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(54) Title: LABELLED SILICA-BASED NANOMATERIAL WITH ENHANCED PROPERTIES AND USES THEREOF

(57) Abstract: The present invention relates to labelled silica-based nanoparticles with enhanced properties, to process for preparing them and to uses thereof.



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LABELLED SILICA-BASED NANOMATERIAL WITH ENHANCED PROPERTIES AND USES THEREOF

FIELD OF INVENTION

- 5 The present invention is oriented to a novel labelled silica-based nanomaterial, in particular a novel fluorescent silica-based nanomaterial with enhanced properties.

BACKGROUND OF THE INVENTION

10 The ideas developed in this patent are in the field of the fluorescent materials developed these last years for many applications. The luminescent properties of different materials were developed in many application fields like laser dyes, chemicals stains or biological labeling. Photo-luminescent properties like life time, FRET, polarization fluorescence, multiphotons excitation, phosphorescence or quenching were studied and lead to specific developments that enlarged the number of applications especially in biochemistry, in physics and telecom.

15 A non exhaustive list of the materials available for the different applications can be: organic dyes, organo-metallic molecules, metallic nanoparticles, rare earth complexes, lanthanides or metallic alloys, used as free molecules or as colloidal dispersions.

Each kind of material has its specificities, its properties and its limitations. Usually the choice of material is conditioned by the application requirements.

20 The idea here is to propose the most versatile material that should combine most of the advantages coming from the different materials, and that will considerably reduce the known disadvantages, like photostability, chemical stability or no expected biological interactions. In this application, the proposed labelled material, in particular fluorescent material, is based on silica nanoparticles. A lot of literature references are already describing fluorescent silica nanoparticles, but as they used the TEOS (tetraethyl orthosilicate) route, the nanoparticles are porous, which affect the chemical stability and the physical properties. In addition, the TEOS route will never allow the synthesis of particles with less than 15nm diameter.

25 Silica nanoparticles were described for the first time by Stöber in 1968, and different patents followed (Unger and al in 1988, Border and al in 1991) to improve the particles synthesis, using the TEOS route. The main improvement was the reduction of porosity (but not fully eliminated), and the reduction of polydispersity. The method was extensively used to encapsulate different materials like organic dyes, inorganic dyes, metallic nanoparticles (iron oxide, ZnO...), and the silica porosity was used as an advantage in silver (or iron oxide) core-

silica shell structures, to dissolve the core and obtain hollow silica shells. In our case, this porosity is a real issue for the long term stability of the materials we target.

More recently, new silica based nanoparticles using silicate route were optimized by Persello (1994, then 1999). In this work, Persello demonstrated the capability to produce highly dense nanoparticles of different sizes from 1 nm to few tenths of nm in diameter. The particles are then non porous, and very well defined; the author claims applications in catalysis, and solid support chemistry. In his whole work, the functionalization was done on the surface of the particles, and never in the core. Moreover, he never functionalized his particles with fluorophores.

SUMMARY OF INVENTION

We claim the invention of silica nanoparticles with a labelled core, in particular fluorescent core, using the silicate route for the particles synthesis, the process for preparing them and the uses thereof.

The present invention relates to a method for making a nanoparticle comprising a core and a first label, comprising the steps of:

(a) providing a first label bound to a first molecule comprising silane thereby forming a silane functionalized label;

(b) providing a first solution comprising free silicon-containing molecules;

(c) mixing the silane functionalized label and the first solution to form a first mixed solution;

(d) reducing the pH of the first mixed solution thereby allowing conditions for the formation of covalent bonds among the silicon-containing molecules to form silica within which the first label is covalently bound, thereby nucleating the core;

(e) allowing sufficient time for the core to grow until stopped.

Preferably, step (d) includes adding an ion-exchange resin to the first mixed solution, thereby to reduce the pH.

Preferably the silane comprises APTES, APTMS, MPTMS, and/or MPTES.

The method may further comprise several steps:

(f) mixing the grown core with a second solution comprising free silicon-containing molecules and preferably comprising a second label to form a second mixed solution, ;

(g) reducing the pH of the second mixed solution thereby growing a shell;

(h) allowing sufficient time for the shell to grow until stopped.

Optionally, the second solution may further comprise a second label.

Steps (f) through (h) may be repeated one or more times, and the second solution may comprise a label different than the first or preceding labels during each repeat.

The method may further comprise the steps of

(i) optionally adding a functionalizing agent surrounding the shell to form a functionalized shell, the functionalizing agent preferably comprising silanes, maleimides, thiols, and/or amines, and

(j) tuning one or more properties of the nanoparticle by grafting one or more surface molecules, preferably comprising a polymer, protein, antibody, antigen, sugar, PEG, organic molecule, and/or enzyme, to the shell or, if present to the functionalized shell.

The free silicon-containing molecules may comprise sodium trisilicate, sodium orthosilicate, sodium pyrosilicate, or hydrates thereof.

The growth of the core and/or the shell may be stopped by depletion of a constituent, addition of a quenching reagent, changing temperature, and/or changing pH.

The present invention further relates to a nanoparticle obtainable or obtained by the method above. The nanoparticle may have a diameter of 500 nanometers or less, 400 nanometers or less, 300 nanometers or less, 200 nanometers or less, 100 nanometers or less, 50 nanometers or less, 25 nanometers or less, 15 nanometers or less, 10 nanometers or less, or 5 nanometers or less, 2.5 nanometers or less, or less than 1 nanometer.

The first label, and more generally the label, may comprise an organic molecule, an organic dye, an inorganic dye, an inorganic molecule, a magnetic particle and/or a radioactive compound. In an embodiment, the first label is a fluorescent dye, or phosphorescent dye, preferably a fluorescent dye.

The core of the nanoparticle may further comprise a second label bound to silica and/or the shell, if present, may further comprises a third label and/or, optionally, a fourth label or no label. The first label, second label, third label, and fourth label each differ from one or more of the other. Alternatively, any two or more of the first label, second label, third label, and fourth label are the same.

The first label, and more generally the label, may be a fluorescent label having a tunable fluorescence polarization, in particular (a) by controlling a density of, or distances between, the fluorophores; (b) by adding a metal to the core and/or the shell; (c) based on the diameter; and/or (d) by controlling a ratio of the first label and a second label that is bound to the silica in the core, in the shell, and/or on a surface of the shell.

The first label, and more generally the label, may be covalently bound to the silica, for instance via a precursor made by the reaction between an aminopropylsilane and dye-NHS or dye-ITC or a precursor made by the reaction between a mercaptopropylsilane and dye-maleimide. Alternatively, the first label, and more generally the label, may be non-covalently bound to the silica, for instance via an electrostatic interaction.

The core, the shell, or both may be non-porous.

The nanoparticle preferably has a diameter of between 2 nanometers and 15 nanometers.

The nanoparticle may comprise an additional layer of functionalizing molecules, for instance selected from the group consisting of silanes, maleimides, thiols, amines functions, polymers, proteins, antibodies, antigens, sugars, PEGs, organic molecules, and enzymes, or be grafted with functionalizing molecules.

The present invention further relates to a method, comprising the steps of:

(a) attaching the nanoparticle according to the invention to a biological entity, preferably comprising tissue, tumor, eukaryotic, archaea or prokaryotic cell - such as bacterium - protein, antigen, DNA, RNA, or virus, via the surface functionalizing molecules; and

(b) detecting the label.

The present invention further relates to a method, comprising the steps of:

(a) providing a microfluidic device;

(b) providing at least a first nanoparticle of any one of claims 12-28 having the first label within a first droplet within the device and a second nanoparticle of any one of claims 12-28 having a second label within a second droplet within the device;

(c) detecting the first label and second label within the device, the detection being preferably done within the device.

The first and the second labels, and more generally the labels, may have a different property, such as intensity; excitation and/or emission wavelength; and/or fluorescence polarization, absorption, and/or fluorescence lifetime.

The present invention further relates to a microfluidic droplet comprising nanoparticles according to the invention. The droplet may be disposed within a microfluidic device.

The present invention further relates to a composition comprising nanoparticles according to the invention.

5 The nanoparticles or compositions according to the invention may be used for sample coding (in particular microfluidic droplets coding), for material doping, for labelling a molecular probe or tagging a molecule or compound of interest.

10 The present invention further relates to a nanoparticle, wherein the nanoparticle comprises a core comprising silica and a first label bound to silica and has a diameter of 15 nanometers or less. The nanoparticle may further comprise a shell, preferably comprising, in particular consisting of, silica, surrounding the core. The core may further comprise a second label bound to silica and/or the shell, if present, may further comprise a third label and/or, optionally, a fourth label, or no label.

15 The first label may be a fluorescent label having a tunable fluorescence polarization, in particular (a) by controlling a density of, or distances between, the fluorophores; (b) by adding a metal to the core and/or the shell; (c) based on the diameter; and/or (d) by controlling a ratio of the first label and a second label that is bound to the silica in the core, in the shell, and/or on a surface of the shell.

The core, the shell if present, or both are non-porous.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Illustration of uses of fluorescent marker for microarrays

Figure 2: Synthesis strategy overview to produce stable, fluorescent silica nanoparticles.

Figure 3: Continuous synthesis setup.

25 Figure 4: Steady state polarization versus interfluorophore distance R and the number of dimers in nanoparticles of $R_0=55 \text{ \AA}$ in silica nanoparticles.

