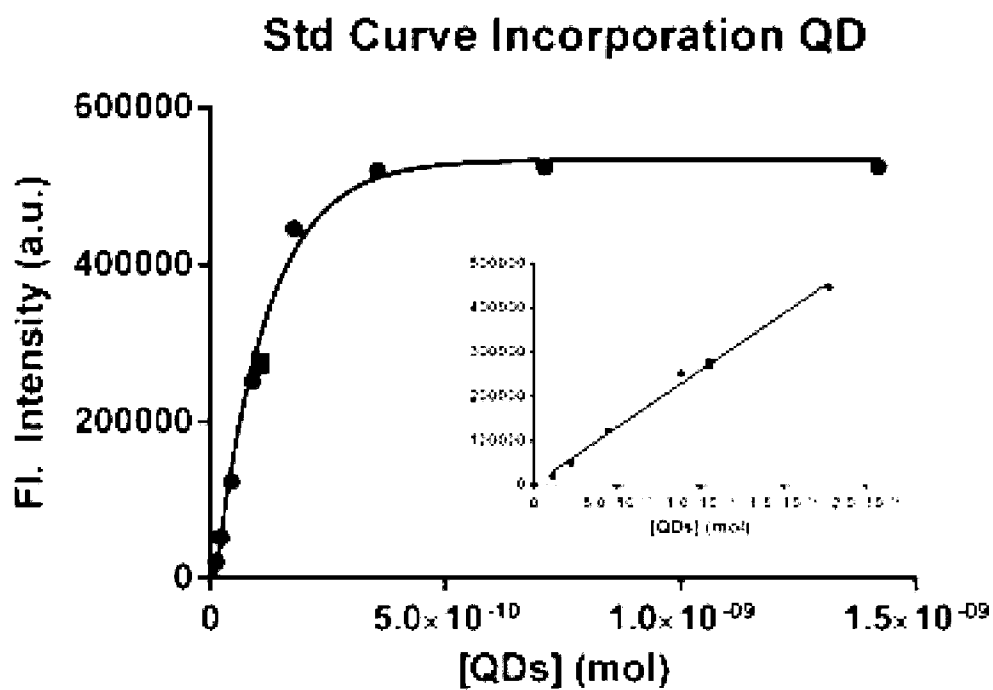


FIG 1 (i)

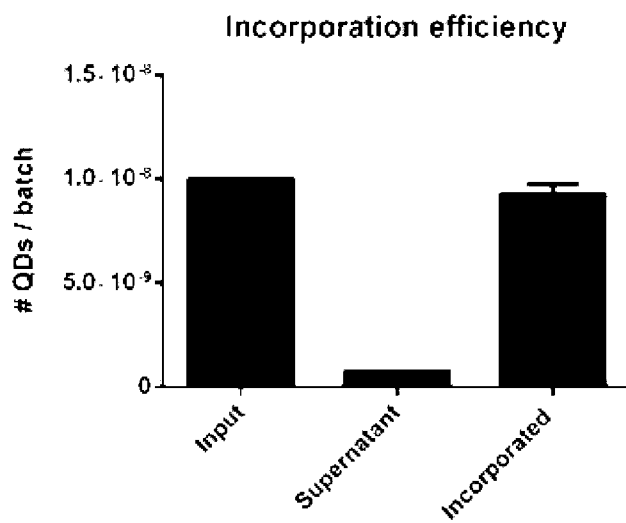


FIG 1 (ii)

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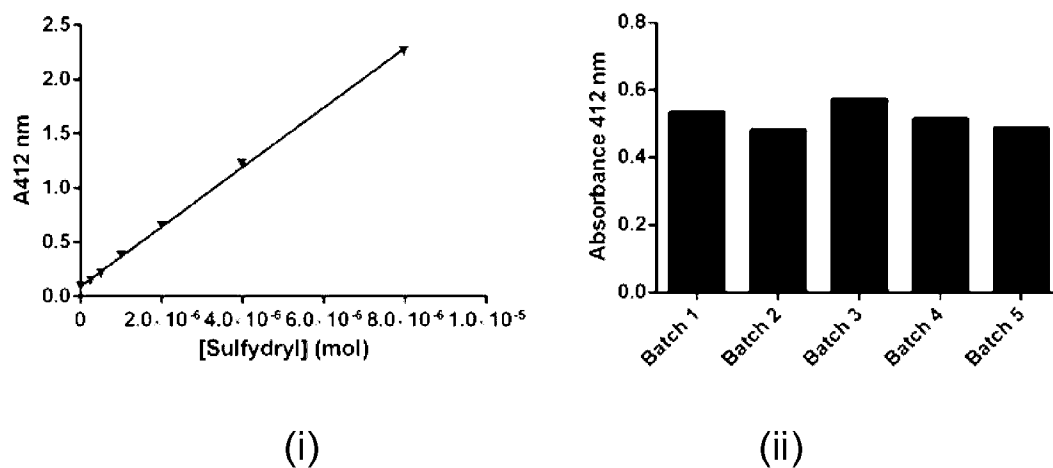