Figure 5: Calculation of the mean distance between fluorophores in nanoparticles as a function of the particles size and the number of fluorescent molecules.

30 Figure 6: Fluorescence Intensity - Polarization mapping by varying beads and fluorescein concentration.

Figure 7: Size distribution by number: influence of the dye loading on the final diameter of the nanoparticles.

Figure 8: Influence of silica wt percentage on final diameter.

Figure 9: Evolution of particle size upon successive overcoatings, measured by double exponential fit.

Figure 10: Size distribution by number of particles: influence of the amount of silicate added for overcoating on the final particle size.

5 Figure 11: Brightness ratios of the silica nanoparticles as a function of their fluorophore concentration.

Figure 12: Fluorescence polarization at maximum emission wavelength of 2.5 nm diameter particles as a function of their fluorophore concentration.

Figure 13: Emission spectra of different silica nanoparticles prepared using the protocol 1.

10 Figure 14: Absorbance spectra of free and encapsulated rhodamine B ITC.

Figure 15: Fluorescence spectra of free and encapsulated rhodamine B ITC.

Figure 16: Absorbance spectra of free and encapsulated Dylight 680-NHS.

Figure 17: Fluorescence spectra of free and encapsulated Dylight 680-NHS.

Figure 18: Chemical structures of Dylight® 680-NHS, Dylight® 800-NHS and DY-782.

15 Figure 19: Histogram of number of droplets in library vs green fluorescence intensity, 3h after library generation

Figure 20: Histogram of number of droplets in library vs infrared fluorescence intensity, 3h after library generation.

20 Figure 21: Histogram of number of droplets in library vs green fluorescence intensity, 18h after library generation.

Figure 22: Histogram of number of droplets in library vs infrared fluorescence intensity, 18h after library generation.

Figure 23: Scatterplot of red vs green fluorescence intensity of droplet library, 3h after its generation.

25 Figure 24: Scatterplot of red vs green fluorescence intensity of droplet library, 18h after its generation.

Figure 25: Emission spectra of several infrared dyes, upon excitation at 680nm.

Figure 26: Influence of silica encapsulation on the photobleaching of FITC and rhodamine B ITC.

30

DETAILED DESCRIPTION OF INVENTION

In a particular embodiment, we propose in the following description a silicate route to prepare dense nanoparticles between 2 and 15 nm in diameter that embed labels or dyes, such as fluorescent molecules. The resulting materials get an incomparable photostability and

chemical stability. The silica surface gives the flexibility to tune the properties of the material by improving its colloidal stability in buffer solutions by grafting polymers like PEG (polyethylene glycol), or in organic solvents by grafting short hydrophobic chains. In polymer-based fiber applications, the nanoparticles could be used as doping materials, the contents and the surface treatment can be easily tunable to improve properties.

Therefore, the present invention relates to a nanoparticle, wherein the nanoparticle comprises a core comprising silica and a first label bound to silica, and has a diameter of 25 nanometers or less. The nanoparticle may also comprise a core comprising other label(s) bound to silica.

10 In a particular embodiment, the core of the nanoparticle is made of silica and labels.

In a particular embodiment, the nanoparticle may comprise more than one label in its core. For instance, the invention also contemplates nanoparticles comprising two, three, four or five distinct labels. The core may comprise a second label bound to silica and/or the shell may comprise a third label and/or, optionally, a fourth label, or no label. The first label, second
15 label, third label, and fourth label may each differ from one or more of the other or be the same.

Preferably, the different labels included in a nanoparticle can be distinctly detectable. For instance, for two distinct fluorescent labels, their emission wavelengths are sufficiently distinct to be simultaneously detected without significant optical crosstalk. The rules to select
20 such different labels are well-known by the one skilled in the art. For example, a combination of FITC (fluoresceine-ITC), Rhodamine B ITC, Dylight 680-NHS and one dye among Dylight 800-NHS, CF770 NHS, DY-782-NHS may be used.

In an alternative embodiment, the present invention relates to a set of several nanoparticles, the set including nanoparticles comprising different labels. For instance, such a set may
25 include a first group of nanoparticles comprising a first label, a second group of nanoparticles comprising a second label, etc. The set may include from two to ten distinct groups of nanoparticles, preferably from two to seven distinct groups of nanoparticles, more preferably from two to five distinct groups of nanoparticles. More preferably, the labels are fluorescent dyes or molecules. The fluorescent dyes or molecules may be chosen so as to be distinctly
30 detectable. For instance, their emission wavelengths are sufficiently distinct to be simultaneously detected. The rules to select such different labels are well-known by the one skilled in the art. For example, a combination of FITC, Rhodamine B ITC, Dylight 680-NHS and one dye among Dylight 800-NHS, CF770 NHS, DY-782-NHS may be used.

In another embodiment, the present invention relates to a set of several nanoparticles, the set including nanoparticles comprising different interacting labels. For instance, such a set may include a first group of nanoparticles comprising a first fluorescent label and a second group of nanoparticles comprising a second label capable of quenching the first fluorescent label.

- 5 Alternatively, such a set may include a first group of nanoparticles comprising a first fluorescent label and a second group of nanoparticles comprising a second fluorescent label having its absorption wavelength overlapping with the emission wavelength of the first fluorescent label, thereby allowing fluorescent transfer (FRET technology). For example, a combination of Dylight 680 and Dylight 750 may be used, as well as a combination of FITC
10 and RhBITC (Rhodamine B-ITC).

The label comprises an organic molecule, an organic dye, an inorganic dye, an inorganic molecule, a magnetic particle and/or a radioactive compound.

- Among the dyes or label that may be used in the present application, may be cited organic dyes, organo-metallic molecules, inorganic dyes, magnetic particles, radioactive compounds,
15 metallic nanoparticles, rare earth complexes, lanthanides or metallic alloys. In the present invention, the terms “dye” and “label” may be indifferentially used to qualify these molecules. These terms are intended to refer to detectable molecules.

- Preferably, the label is a fluorescent molecule or dye. In particular, the organic and/or inorganic dyes or molecules may be chosen among fluorescent dyes such as rhodamine B
20 ITC, fluorescein ITC, biphotonic fluorophores such as fluorenyl based dyes and amine-reactive dyes such as dyes of the Dylight[®] and Alexa[®] series. Specific dyes that may be used are Dylight 680 NHS, Dylight 800 NHS, IRDye 800CW NHS, CF 770 NHS, DY-782, IRDye 800RS NHS, CF 790 NHS, and mixtures thereof. The chemical structures of Dylight[®] 680-NHS, Dylight[®] 800-NHS and DY-782 are provided on figure 18.

- 25 Preferably, the dye is chosen among rhodamine B ITC, Dylight 680 NHS, DY-782-NHS and CF 770-NHS.

- The dye may alternatively be chosen among amine or maleimide derivatives of ATTO488 (Sigma-Aldrich Co, Missouri, USA), BODIPY FL (Invitrogen Corp. California, USA), DyLight 488 (Pierce Biotechnology, Inc. Illinois, USA), Sodium fluorescein, DY-682
30 (Dyomics GmbH, Jena, Germany), green fluorescent protein (GFP) and derivatives such as EGFP, blue fluorescent proteins (EBFP, EBFP2, Azurite, mKalamal), cyan fluorescent proteins (ECFP, Cerulean, CyPet) and yellow fluorescent proteins (YFP, Citrine, Venus, YPet), DsRed and derivatives thereof, Keima and derivatives thereof.

The dye may alternatively be chosen among fluorescent dyes based on xanthene, benzo[a]xanthene, benzo[b]xanthene, benzo[c]xanthene, coumarin, benzocoumarin, alizarin, azo, phenoxazine, benzo[a]phenoxazine, benzo[b]phenoxazine, benzo[c]phenoxazine, naphthalimide, naphtholactam, azolactone, methyne, oxazine, thiazine, diketopyrrolopyrrole, quinacridone, thioepindoline, lactamimide, diphenylmaleimide, acetoacetamide, imidazothiazine, benzanthrone, phthalimide, benzotriazole, pyrimidine, pyrazine, triazine, acridin, oxazine, cyanine, thiazol, anthraquinone, azamethine, polyene, oxonol, benzimidazol or indolenine.

Another preferred label is also a fluorescence quencher, for example belonging to the following families: Dabcyl, QXL, IRDye QC or QSY.

The label may alternatively be chosen among phosphorescent dyes or molecules such as Rhodamine 6G, eosin, platinum porphyrins, organometallic complexes containing osmium, ruthenium, iridium or platinum.

In a first embodiment, the nanoparticle's label may be covalently bound to the silica. More preferably, the label is covalently bound to the silica via an aminopropylsilane + dye-NHS or dye-ITC precursor or a mercaptopropylsilane + dye-maleimide precursor. In an alternative embodiment, the nanoparticle's label may be non-covalently bound to the silica. Preferably, the label is non-covalently bound to the silica via an electrostatic interaction.

More specifically, fluorescent properties may be obtained by including fluorescent organic or inorganic dyes. The dyes may be bound to the silica:

- With a covalent bound, for instance *via* an (aminopropylsilane + dye-NHS (N-hydroxysuccinimide) or dye-ITC (isothiocyanate)) precursor or a (mercaptopropylsilane + dye-maleimide) precursor,
- Or with a non covalent bound, for instance *via* electrostatic interactions (silica is minus charged, the embedded molecule can be plus charged).