FIG 2

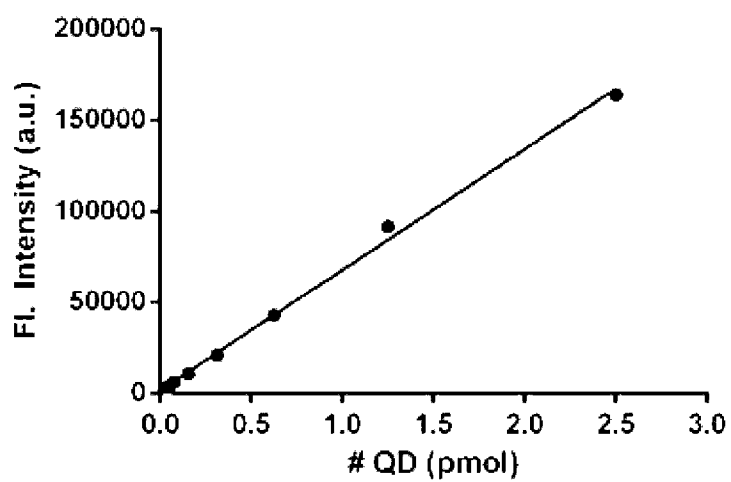


FIG 3 (i)

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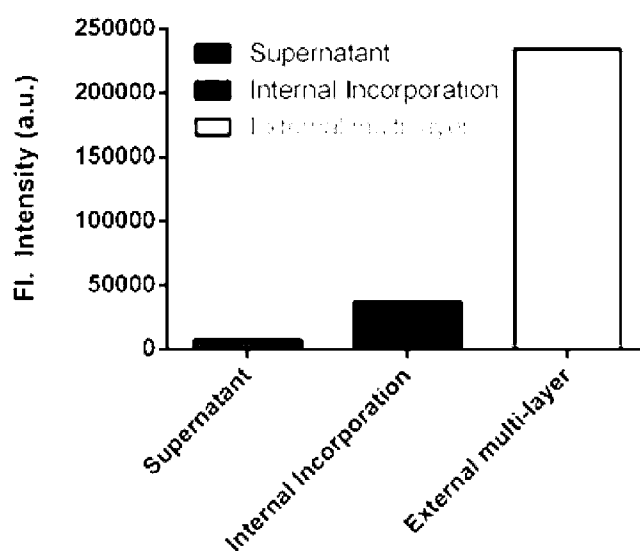


FIG 3 (ii)

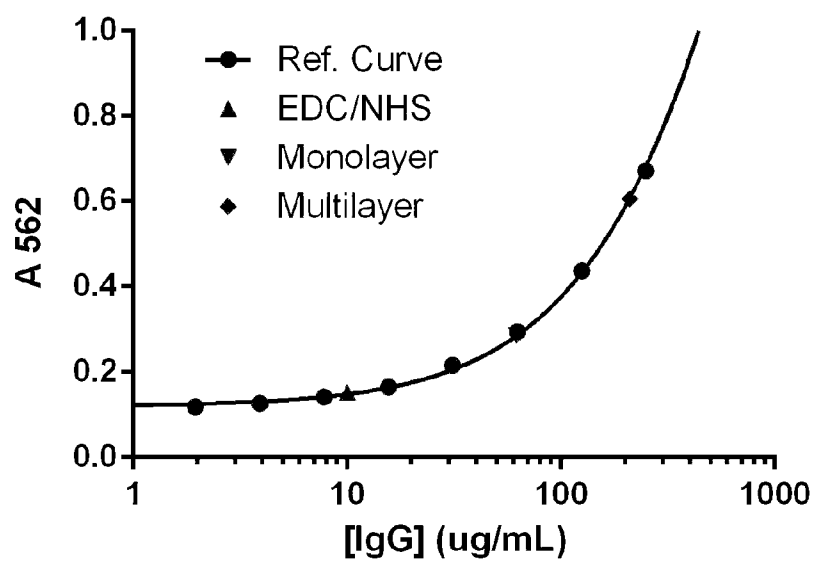


FIG 4 (i)

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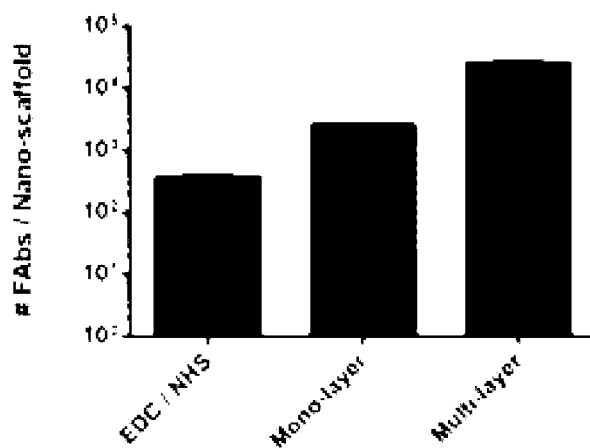


FIG 4 (ii)

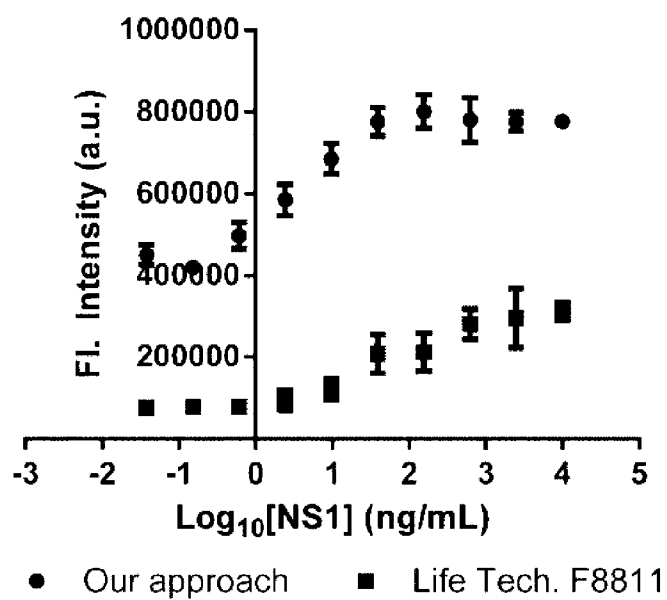


FIG 4 (iii)

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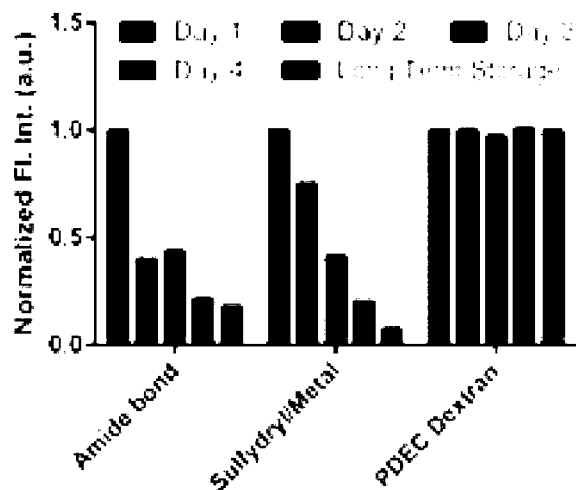


FIG 5

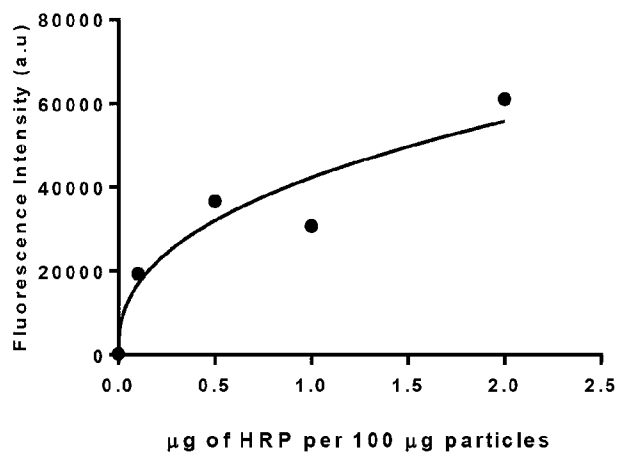


FIG 6 a)

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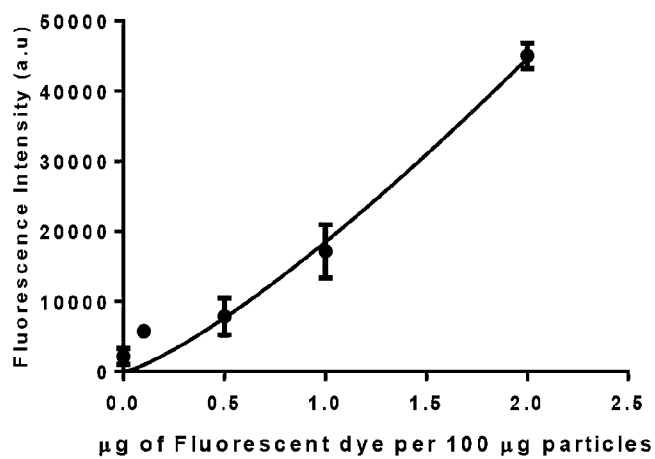


FIG 6 b)

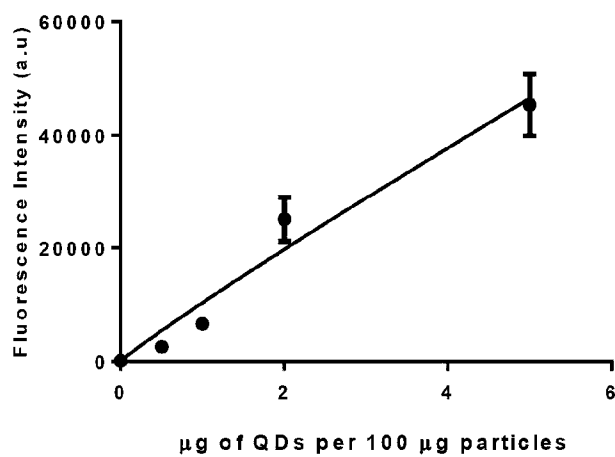


FIG 6 c)