By (X + Y) precursor is designed a precursor made by the reaction between X and Y entities. For instance, X may be aminopropylsilane and Y dye-NHS (N-hydroxysuccinimide) or dye-ITC (isothiocyanate). Alternatively, X may be mercaptopropylsilane and Y dye-maleimide.

Different strategies are described in the literature to produce fluorescent materials using organic or inorganic dyes. Some of them used big particles, for instance with a diameter superior to 25 nanometers, others used nanoparticles based on metals, rare earth, organometallic, or organic dyes.

The quantity of dye or label to be used in the core of the nanoparticle can vary in a wide range depending on the desired nanoparticles properties. For instance, the [dye]/[silane] molar ratio may be comprised between 1/160 and 1/10, in particular around 1/20.

Preferably, for a better quality of the nanoparticles, the label or dye of the nanoparticle core is
5 homogenously dispersed within the silica.

In a preferred embodiment, the nanoparticle further comprises a shell surrounding the core. By a shell is intended at least a first layer covering the silica core. Preferably, the shell comprises or consists in silica. Alternatively, the shell comprises or consists in a polymer. Optionally, the polymer may be functionalized. Such a polymer may be, for instance, an
10 acrylic polymer or a copolymer including acrylic monomers, poly-L-lysine, polystyrene, PMMA (polymethyl methacrylate), polybutadiene, biopolymers.

In a first embodiment, the shell is devoid of label or dye. In particular, when the core label is a fluorescent molecule or label, the shell does not include a fluorescent label.

In an alternative embodiment, the shell may include a label, distinct or not from the core label.
15 Preferably, the label is homogenously dispersed in the shell. In particular, the nanoparticles may have a shell comprising several successive layers (i.e., silica layer), each layer including a label (preferably, a fluorescent label or dye) distinct or not from the labels included into the nanoparticles core and into the other shell layers. The rules for selecting the shell label are the same than those used for selecting several labels to be included in the core. The nanoparticles
20 may also have a shell comprising several successive layers (i.e., silica layer), some including a label and other(s) not.

Optionally, the nanoparticle comprises an additional layer of functionalizing molecules or is grafted with functionalizing molecules. The functionalizing molecules may be selected from the group consisting of silanes, maleimides, thiols, amines functions, polymers, proteins,
25 peptides, aptamers, folic acid, antibodies, antigens, sugars, PEGs, organic molecules, enzymes, mixtures thereof and derivatives thereof.

In particular, the shell may be treated for functionalizing its surface. Then, the shell surface may be functionalized with molecules to increase the nanoparticles solubility in a solvent (e.g., water or organic solvents), their colloidal stability (e.g., in a buffer solution), or for
30 providing them to a binding ability to molecules of interest. For increasing the solubility in organic solvents, the nanoparticles may be functionalized with short hydrophobic chains. For increasing the colloidal stability, the nanoparticles may be functionalized with polyethylene glycol (PEG) chains. The short hydrophobic chains or PEG chains may be themselves functionalized for providing them to a binding ability to molecules of interest. Molecules for

providing a binding ability to molecules of interest may be selected from the group, but are not limited thereto, consisting of a protein, an antibody, a fragment or a variant thereof (i.e., having a binding specificity to molecules of interest), a nucleic acid (e.g., single or double chains, RNA, DNA, RNA/DNA hybrid, analogs thereof; a probe or primer), an antigen (e.g.,
5 tumor antigen, viral antigen, bacterial antigen, and the like), an enzyme (e.g., luciferase, β -galactosidase, phosphatase), an enzyme substrate, a reporter molecule, a ligand (e.g., cell ligand, receptor ligand), streptavidin, avidin, biotin and the like, a drug and a sugar (e.g., polysaccharide).

Accordingly, the nanoparticles of the invention are preferably formed of:

- 10 - a core obtained by including at least one label in silica,
- a first cover layer of silica, also designated as a shell, and
- optionally an additional layer of functionalizing molecules.

The structure of particles is detailed on figure 2.

In an embodiment, the diameter of the nanoparticles (including the core + silica shell) is 25
15 nm or less. For instance, the diameter of the nanoparticles is of 20 nanometers or less, 15 nanometers or less, 10 nanometers or less, 5 nanometers or less, 2.5 nanometers or less, or less than 1 nanometer. In particular, the diameter of the nanoparticles is comprised between 2 and 15 nm, preferably between 2 and 10 nm, in particular between 2.5 and 4.5 nm. The diameter can be modulated by modulating the number of silica overcoatings (OC) or the
20 thickness of the shell.

In another embodiment, the diameter of the nanoparticles is 500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 25 nm or less, 15 nm or less, 10 nm or less, 5 nm or less, 2.5 nm or less, or less than 1 nm.

Preferably, the nanoparticles of the invention have a core or a shell which is non-porous.

25 More preferably, the nanoparticle core is non-porous silica. Alternatively, the shell may be non-porous, in particular non-porous silica. Still more preferably, both the core and the shell of the nanoparticle are non-porous, in particular non-porous silica.

Preferably, the nanoparticles are essentially spherical.

The nanoparticles of the invention are obtainable, or obtained, by the synthesis process
30 described in the "synthesis protocol" section below.

When the dye is fluorescent, the main application of this kind of material uses its enhanced fluorescence properties.

The nanoparticle of the invention may be used in a large number of applications. In particular, all the applications involving the detection of a label, in particular a fluorescent label or dye,

are relevant for the nanoparticles. Briefly, the nanoparticles of the invention may be used for sample coding (in particular coding of microfluidic droplets), for material doping, for labelling a molecular probe or tagging a molecule or compound of interest. Examples of applications are detailed below. The nanoparticles may be used as marker, in particular biomarker, for in-vivo imaging, in microscopy (in particular fluorescence microscopy), as biosensor, in FRET, ELISA, quenching, fluorescence polarization, immunofluorescence, immunohistochemistry experiments, as optical probes, as diagnostic tools or for bio-analytical applications, as luminescent signal amplifiers, for mechanical reinforcement, in LED lighting, in LASERS, displays and the like. Those applications are well-known by the one skilled in the art.

More particularly, the present invention relates to a composition comprising nanoparticles of the invention, or a set of nanoparticles as disclosed above. The composition may be a diagnostic composition. It also relates to microfluidic droplets including nanoparticles of the invention, or a set of nanoparticles as disclosed above.

In particular, the present invention relates to a method, comprising the steps of:

- (a) attaching the nanoparticle of the invention to a biological entity via the surface functionalizing molecules, or creating an interaction between both; and
- (b) detecting the label.

Similarly, the present invention relates to a method for detecting a biological entity in a sample, comprising

- (a) providing a nanoparticle of the invention functionalized with a moiety capable of binding the biological entity to be detected;
- (b) contacting the nanoparticle with the sample; and
- (c) detecting the binding of the nanoparticle to the biological entity in the sample.

The biological entity may comprise tissue, tumor, eukaryotic, archaea or prokaryotic cell (such as bacterium), protein, peptide, antibody, receptor, antigen, antibody, enzyme, nucleic acid (such as DNA or RNA) or virus. The moiety is preferably capable of specifically binding the biological entity. The sample may be immobilized on a solid support. Typical pairs of binding moiety/biological entity can be streptavidin/biotin, antigen/antibody, ligand/receptor, enzyme/substrate, nucleic acid/nucleic acid, nucleic acid/DNA or RNA binding protein, and the like.

Enhanced fluorescence emission

Fluorescent dye encapsulation in this non porous silica beads induce an enhancement of the emission rate, which was never previously observed in a bead that small, as small as the beads of the present invention. Indeed, a decrease of the fluorescence lifetime ($\tau = 1/(K_{rad} + K_{non-rad})$) increases the quantum yield of the dye embedded in the nanoparticles ($Q = K_{rad}/(K_{rad} + K_{non-rad})$).

Figures 14 to 17 present the absorbance and fluorescence spectra obtained for different dyes both free and embedded in silica nanoparticles according to the invention. These spectra clearly evidence the fluorescence of the embedded dye is enhanced.

High FP

The fluorescence polarization (FP) properties of nanoparticles of the invention are also an interesting feature.

The principle of FP in fluorescent SNPs (silica nanoparticles) has been previously reported in specific literature. Without wishing to be bound to any theory, the immobilization of the fluorophore in a very dense particle decreases its mobility drastically, which leads to the increase of the steady state fluorescence polarization. Furthermore, the tiny size of the nanoparticles and the NIR emission greatly reduces uncontrolled depolarization induced by the light scattering.

FP tunability by FRET

The fluorescence polarization can be tuned by playing on Förster Resonance Energy Transfer (FRET) within 2 fluorophores A and B, the emission spectrum of A overlapping the absorption spectrum of B. Thus, chromophore A (donor) may transfer energy to chromophore B (acceptor) with another orientation, which contributes to depolarize the signal. Note that A and B can be the same fluorophore if its absorption and emission spectra overlap: in this case the phenomenon is called Homo-FRET.

The theoretical calculation of the depolarization fluorescence by HomoFRET properties was described in the literature by L. W. Runnels and S. F. Scarlata. The calculation takes into account the spectral properties of the fluorophore, the random orientation of the dyes, the refractive index of the dispersant media, and the number of molecules pairs (clusters) interacting by FRET.