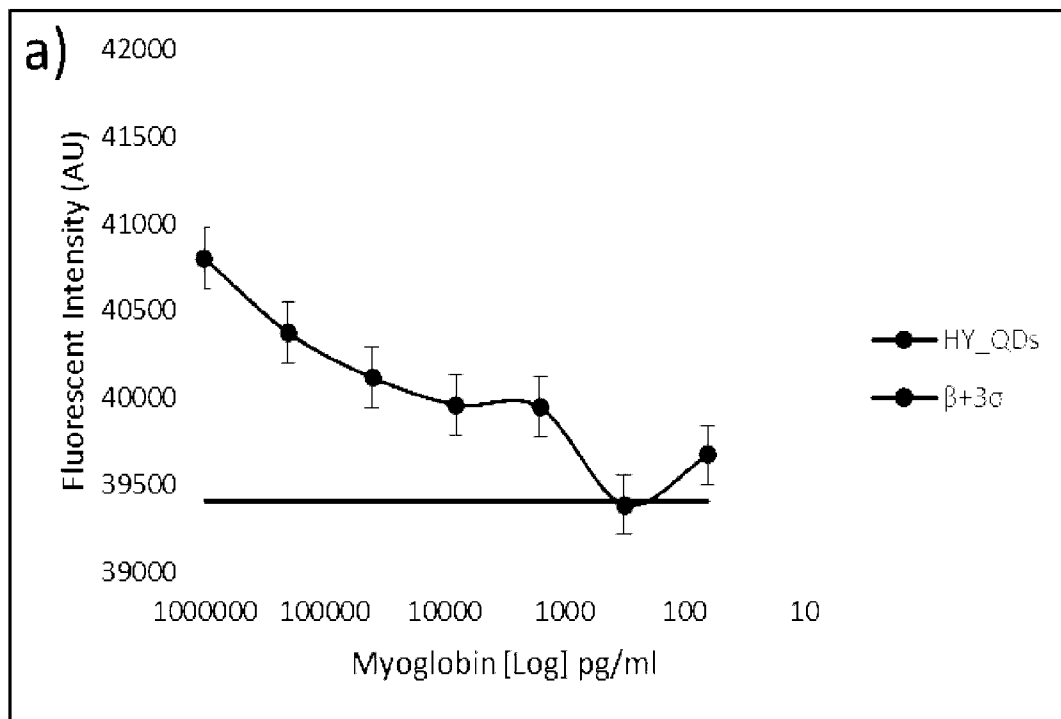


FIG 7 a)

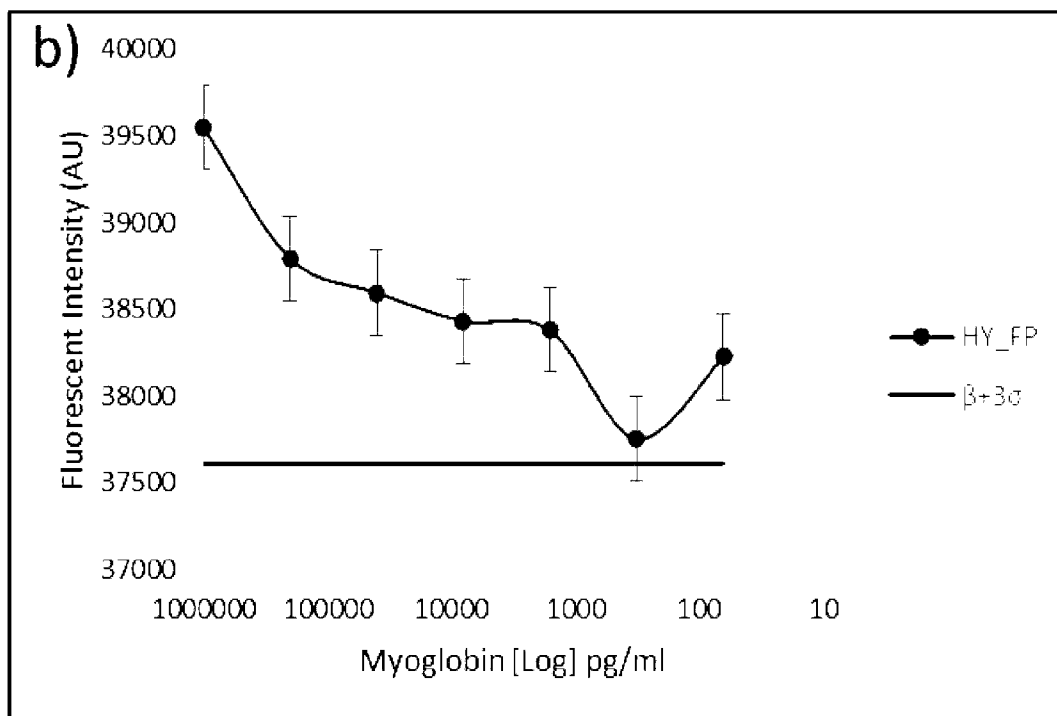


FIG 7 b)

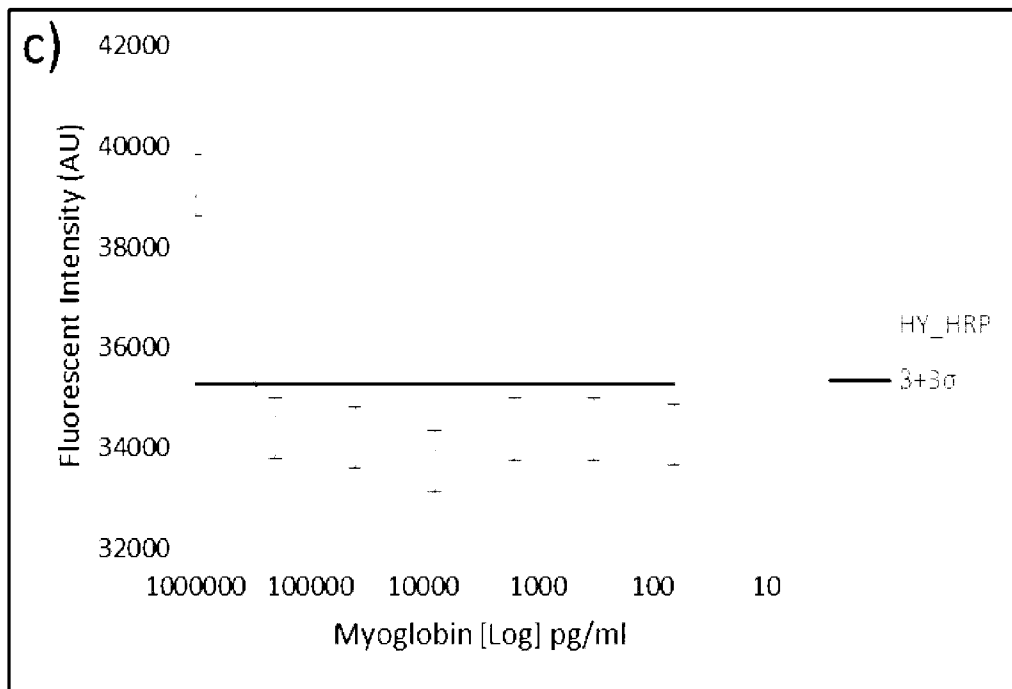


FIG 7 c)

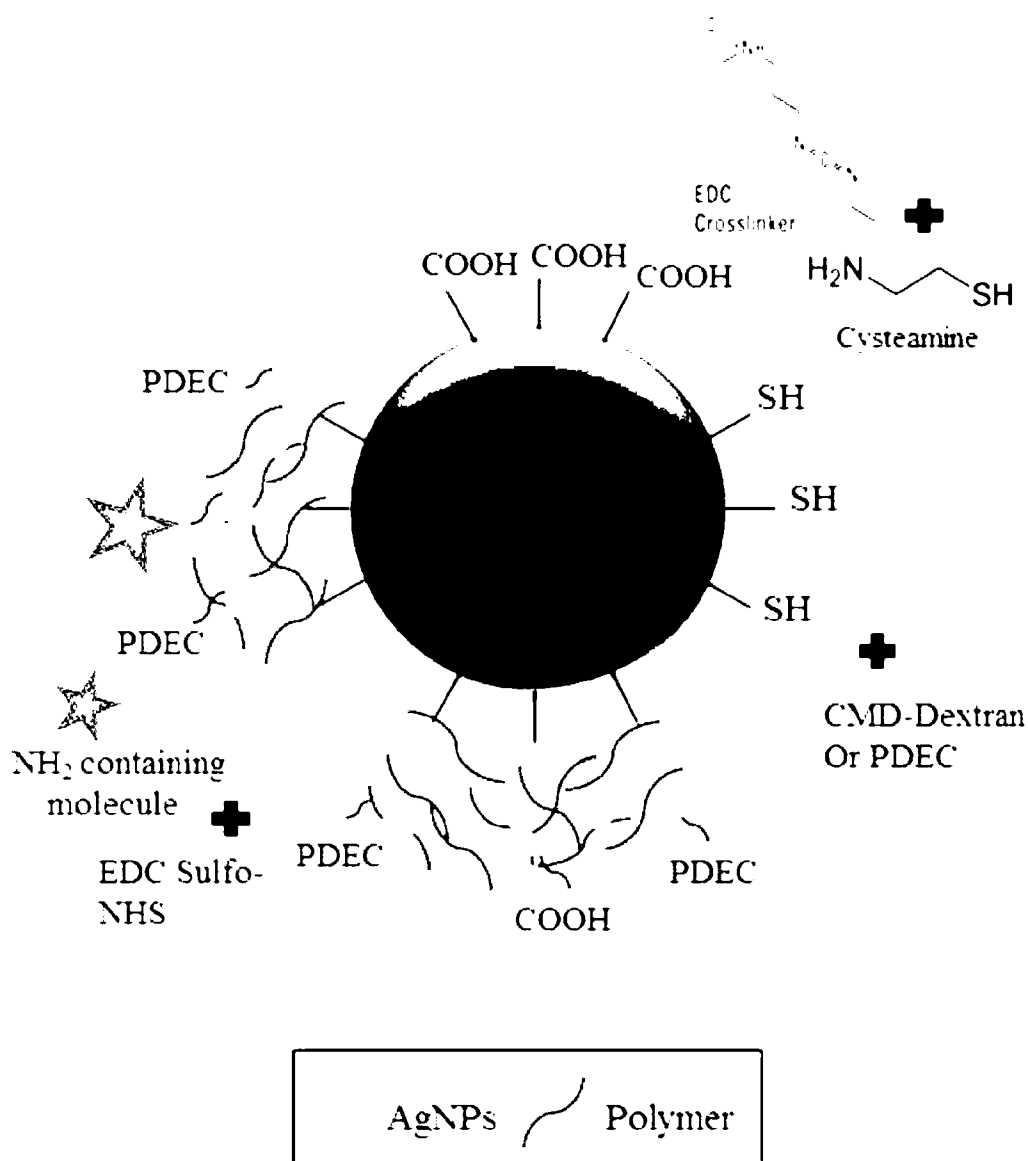


FIG 8

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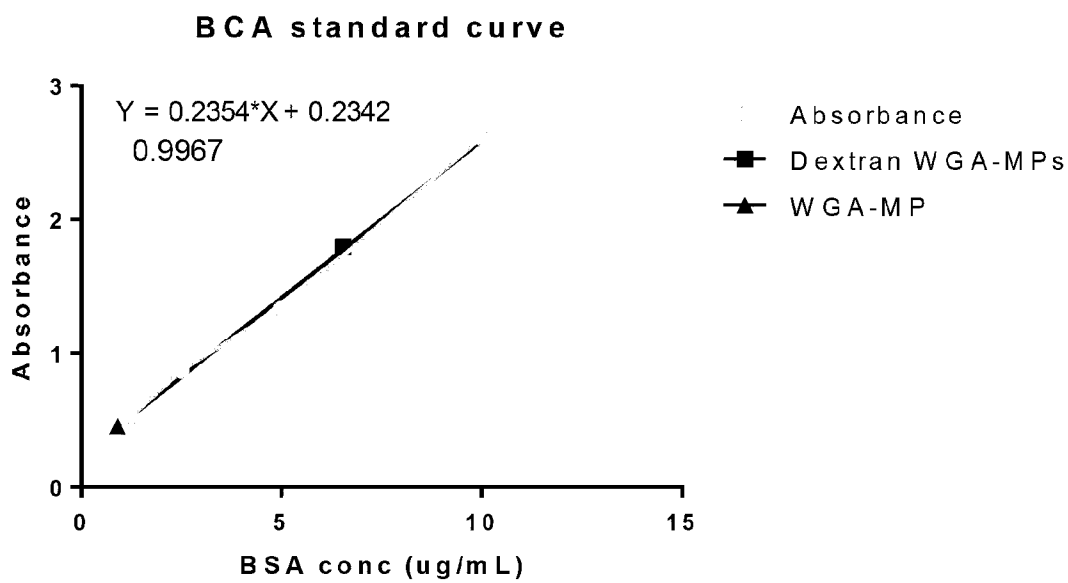