A full description of this FRET phenomena in nanoparticles was already described these last years, but the particles used were always prepared by TEOS route. That means that the long

term storage properties of this kind of porous material cannot be guaranteed, and the porosity can induce long term photodestabilization of the dyes encapsulated in the nanoparticles. In addition, in the literature, authors who tried to observe the fluorescence polarization decrease with tiny particles (<10nm in diameter) never managed to achieve it.

- 5 Based on the theoretical simulation (see details in the examples), we can estimate the minimum number of fluorescent molecules per bead of a certain size necessary to drop down the FP signal around 50mP.

Via the silicate route for particles of 2.5 nm diameter, the FP can be decreased from 410mP to 350mP, by increasing the dye concentration. Additionally, increasing the size of nanoparticles
10 up to 15 nm affords a decrease in FP to 50mP. This allows filling more fluorescent molecules per particle.

The theoretical calculations estimate the best conditions to maximize the HomoFRET in each particle, and lower the FP value to 50mP: a good working range appears to be 20 dye molecules in 15 nm diameter nanoparticles. 15 nm size particles are accessible by the silicate
15 route synthesis. And including 20 molecules in the beads means that we will get enough clusters (>4) for all the beads, especially if we consider the distribution of molecules through the particles as following the Poisson distribution. And the mean the distance between clusters will be in average around 4.4nm, which is around $0.8 \times R_0$ (the best compromise between FRET and self quenching).

- 20 Accordingly, the present invention relates to a method for detecting binding or short distance between two elements in a sample, comprising:

- providing a first nanoparticle of the invention with a first fluorescent label, said nanoparticle being functionalized with a first moiety capable of binding to one of the two elements;
- 25 - providing a second nanoparticle of the invention with a second fluorescent label, said nanoparticle being functionalized with a second moiety capable of binding to the other of the two elements, wherein the emission spectrum of the first fluorescent label overlaps the absorption spectrum of the second fluorescent label;
- contacting the sample with the first and second nanoparticles; and,
- 30 - detecting the fluorescence of the second fluorescent label after an excitation of the first fluorescent label, the fluorescence detection being indicative of the short distance between two elements.

The invention further relates to a kit for detecting the distance between two elements, the kit comprising first nanoparticle of the invention with a first fluorescent label, said nanoparticle

being functionalized with a first moiety capable of binding to one of the two elements; and a second nanoparticle of the invention with a second fluorescent label, said nanoparticle being functionalized with a second moiety capable of binding to the other of the two elements, wherein the emission spectrum of the first fluorescent label overlaps the absorption spectrum of the second fluorescent label.

Hetero FRET assays

The previous description described only the HomoFRET phenomenon, that can be used to reduce the FP value of the particles. But as the seeds particles diameter (around 2.5 nm) is smaller than Förster distance (5 to 10 nm), the nanoparticles of the invention may be used for any FRET application usually used in biosensor, and bio-analytical applications. A non exhaustive list of applications is proposed in the “applications” section of this document.

Synthesis protocol

The same protocol is used to produce the full variety of silica nanoparticles materials needed to target the multiples filed applications, such as multidimensional encoding, using fluorescence intensity and fluorescence polarization, biosensor, fiber doping, or bio-analytical applications.

The synthesis protocol followed in this work is innovative by the flexibility and the accuracy of the beads that are produced. The synthesis is split in two parts: the silica seed creation, followed or not by a shell growing on the particles, preferably using an automated system.

The seeds or cores already contain labels, preferably fluorescent molecules, (1 to 10 per seed) by including in the silicate solution the right concentration of dye or label pre-bound to a silane, in particular an alkoxysilane, such as a trimethoxysilane or a triethoxysilane, in particular APTES, APTMS, MPTMS and/or MPTES. The label or dye may be bound to the silane by a covalent bond or a non-covalent bond. Preferably, the label or dye is bound to the silane by a covalent bond. The silane is consecutively covalently bound to the silicate during the polycondensation process. Any positively charged molecule can be embedded as a label in the nanoparticles, as well as any succinimidyl ester, maleimide or silane functionalized molecule. Additional molecules that are not labels, chosen among positively charged molecules, succinimidyl esters, maleimides and silane functionalized molecules may also be embedded in the nanoparticles. The silicate may be chosen in particular among sodium trisilicate, sodium orthosilicate, sodium pyrosilicate, and hydrates thereof.

During the growing process, preferably the automated growing process, the same silane-dye molecule can be added gradually to be uniformly embedded in the whole bead core. To get a perfect sealing of the bead, the growth will be then continued during few more nm, without silane-dye addition, to create a bare shell that prevents any long-term leakage issue.

- 5 This work is distinguished from the previous ones by the quality of the particles produced, which are functionalized in the core, and the porosity of the nanoparticles is drastically reduced, which improves the chemical and optical stability for long term storage.

The produced nanoparticles are denser than those previously described and produced *via* the TEOS route. The density can be characterized by measuring the volume fraction of particles
10 in solution. For instance, the density of nanoparticles synthesised via the Stöber route may be between 1.5 and 2 g/cm³, in particular 1.8 g/cm³. The density of nanoparticles of the invention is generally more than 2 g/cm³, in particular around 2.2 g/cm³.

In addition, the synthesis protocol is easy to implement since only a rough filtration is required to purify the nanoparticles produced, instead of a full dialysis or ultrafiltration for
15 Stöber protocol.

Accordingly, the present invention relates to a method for making the nanoparticle of the invention, comprising the steps of:

- (a) providing a first label bound to a first molecule comprising silane, thereby forming a silane functionalized label;
- 20 (b) providing a first solution comprising free silicon-containing molecules;
- (c) mixing the silane functionalized label and the first solution to form a first mixed solution;
- (d) reducing the pH of the first mixed solution thereby allowing conditions for the formation of covalent bonds among the silicon-containing molecules to form silica within
25 which the first label is covalently bound, thereby nucleating the core;
- (e) allowing sufficient time for the core to grow until stopped.

In a particular embodiment of the method, in step (a), more than one first label bound to a first molecule comprising silane is provided. Indeed, two, three, four or five distinct labels bound to a first molecule comprising silane may be provided. Preferably, the different labels
30 included in a nanoparticle can be distinctly detectable. For instance, for two distinct fluorescent labels, their emission wavelengths are sufficiently distinct to be simultaneously detected. The rules to select such different labels are well-known by the one skilled in the art. Preferably, the label comprises an organic molecule, an organic dye, an inorganic dye, an inorganic molecule, a magnetic particle and/or a radioactive compound. More preferably, the

label is a phosphorescent or fluorescent molecule or dye, still more preferably a fluorescent molecule or dye.

Preferably, step (d) includes adding an ion-exchange resin to the first mixed solution, thereby to reduce the pH. For instance, the an ion-exchange resin is an acidic exchange resin such as
5 Amberlite[®] IR-120 (Aldrich), Amberlite IRN77, Dowex HCR-W2, Dowex Marathon.
Preferably, the silane is as detailed above and may be selected from the group consisting of APTES, APTMS, MPTMS, and/or MPTES.

Preferably, the free silicon-containing molecules may comprise all common types of silicates, such as metasilicates, disilicates, and favourably alkali metal silicates such as sodium silicate
10 or potassium silicate. This includes sodium trisilicate, sodium orthosilicate, sodium pyrosilicate, or hydrates thereof. If sodium silicates are used, the SiO₂/Na₂O weight ratio is preferably between 2 and 4.

Preferably, in step (c), the quantity of label can vary in a wide range depending on the desired nanoparticles properties. For instance, the [dye]/[silane] molar ratio may be comprised
15 between 1/160 and 1/10, in particular around 1/20.

When the nanoparticle comprises a silica shell, the method further comprises

(f) mixing the grown core with a second solution comprising free silicon-containing molecules to form a second mixed solution;

(g) reducing the pH of the second mixed solution thereby growing a shell;

20 (h) allowing sufficient time for the shell to grow until stopped.

In a particular embodiment, the second solution is devoid of label, in particular fluorescent label. In an alternative embodiment, the second solution further comprises a second label. In particular, steps (f) through (h) may be repeated one or more times, and the second solution may comprise a label different than the first or preceding labels during each repeat.
25 Preferably, the different labels included in a nanoparticle can be distinctly detectable. For instance, for two distinct fluorescent labels, their emission wavelengths are sufficiently distinct to be simultaneously detected. The rules to select such different labels are well-known by the one skilled in the art.

When the nanoparticle is functionalized, the method further comprises the steps of (i)
30 optionally adding a functionalizing agent surrounding the shell (or core when the shell is absent) to form a functionalized shell (or core), and (j) tuning one or more properties of the nanoparticle by grafting one or more surface molecules to the shell or core when the shell is absent) or, if present to the functionalized shell. Functionalization is disclosed in details above. For instance, the functionalizing agent comprises silanes, maleimides, thiols, and/or

amines. The surface molecules may be any molecule of interest and comprise molecules for increasing the solubility of the nanoparticles in a solvent (e.g., organic solvents), their colloidal stability (e.g., in a buffer solution), or for providing them with the ability to bind molecules of interest. For instance, the surface molecules may be a short hydrophobic chain optionally functionalized to be able to bind molecules of interest, a polyethylene glycol (PEG) chain optionally functionalized to be able to bind molecules of interest, a protein, an antibody, a fragment or a variant thereof (i.e., having a binding specificity to molecules of interest), a nucleic acid (e.g., single or double chains, RNA, DNA, RNA/DNA hybrid, analogs thereof; a probe or primer), an antigen (e.g., tumor antigen, viral antigen, bacterial antigen, and the like), an enzyme (e.g., luciferase, β galactosidase), an enzyme substrate, a reporter molecule, a ligand (e.g., cell ligand, receptor ligand), streptavidin, avidin, biotin and the like, a drug and a sugar (e.g., polysaccharide). In particular, the surface molecules comprise a polymer, protein, antibody, antigen, sugar, PEG, organic molecule, and/or enzyme.