FIG 9

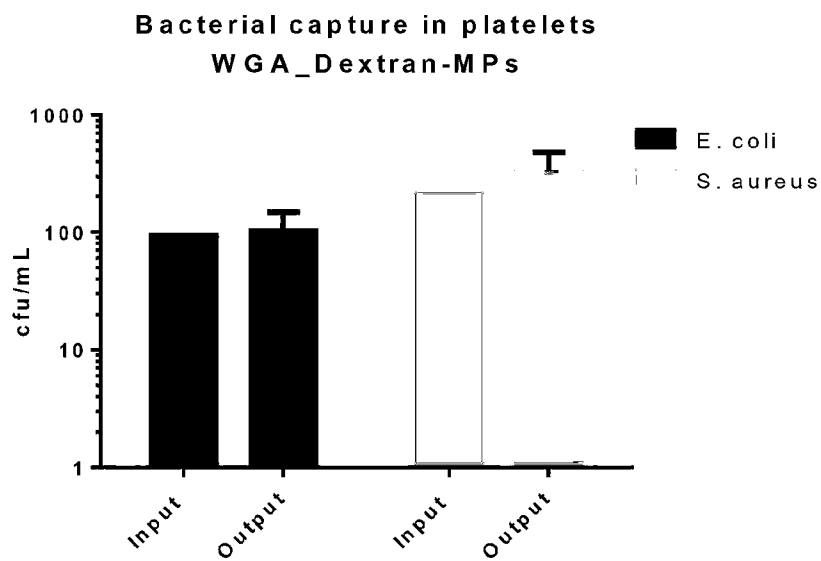


FIG 10

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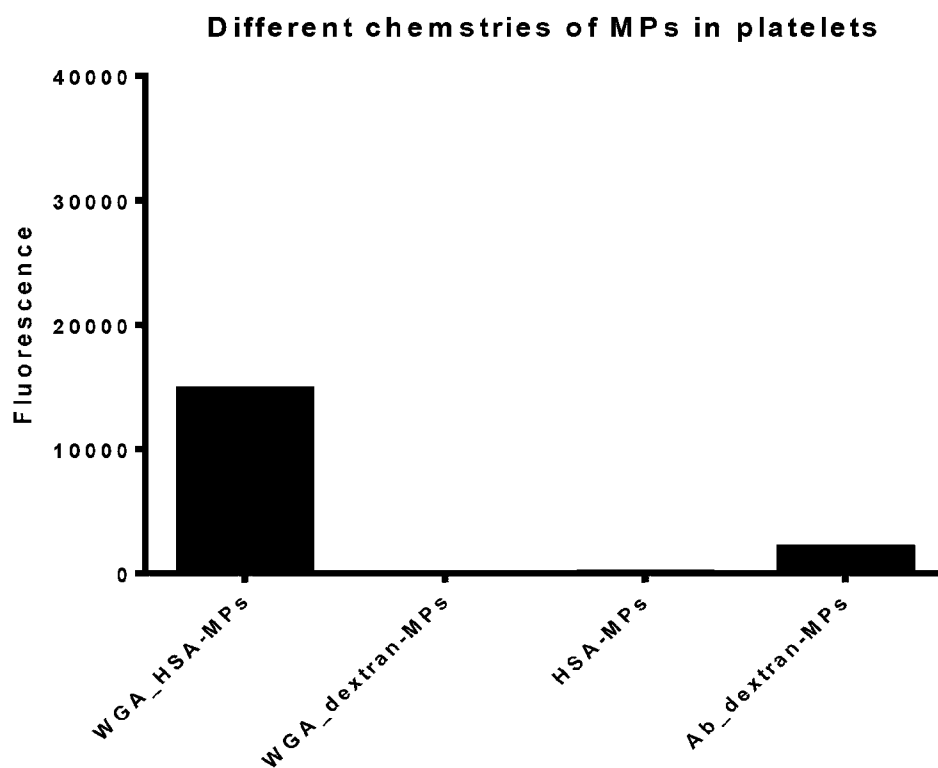