Preferably, growth of the core and/or the shell is stopped by depletion of a constituent, addition of a quenching reagent, changing temperature, and/or changing pH.

One object of the present invention is particles obtainable or obtained by the synthesis process described above.

Colloidal Stabilization

The nanoparticles produced (core + silica shell) are stable in deionized water by electrostatic repulsion. But the screening length decreases in buffer solutions due to the presence of salts. To improve the nanoparticles stability in buffered solution, the nanoparticles are stabilized via steric repulsion, using functionalizing molecules such as polymers. The best biocompatible and water soluble polymer being polyethylene glycol (PEG), different strategies are proposed for the nanoparticles surface functionalization.

The first way is by functionalization of the silica surface using APTES or MPTES (silanes) to get primary amines or thiol functions. Those functions react specifically and respectively with PEG-NHS and PEG-maleimides. It is also possible to directly graft PEG alcoxysilanes on the silanol groups at the surface. The particles become crowned with PEG, which doesn't affect the water solubility but improves the stability in buffer solution. In addition by using bifunctional PEG-polymers, the nanoparticles surfaces can be modulate to meet the application requirements, by keeping good binding properties to IgG, proteins, or cells (using NHS, maleimide, lysine, avidin functions ...).

The second way, is by growing the acrylic polymer, with PEG in side chain, using the radical polymerization (controlled or not), that allows the grafting of longer chains. This dense core (silica)/shell (polymer) structure is probably the most stable for long term storage. As previously described the end chain can be also functionalized to keep the same reactivity.

5 In order to favour their solubility in organic solvents, the NPs may be alternatively functionalized with hydrophobic molecules, in particular short hydrophobic chains. Among short hydrophobic chains that may be used to functionalize the nanoparticles may be cited short chains of triethoxy-functionalized polyethylene, polypropylene, polypropylene oxide, polyvinyl chloride, polyvinyl acetal. One of ordinary skill in the art will chose the appropriate
10 hydrophobic chain to use in function of the properties of the solvent wherein the particles are dispersed or solubilised.

Other functionalizing molecules may be molecules containing silanes, maleimides, thiols, and amines functions, polymers, proteins, antibodies, antigens, sugars, PEGs, organic molecules, and enzymes.

15 **Other advantages**

By varying the fluorescent molecule structure, the excitation and emission wavelengths can be tuned. By adjusting the concentration of dye molecules par particles the polarization fluorescence properties can be modulated.

20 Accordingly, the nanoparticles of the invention may comprise a fluorescent label having a tunable fluorescence polarization. The fluorescence polarization can be tuned though different ways, in particular (a) by controlling a density of, or distances between, the fluorophores; (b) by adding a metal to the core and/or the shell; (c) based on the diameter; and/or (d) by controlling a ratio of a first label and a second label that is bound to the silica in the core, in
25 the shell, and/or on a surface of the shell.

In addition, encapsulating the dye or label in silica has two positive consequences: photobleaching is considerably reduced and quantum efficiency is enhanced. Figure 26 compares the photobleaching of free rhodamine B ITC with that of the same dye encapsulated in silica according to the invention. The photobleaching time constant is significantly
30 increased.

As far as barcoding in droplets, silica nanoparticles prove to be compatible with droplets, as showed on figures 19 to 24: the barcodes remain stable in time.

Summary of the advantages

To summarize the specifications for this innovative material the following arguments are advanced:

- The synthesis protocol is very easy
- 5 - The dyes available for this kind of applications are very diverse
- The particles size is very small, and can be tuned to reach specific needs
- As they are small, the number of objects per volume units is higher
- As the particles are small, they are less heavy for their guest
- The particles are non-porous
- 10 - The surface functionality is adjustable
- The particles can be used for FRET characterization
- The excitation time and the response time are in less than microsecond time scale
- Stability of the material in any chemical media (different pH buffers between 2 and 12, organic solvent like dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol,
- 15 ethanol, acetonitrile...)
- Inert material that will not affect the media in which they are immersed
- Stability over short and long term storage (no chemical degradation, no colloidal destabilization)
- High chemical resistance
- 20 - The material are well dispersed in the media, resulting in a homogenous signal in sub- μm dimensions
- They are compatible with the biology
- The interactions with the biological systems is limited, and do not affect the kinetics or the mechanisms
- 25 - The fluorescence signal is strong
- Photo-stability over short and long term storage for the coding.

APPLICATIONS**30 Application 1: barcodes for HTS of drug candidates in droplet microfluidics**

Droplet-based microfluidics is the most promising technology to increase the sampling rate and the throughput for drug screening, diagnostics or multi-component reactions. Unfortunately, it is impossible to spatially or temporally encode the information contained within the droplets once the droplets are collected out of the chip and mixed all together.

To extend the capability of this technology, fluorescent nanoparticles according to the invention can be used as a labelling system of each reacting volume to identify the drug candidate species it contains. Assuming this volume is confined in liquid droplets mixed with millions of other droplets, it is important to identify the droplets containing the same drug candidate, and distinguish the different compositions.

For that, a digital coding system can be proposed, using fluorescent intensity. The number of codes generated by this kind of strategy is proportional to the number of discrete intensity levels that can be used, multiplied by the number of colors. Since the main applications targeted are biological systems that will be studied in the droplets, it is important to leave the whole UV-visible part of spectrum for the application. Thus the fluorescent coding systems have to be shifted as much as possible in the Near IR part.

As the window becomes very restrictive, the number of colors becomes limited. To increase the coding capability it is possible to add one more freedom axis (variable), by using the fluorescence polarization in addition to the fluorescence intensity.

The application constraints, that are the use of biological and chemical systems in high throughput droplets-based microfluidics, dictate the specifications of the nanoparticles to be developed.

An example of nanoparticles according to the invention that would be suitable for such application combines the following properties:

- The excitation time and the response time are less than microsecond scale to not affect the throughput targeted;
- 2-10 nm particles are well dispersed in their media, giving an homogenous signal in sub- μm dimensions;
- The fluorescence signal is strong enough to cover at least 20 discrete intensity levels;
- For each labeling color, at least two polarizations states can be produced to create polarization levels used in the multidimensional encoding;
- Silica exhibits high chemical resistance to harsh conditions (pH, temperature);
- nanoparticles of the invention are inert and will not affect the chemical/biological reactions occurring in the droplet;
- Once functionalized, the nanoparticles exhibit long-term colloidal stability in a broad range of chemical media (different pH buffers between 2 and 12, organic solvents like DMSO (dimethylsulfoxide), Methanol...);
- The covalent binding of dyes in small nanoparticles enables short and long term storages in bulk and in the droplets (no exchange between droplets, no chemical degradation);

- Nanoparticles of the invention show good photostability at short term (illumination by laser in droplets) and long term (storage): the coding is not degraded.

Therefore, the present invention relates to the use of nanoparticles of the invention for coding microfluidic droplets. In particular, it relates to the use of a set of nanoparticles of the invention for barcoding microfluidic droplets.

The present invention further relates to a method, comprising the steps of:

(a) providing a microfluidic device;

(b) providing at least a first nanoparticle of the invention having the first label within a first droplet within the device and a second nanoparticle of the invention having a second label within a second droplet within the device;

(c) detecting the first label and second label within the device.

Preferably, the first and second labels are fluorescent dyes or molecules. In particular, for the two distinct fluorescent labels, their emission wavelengths are sufficiently distinct to be simultaneously detected. The rules to select such different labels are well-known by the one skilled in the art. Of course, the method is not limited to two different labels. The system may be incremented to three, four, five or more droplets with the corresponding labels.

In an embodiment, the first and second labels, and more generally the labels, have a different property, for instance comprising intensity; excitation and/or emission wavelength; and/or fluorescence polarization, absorption, and/or fluorescence lifetime.

Preferably, detection step (c) is performed within the device.

Another object of the invention is a microfluidic droplet comprising a nanoparticle according to the invention, that may be disposed within a microfluidic device.

Application 2: Optical probes for fluorescent polarization microscopy

Nanoparticles with different FP levels but same diameter and same maximum emission wavelength may be synthesised by a process according to the invention.

Particles of each FP level can be functionalized differently (with more or less hydrophobic surfactants, or distinct antibodies, or different DNA strands for instance), then mixed and be all be injected into cells or whole organisms.

Each type of particle will go to its different target, and FP microscopy will allow differentiated visualization of each of them, using only 1 excitation wavelength.

Given that nanoparticles of the invention can be spherical and of uniform size, they are more reliable than using mixed quantum rods of different FP levels (varying their aspect ratio) whose different geometries induce different diffusion and rotation properties.

Application 3: Immunology tests by FRET signal variation

Nanoparticles of the invention may be used in immunology tests.

For instance, specific antibodies may be bound to 2 nm diameter particles encapsulating 1 fluorophore A (ex: fluorescein), and all antigens may be bound to other 2 nm diameter particles containing fluorophore B (ex: rhodamine B) whose absorption spectrum overlaps the emission spectrum of fluorophore A. The other necessary condition is that fluorophore B must not be excited by the wavelength used to excite fluorophore A (ex: 488 nm).