FIG 11

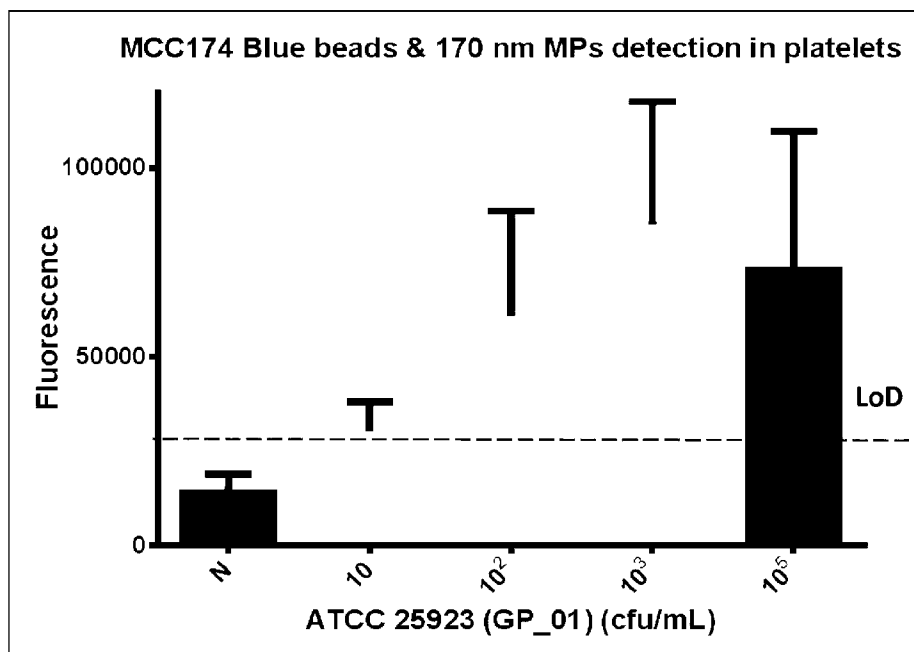


FIG 12

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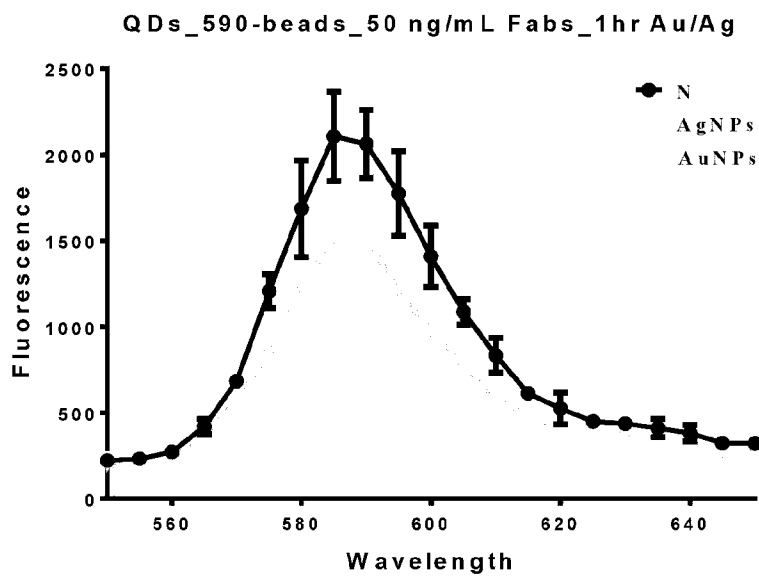


FIG 13 (a)

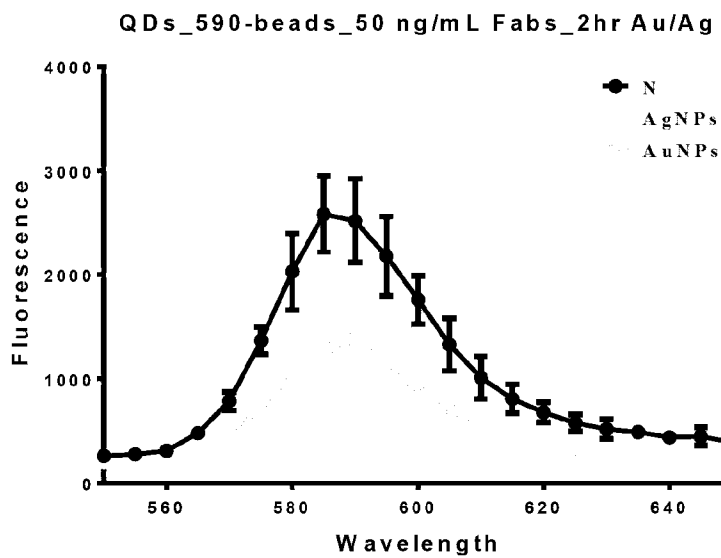


FIG 13 (b)

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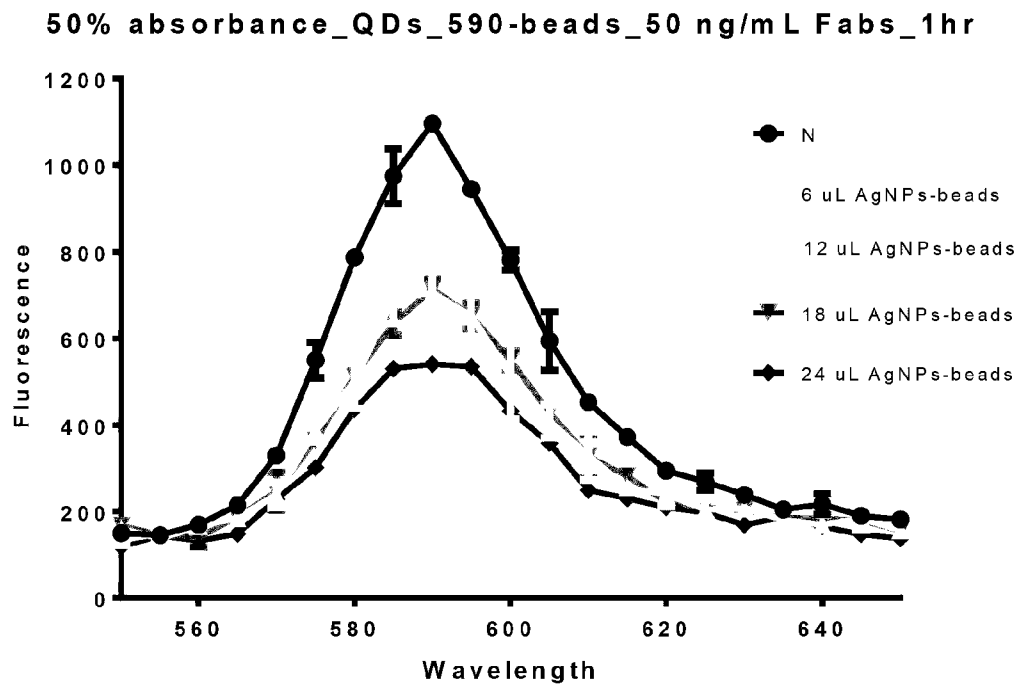