The antigen corresponding to the chosen antibody will bind to it, the distance between both will go under FRET distance, and fluorophore energy transfer will take place, enabling fluorescence emission from fluorophore B.

Using a filter centered around fluorophore B maximum emission wavelength, the bright regions will locate the antigen specific to the chosen antibody. Moreover, the variation in fluorescence intensity will be dependent on the antibody-antigen distance.

Nanoparticles of the invention are especially suitable for this application, because once close to each other, their center-to-center distance is around 2 nm, which is below FRET distance (contrary to commercial 30 nm radius core-shell nanoparticles which could not be used). In addition, their brightness is higher and they are less sensitive to photobleaching than the free fluorophore, which is a real asset during long observations.

Application 4: Molecular tagging to follow dynamic phenomena by fluorescence microscopy

A 2-nm fluorescent nanoparticle according to the invention may be functionalized with a marker specific to the moving target that is aimed to be observed. It binds to the target and enables the observation of its movement.

Given its small size, the fluorescent nanoparticle will have a reduced impact on the target motion, minimizing the bias. Moreover, its small size enables use of fluorescent microscopes with high-resolution in X, Y and Z axes. Finally, it will be brighter and more photostable than the corresponding free dye, and will not blink like the majority of quantum dots.

Application 5: Fluorescent ELISA tests

Fluorescent nanoparticles of the invention may be functionalized with NHS endgroups, and then added to the blood plasma sample investigated. The nanoparticles bind to all the antigens. The mixture is added into wells functionalized with the antibody specific to the

antigen that is being sorted. Antibody-antigen binding takes place; the well is then washed to remove all non-specific antigens, then a fluorescence measurement is performed. The fluorescence intensity will be proportional to the number of fluorescent-labeled bound antibodies.

5

Application 6: Fluorescent marker for microarrays

Fluorescent nanoparticles according to the invention may be used as fluorescent markers for microarrays. Nanoparticles covering antibodies or streptavidin or any other specific marker can be for instance used (see example on figure 1, from en.wikipedia.org/wiki/Antibody_microarray).

10

Using 2 nm particles will enable to bind more particles by antibody than with classically used 30 nm particles of the same brightness for each (containing the same number of fluorophores): the fluorescence intensity will consequently be increased, as well as the assay sensitivity.

15

Application 7: Near-infrared (NIR) emitting probe for in-vivo imaging

2-5nm nanoparticles containing covalently bound NIR fluorophores are potential powerful fluorescent markers for *in vivo* imaging: once properly functionalized, their small size will enable them to cross all types of membranes that would be impassable by particles of $d > 50\text{nm}$.

20

Moreover, NIR organic fluorophores commonly have FWHM (full-width half-maximum) values around 80 nm at 800 nm maximum emission, which is better than the ~120 nm FWHM obtained with CdTeSe/CdZnS core-shell QD at the same wavelength (ref Pons T. *et al*, CHEMISTRY OF MATERIALS, 21, 8, 1418-1424, 2009), enabling more multiplexing. Otherwise, CdSe/CdTe QDs with similar FWHM (ref Kim S. *et al*, J. AM. CHEM. SOC. 2003, 125, 11466-11467) are not biofriendly because of Cd, contrary to biocompatible silica.

25

Application 8 : Enzymatic assays using FRET or quenching.

Enzymatic assays can be performed using nanoparticles of the invention by 2 ways :

30

- using FRET : one set of nanoparticles encapsulating fluorophore A and another set encapsulating fluorophore B (same absorption/emission spectra properties as in application 3), are functionalized separately with chemical functions so that they can later be bound 1 by 1 (ex : first set functionalized by alcohol and the second one functionalized by an activated acid, bonding of nanoparticles 1 by 1 will happen *via* formation of ester bonds).

Once coupled, FRET can be observed between the 2 particles : B dye is emitting fluorescence. Then, enzymatic activities of several enzymes (for instance esterases) can be monitored by measuring the decrease of fluorescence by B upon ester function cleavage, the separation of the 2 FRET moieties.

- 5 - using a quencher : with the same principle, nanoparticles encapsulating quencher dyes can be covalently bound to nanoparticles encapsulating the corresponding fluorophore : fluorescence emission is measured and is indicative of the cleavage of the chemical functions. Upon enzymatic cleavage, the quencher will be released and emission from the fluorophore will increase.

10

Applications 9 and 10: Doped polymeric fibers and doped dye laser

Very tiny fluorescent silica nanoparticles according to the invention are promising candidates for doped materials based on new low cost polymers or sol-gel, in optical amplification phenomenon.

- 15 Indeed, fluorescence emission is largely used to induced amplified spontaneous emission or light amplification by stimulated emission of radiation (LASER) in Liquid Dyes Laser. But recently, the interest for low cost solid polymers components (Somasundaram *et al.* Journal of photochemistry and photobiology, 1999, vol. 125, n°1-3, pp. 93-98 (10 ref.), Yu et al. Optics Express 2007, Vol. 15, No. 16, p. 9989), instead of silica gels (Altman et al. IEEE Photonics technology letters 1991, vol. 3, n°3, pp. 189-190 (5 ref.)) (as the host media) for dye lasers or
20 optical amplification grows up. For this kind of application it is important to get read of the common issues associated to the liquid solution of free dyes, such as: concentration variation, useless solvents or photobleaching. The tiny silica nanoparticles can address the cited problems since they are water soluble, can be used at very high concentration, and the photo-
25 bleaching is very limited. Using very tiny silica nanoparticles the scattering effect is considerably reduced. In addition, the silica surface can be easily functionalized to make it compatible with the polymeric matrix or another sol-gel thin film.

The doping with silica fluorescent nanoparticles application can be useful in the following applications, among others:

- 30 - Doped Polymer Optical fiber component for optical amplification in telecommunication or in new low cost polymer LASER fiber.
- New generation of Active optical waveguide or integrated components based on Sol- Gel technology for optical amplification or integrated low cost LASER.

The other very useful application field is liquid Laser. The fluorescent silica nanoparticles are one of the most promising candidates for stable dye laser. The microelectronic expansion opens the access to the droplets based microfluidic, which can generate the new generation of dye LASER. Indeed, droplets can be used like optical micro cavity if there is a high contrast index (0.2 to 0.3) between the droplets and the media, which induced whispering modes that can produce the amplification emission signal (Tang *et al.* Lab Chip 2009, 9, 2767–2771). The concentration variation reduction, confirmed by a better photo-stability and a reduction transport effect between droplets encapsulating the silica fluorescent nanoparticles will allow better stability in microfluidic droplets using the dyes laser phenomenon.

EXAMPLES

Example 1: Experimental methods – Protocols

Protocol 1:

Synthesis strategy overview

The synthesis optimizations were done with inexpensive fluorophores like Fluorescein isothiocyanate, or Rhodamine B isothiocyanate. Then, the protocols were transferred to the Dylight® NIR dyes.

Silane-dye precursor synthesis

A stock solution of dye (Fluorescein isothiocyanate or Rhodamine B isothiocyanate, both Sigma Aldrich, or Dylight® 680-NHS and Dylight® 800-NHS, Thermo Scientific) functionalized with isothiocyanate, NHS or maleimide, in 99,9% anhydrous ethanol (Merck) or DMSO was prepared under nitrogen in a dry 1.7mL microcentrifuge tube (Axygen); the final concentration was in the range of $5 \cdot 10^{-4}$ - 10^{-2} M. Between each use, the vial was stored at 4°C away from light and moisture. If necessary, this stock solution was diluted to $5 \cdot 10^{-4}$ M with anhydrous ethanol under nitrogen before use.

A sample of this diluted dye solution (in the range of 0.1 to 1 mL) was mixed under nitrogen with 3-aminopropyltriethoxysilane or 3-thiopropyltriethoxysilane, in the 10^{-5} to 10^{-4} mol/L range, keeping a ratio [dye]/[silane]=0.05. The reaction mixture was kept closed hermetically in the dark at 50°C for 30 min.

Fluorescent seed particles synthesis

A pH meter (pH 211, Hanna Instruments) was used to monitor the pH all along the reaction steps. In an opaque plastic beaker, a sodium trisilicate solution (Sigma-Aldrich) was diluted to 10% w/w with MiliQ water (Millipore SAS, Molsheim, France) under stirring: the pH

- 5 reached 11.65. Then, the silane-dye reaction mixture was added: the pH did not change.
12.5g of Amberlite[®] IR-120 acidic exchange resin (Aldrich) were added: the pH started dropping continuously. When pH reached 9.00, the mixture was filtered on cotton pad and the filtrate was kept under stirring in the dark overnight. After 16 hours of reaction, the pH had raised to ~10, so 0.7g Amberlite[®] were added to lower the pH back to 9.00. Once this value
- 10 was reached, the mixture was filtered on cotton and the filtrate kept away from light.

Dialysis

To get rid of the ungrafted dye molecules, the seed particles were dialysed in ~30 times their volume of miliQ water with a MWCO=4000 (molecular weight cutoff) cellulose dialysis

- 15 membrane (Carl Roth). During dialysis, samples were stirred in the dark. This step is not compulsory for the process but was performed to check if any ungrafted dye molecules remained.

Layer-by-layer fluorescent cores particles growth

The particles growth had to be performed quickly after seed dialysis, to prevent long term loss of fluorophores from the seeds. It was performed using the same principle as the seed synthesis (condensation of silicates by acidification), adding one equivalent of silicate and silane+dye, then the appropriate amount of Amberlite[®] to reach pH 9. The mixture was filtered and left to react during 15 minutes, then the same operation was repeated. After

- 25 adding 6 equivalents, particles had grown from 2.5 nm radius to 4.5 nm.

Continuous fluorescent cores particles growth

The previous protocol being quite time-consuming if used for particles with a greater diameter, such as 15 nm diameter particles, to the growth reaction was automated and improved by continuously adding both reagents on the seed particles solution, keeping the pH

- 30 constant around 9.00 to avoid secondary nucleation or precipitation.

The setup used is drawn on figure 3: Amberlite[®] is continuously pushed through a pierced 50 mL centrifuge tube (Corning) by a plastic spatula rotated at a fixed speed with a motor. The silicate is dispensed through a syringe and a PTFE 0.7 mm tubing pushed by a OEM syringe

pump (Harvard Apparatus, Holliston, MA). The flow is regulated automatically to keep the pH around 9.00. To do so, a pH control program was written in Labview (National Instruments, Austin TX).

5 Protocol 2: Synthesis of fluorescent SNPs in 4 colors

First step: dissolution of the fluorophore in solvent

For each fluorophore, a certain amount of powder was weighed in a 1.7mL tube (Axygen) or taken as packaged (see table below); an appropriate volume of anhydrous ethanol (Merck) was added to it, to make a 5mM stock solution. Between each use, the tubes were kept in the dark at 4°C, away from light and moisture.

	Provider	m ($\times 10^{-3}$ g)	M (g/mol)	V _{solv} (mL)
FITC	Sigma	4.5	389.4	2.32
RhBITC	Aldrich	8.7	536.1	3.24
Dylight 680 NHS	Thermo Scientific	1.0	927.0	0.214
Dylight 800 NHS	Thermo Scientific	1.0	1027	0.195

Second step: coupling with aminosilane

The ratio $n_{\text{APTES}}/n_{\text{dye}}$ used was 20/1. 2.28 μ L ($=9.7 \times 10^{-6}$ mol) of aminopropyltriethoxysilane (further referred as APTES, Aldrich) were added to 97.0 μ L of fluorophore stock solution ($=4.85 \times 10^{-7}$ mol of fluorophore) and vortexed. Then, this mixture was incubated at 50°C for 30min in the dark.

Third step: synthesize the fluorescent SNP cores

A pH meter (pH 211, Hanna Instruments) was used to monitor the pH all along the reaction steps.

In an opaque small glass vial, 1.00g of a sodium trisilicate solution (Sigma-Aldrich) was diluted to 10% w/w by adding it dropwise to 9.0mL of MilliQ water (Millipore SAS, Molsheim, France) under stirring at 600rpm: the pH reached 11.65. Then, the APTES-dye reaction mixture was added, still under stirring: the pH did not change. The final fluorophore concentration of this solution was 50 μ M.

1.25g of Amberlite IR-120 acidic exchange resin (Aldrich) was added: the pH started dropping continuously. When pH reached 9.00, the mixture was filtered on cotton pad and the filtrate was kept under stirring at 400rpm in the dark overnight.

After 16 hours of reaction, the pH had raised to ~10, so 0.07g Amberlite were added to lower the pH back to 9.00. Once this value was reached, the mixture was filtered on cotton and the filtrate kept away from light.

Fourth step: growth of the bare silica shell

To the previous fluorescent cores mixture, 720 μ L of sodium silicate were added to the reaction vial under stirring at 600rpm. pH increased to about 11.35. Then, 1.25g Amberlite IR 120 acidic exchange resin was added to lower the pH. Once it reached 9.00, the mixture was filtered on cotton and the filtrate stirred at 400 rpm during 30' in the dark. Finally, the particle diameter was measured by Dynamic Light Scattering (LB550, Horiba), giving a value of 4nm. In this synthesis, this step was only performed once, but it is possible to repeat it several times to grow a thicker shell.

Fifth step: PEG grafting

First, a solution of borate buffer pH 9.2 and ionic strength 0.01M was prepared. To do so, a 0.1M solution of HCl was prepared by diluting a 1.0M stock solution (Sigma), and a 0.01M solution of sodium tetraborate was prepared by dissolving 1.006g of anhydrous sodium tetraborate (Sigma) in 500mL milliQ water. Then, the 0.1M HCl solution was added dropwise under pH monitoring, until pH reached a value of 9.20 (about 5.5mL of HCl solution were needed). Finally, water was added to reach a total volume of 1.00L.

Then, each solution of fluorescent core-shell SNPs was diluted to 0.48%w/w of silica by mixing 2.0mL of it to 18.0mL of borate buffer pH 9.2 in a 50mL round bottom flask (Chemglass).

The appropriate quantity of PEG700 triethoxysilane (Gelest) to add was calculated, based on the mean surface of the core-shell nanoparticles, the volume of the solution and the surface coverage desired (here, we chose to have a coverage of 0.5 PEG chain/nm²). The final calculated weight of PEG700 silane to add was 0.128g. This quantity was added to the diluted core-shell nanoparticles under stirring.

Finally, the round bottom flask was connected to a Dimroth condenser (Chemglass) and the mixture was stirred at 500rpm and heated under reflux at 120°C during 2 hours in the dark. Finally, the reaction mixture was allowed to cool down to room temperature.

The global synthesis scheme according to protocol 2 is provided on figure 2.

Protocol 3: Synthesis of fluorescent silica nanoparticles with successive overcoatings

A stock solution of 10mM FITC was prepared by dissolving 15.0mg of FITC in 3.85mL anhydrous DMSO (Merck). Then, this stock solution was further diluted in anhydrous ethanol to a concentration of 5×10^{-4} M. Then, 2.36 μ L of APTES was added to 1mL of this solution, giving a $n_{\text{APTES}}/n_{\text{dye}}$ ratio of 20/1. The tube was vortexed and left to incubate in the dark at 50°C for 30min to complete the coupling reaction.

A pH meter (pH 211, Hanna Instruments) was used to monitor the pH all along the following reaction steps.

In an opaque 250mL plastic bottle, 10.0g (7.20mL) of a sodium trisilicate solution (Sigma-Aldrich) were diluted to 10% w/w by adding it dropwise to 90.0mL of MilliQ water (Millipore SAS, Molsheim, France) under stirring at 600rpm: the pH reached 11.65. Then, the APTES-dye reaction mixture was added, still under stirring: the pH did not change.

12.5g of Amberlite IR-120 acidic exchange resin (Aldrich) were added: the pH started dropping continuously. When pH reached 9.00, the mixture was filtered on cotton pad and the filtrate was kept under stirring at 400rpm in the dark overnight to enable growth of the fluorescent silica cores.

After 16 hours of reaction, the pH had raised to ~ 10 , so 0.7g Amberlite IR 120 were added to lower the pH back to 9.00. Once this value was reached, the fluorescent cores mixture was filtered on cotton and the filtrate kept away from light.

To the previous mixture, 7.20mL of sodium silicate were added to the reaction vial under stirring at 600rpm. pH increased to about 11.35. Then, 1.25g Amberlite IR 120 acidic exchange resin was added to lower the pH. Once it reached 9.00, the mixture was filtered on cotton and the filtrate stirred at 400 rpm during 30' in the dark to complete the shell growth. Finally, the particle diameter was measured by Dynamic Light Scattering (Nanosizer ZS, Malvern).

The previous shell growth/overcoating step was successively repeated again 5 times, and particle diameter was measured after each step 3 times by DLS after previously filtering samples with 0.45 μ m PVDF syringe filters (Millipore).

Protocol 4: Synthesis of silica nanoparticles with several wt% of silicate

A pH meter (pH 211, Hanna Instruments) was used to monitor the pH all along the following reaction steps. Three syntheses were performed simultaneously.

In three 250mL plastic bottles, x grams (see table below) of a sodium trisilicate solution (Sigma-Aldrich) were diluted to $x\%$ w/w (see table below) by adding them dropwise to $(100-x)$ mL of MilliQ water (Millipore SAS, Molsheim, France) under stirring at 600rpm: the pH reached 11.65.

- 5 y grams (see table below) of Amberlite IR-120 acidic exchange resin (Aldrich) were added in each bottle: the pH started dropping continuously. When pH reached 9.00, the mixture was filtered on cotton pad and the filtrate was kept under stirring at 400rpm overnight to enable growth of the silica cores.

Sample	A	B	C
x	10.0	15.0	20.0
y	11.92	17.88	23.84

10

After 16 hours of reaction, the pH had raised to ~ 10 , so 0.7g Amberlite IR 120 were added to lower the pH back to 9.00. Once this value was reached, the fluorescent cores mixture was filtered on cotton.

- 15 The particle diameter was measured by Dynamic Light Scattering (Nanosizer ZS, Malvern) on the three samples previously filtered with $0.45\mu\text{m}$ PVDF syringe filters (Millipore).

Protocol 5: Synthesis of fluorescent silica nanoparticles containing several amounts of fluorophore

- 20 Stock solutions of 10mM FITC and RhBITC were prepared by dissolving 15.0mg FITC or 12.0mg RhBITC in respectively 3.85mL and 2.23mL anhydrous DMSO. Then, these stock solutions were further diluted in anhydrous ethanol to a concentration of $5 \times 10^{-4}\text{M}$. Then, x μL of APTES (see table below) were added to y mL (see table below) of these solutions, keeping a $n_{\text{APTES}}/n_{\text{dye}}$ ratio of 20/1. The tube was vortexed and left to incubate in the dark at 50°C for 30min to complete the coupling reaction.