FIG 14

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/AU2015/050564

A. CLASSIFICATION OF SUBJECT MATTER

C08J 7/00 (2006.01) **C08J 7/04 (2006.01)** **C08J 5/00 (2006.01)** **C09K 11/02 (2006.01)** **C09K 11/88 (2006.01)**
B82B 1/00 (2006.01) **B82B 3/00 (2006.01)**

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)

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 practicable, search terms used)

WPI, EPODOC, MEDLINE, HCAPLUS, BIOSIS and WPIDS keywords: SENSOR; SCAFFOLD; SUPPORT; FRAMEWORK; IMPLANT; MATRIX; SURFACE; LAYER; COATING; METAL; GOLD; SILVER; PLATINUM; COPPER; NANOPARTICLE; QUANTUM DOT; AGAROSE; CELLULOSE; DEXTRAN; SEPHAROSE; HYALURONIC; POLYVINYLALCOHOL; POLYVINYLACETATE; POLYSACCHARIDE; POLYMETHYLMETHACRYLATE; THIOL; SULPHIDE; SILANE; CROSSLINK; MULTI-LAYER POLYMER NETWORK; synonyms and associated terms. GOOGLE SCHOLAR keywords: SENSOR; SCAFFOLD; MULTILAYER POLYMER NETWORK; CROSSLINK; NANOPARTICLE; QUANTUM DOT; RAZONI; COOPER; synonyms and associated terms. ESPACENET keywords: SENSOR; SCAFFOLD; MULTILAYER POLYMER NETWORK; CROSSLINK; NANOPARTICLE; QUANTUM DOT; synonyms and associated terms combined with Inventor names: RAZONI; COOPER; and/or Applicant name: UNIVERSITY OF QUEENSLAND. Applicant/Inventors names were also searched in internal databases provided by IP Australia.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

 Date of the actual completion of the international search
 15 December 2015

 Date of mailing of the international search report
 15 December 2015

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Authorised officer

Steven Zammit
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 (ISO 9001 Quality Certified Service)
 Telephone No. 0399359644

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2015/050564
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRADESS, R. ET AL., "Localized surface plasmon resonance sensor based on Ag-PVA nanocomposite thin films", Journal of Materials Chemistry, 2009 , 19, p9233-9240 see Abstract; page 9233, last paragraph; pages 9234-9235, paragraphs 1-3 of "Results and Discussion"; page 9237, paragraphs 1-3; and Figures 1A, 4A and 4B	1-30
X	GORDON, O. ET AL., "Silver Coordination Polymers for Prevention of Implant Infection: Thiol Interaction, Impact on Respiratory Chain Enzymes, and Hydroxyl Radical Induction", Antimicrobial Agents and Chemotherapy, 2010 , 54(10), p4208-4218 see Abstract; page 4210, column 2, "Results section"; and Figure 1	1-4, 8, 17-18
A	LUO, S. ET AL., "Applications of LPG fiber optical sensors for relative humidity and chemical warfare agents monitoring", Proceedings of SPIE-The International Society for Optical Engineering, 2002 , Volume 4920 (Advanced Sensor Systems and Applications), p193-204 see whole document	1-30
X	CN 101545883 A (EAST CHINA NORMAL UNIVERSITY) 30 September 2009, English machine translation retrieved from ESPACENET database see Abstract; and Example 1	1-30
X	CN 102250619 A (HUNAN UNIVERSITY) 23 November 2011, English machine translation retrieved from ESPACENET database see Abstract; and Examples	1-30
A	KR 20140100662 A (EWHA UNIVERSITY - INDUSTRY COLLABORATION FOUNDATION) 18 August 2014, English machine translation retrieved from ESPACENET database see whole document	1-30
A	YAO, X. ET AL., "Sub-attomole oligonucleotide and p53 cDNA determinations via a high-resolution surface plasmon resonance combined with oligonucleotide-capped gold nanoparticle signal amplification", Analytical Biochemistry, 2006 , 354, p220-228 see whole document	1-30
Form PCT/ISA/210 (fifth sheet) (July 2009)		

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2015/050564	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
CN 101545883 A	30 September 2009	CN 101545883 A CN 101545883 B	30 Sep 2009 08 Aug 2012
CN 102250619 A	23 November 2011	CN 102250619 A CN 102250619 B	23 Nov 2011 17 Apr 2013
KR 20140100662 A	18 August 2014	KR 20140100662 A KR 101436823 B1	18 Aug 2014 03 Sep 2014
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			