- 25 A pH meter (pH 211, Hanna Instruments) was used to monitor the pH all along the following reaction steps. Three syntheses were performed simultaneously.

- In eight opaque 100mL plastic bottles, 5 grams of a sodium trisilicate solution (Sigma-Aldrich) were diluted to 10% w/w (see table below) by adding them dropwise to 45 mL of MilliQ water (Millipore SAS, Molsheim, France) under stirring at 600rpm: the pH reached 11.65. Then, the APTES-dye reaction mixtures were added to each vial, still under stirring: the pH did not change.

30

11.92 grams (see table below) of Amberlite IR-120 acidic exchange resin (Aldrich) were added: the pH started dropping continuously. When pH reached 9.00, the mixture was filtered on cotton pad and the filtrate was kept under stirring at 400rpm overnight to enable growth of the silica cores.

5

Sample	A1 (FITC), A2 (RhBITC)	B1 (FITC), B2 (RhBITC)	C1 (FITC), C2 (RhBITC)	D1 (FITC), D2 (RhBITC)
x	1.05	2.11	10.55	21.10
y	0.072	0.143	0.716	1.432

After 16 hours of reaction, the pH had raised to ~10, so 0.35g Amberlite IR 120 were added to lower the pH back to 9.00. Once this value was reached, the fluorescent cores mixture was filtered on cotton.

10

Protocol 6: Silanization reaction between APTES and several infrared fluorophores

Stock solutions of 5mM Dylight 800-NHS, DY-782 NHS, DY 800-NHS, CF 770-NHS and IRDye 800 CW-NHS were prepared in anhydrous DMSO. Then, 1 μ L of APTES was added to 21.4 μ L of these solutions, keeping a $n_{\text{APTES}}/n_{\text{dye}}$ ratio of 20/1. The tubes were vortexed and left to incubate in the dark at 50°C for 30min to complete the coupling reaction.

15

Then, 7.4 μ L of each reaction mixture were added to 993 μ L of milliQ water, and the fluorescence spectra of these samples were measured on a spectrophotometer (Spectramax, Molecular Devices), using an excitation wavelength of 680nm and a cutoff of 695nm. These spectra are shown on figure 25.

20

Example 2: Steady state anisotropy calculations

The calculations of the steady state anisotropy are given in the following equation :

$$r_n = r_{\text{mono}} \frac{1 + \left(\frac{R_0}{R}\right)^6}{1 + N\left(\frac{R_0}{R}\right)^6} + r_{\text{et}} \frac{(N-1)\left(\frac{R_0}{R}\right)^6}{1 + N\left(\frac{R_0}{R}\right)^6}$$

The relation between the anisotropy r_n and the polarization P , is given by the following equation:

25

$$P = \frac{3r_n}{2 + r_n}$$

N is the number of dimers in the nanoparticles.

r_{mono} is the steady state anisotropy for one fluorophore when the energy transfer between two dyes goes down to zero.

r_{et} is the theoretical minimum value if the energy transfer is equal 100% between dyes. r_{et} is equal to 0.016.

R is the distance between donor and acceptor.

R_0 is the Förster distance at which the energy transfer efficiency is 50% and given by the equation:

$$K^2 = (\cos\theta - 3\cos^2\beta)^2$$

In our case, Q is the quantum field of the fluorophore in the nanoparticles.

n is the refractive index of the silica assumed equal to 1.475,

K^2 the orientation factor of the fluorophore. $K^2 = 2/3$ is often assumed. This value is obtained when both dyes can be considered to be isotropically oriented during the excited state lifetime.

J is the spectral overlap integral between the absorption and the emission spectra of the dyes.

The figure 4 describes the steady state versus the inter fluorophore distance R , and the number N of dimers if the Förster distance R_0 is equal to 5.5 nm.

Based on the theoretical simulation, we can approach the number of fluorescent molecules per bead of certain size, necessary to drop down the FP signal around 50mP. A good working range is shown to be 20 molecules in 15 nm diameter nanoparticles. 15nm size particles are accessible by the silicate route synthesis. And including 20 molecules in the beads, mean that we will get enough clusters (>4) for all the beads especially if we consider the distribution of molecules through the particles as following the Poisson distribution. And the mean the distance between clusters will be in average around 4.4 nm that's around $0.8 \times R_0$ (the best compromise between FRET and self quenching).

The measurements of the steady intensity I_t and of the state polarization P are made by a multispectral confocal fluorescence microscope by the following equations:

$$I_t = I_{par} + 2I_{per}$$

$$P = \frac{I_{par} - I_{per}}{I_{par} + I_{per}}$$

with I_{par} and I_{per} being the emission parallel and perpendicular respectively to the excitation polarization direction

Example 3: Calculation of the mean distance between fluorophores in nanoparticles as a function of the particles size and the number of fluorescent molecules.

The table presented in figure 5 presents the mean distance between fluorophores in nanoparticles as a function of particles size and number of dyes per particle.

- 5 The shadowed zone comprises distances lower than the Förster distance R_0 but higher than the quenching distance, that enable FRET effect to take place. This table gives the experimental conditions for this to happen: the radius of the nanoparticles has to be between 6 and 10nm, and the dye content must be between 15 and 22 molecules per particle. Based on this, it is now possible to set the amount of dye to add in the synthesis, and the growth protocol to use.

10

Example 4: Polarization / Intensity 2D encoding study.

Studying the multiplexing capability of the system demonstrated the relation between the intensity and the polarization together, are not linear with the concentration of the corresponding dye. The explanation lies in the following equations:

Mixture of free and bound dye : $(FP ; I) = f(c_f ; c_b)$

$$I = \varepsilon'_f \cdot c_f + \varepsilon'_b \cdot c_b$$

$$P = \frac{P_{\min} \cdot \varepsilon'_f \cdot c_f + P_{\max} \cdot \varepsilon'_b \cdot c_b}{\varepsilon'_f \cdot c_f + \varepsilon'_b \cdot c_b}$$

$$\varepsilon' = 2,3 \varepsilon l c \phi k P_o$$

15

I is the fluorescent intensity, P is the fluorescence polarization, c_f and c_b are the concentrations of free and bound dye, l is the optical length of the sample, ε is the extinction coefficient of the dye, ϕ is the quantum yield and k is a factor.

- 20 The plot illustrating the bi-dimensional coding obtained by mixing two kinds of material having high FP and low FP demonstrate that the error coming from solution handling (pipetting), is affecting a lot the resolution of the discrete code generated. To improve the resolution, we propose to do the synthesis of intermediate FP values nanoparticles that will be just diluted to create the different levels in intensity. Two closest FP nanoparticles can be
- 25 combined to get an intermediate FP level, with lower deviation.

Figure 6 represents the fluorescence intensity/polarization mapping by varying fluorescein in nanoparticles and free fluorescein concentrations.

Example 5: Nanoparticles characterizations**1. Size characterization of the nanoparticles**

Silica nanoparticles size distribution is measured by dynamic light scattering. Results are provided on figure 7. The nanoparticles were prepared by silicate condensation following the protocol 5. The size is independent of the dye concentration. These nanoparticles are especially small, and were never described earlier within this size range.

2. Study of the effect of the initial silicate concentration on the particles size:

Results of this study are presented on figure 8. The initial silicate concentration effect on the particles size, following the protocol 4 seems not to change drastically the final particle size, which stays between 2.5 nm and 3 nm.

3. Manual layer-by-layer growth of SNPs by successive silicate additions

Figure 9 presents the evolution of particles size measured by double exponential fit in function of the number of overcoatings (OCs).

The silica nanoparticles were grown using the layer by layer method described in the protocol 3.

The dynamic light scattering measurements affords the size characterization and demonstrate the increase of the particles diameter, from 2.5 nm to 4.5 nm as a function of the number of silicate equivalents that was added. It is a significant increase considering the repeatability of the experiments, and the continuity of the data.

We demonstrate also that we can grow the nanoparticles using the protocol 3 up to 10 nm without affecting the polydispersity. As an example, figure 10 compares the polydispersity of nanoparticles with different number of OCs and thus different diameters by dynamic light scattering. The polydispersity is not affected by the diameter increase.

Nevertheless, as the growth is not linear with the silicate weight added (the diameter is proportional to $(\text{silicate mass/density})^{(1/3)}$), more than 60 additions are necessary to reach 20 nm particles size by using this protocol. An automated system would be useful for such sizes.

4. Quantum yield and FP properties (2.5 nm diameter particles)

Nanoparticles were synthesised according to protocol 5.

The absorbance and fluorescence spectra were measured on a UV-vis spectrophotometer (Spectramax E5, Molecular Devices), as well as the FP value at maximum emission

wavelength. The brightness ratios and FP values of each sample are plotted on fig 11 and fig 12.

In this preliminary study we demonstrate also for these tiny nanoparticles that we can observe an enhancement of the fluorescence brightness (increase of the quantum yield efficiency compare to the free dye) at least 2 times the free dye, as long as we do not reach the quenching condition (molecules too close from each other).

We demonstrate also an increase of the fluorescence polarization properties, and these properties can be tuned by playing on the number of fluorescent molecule per particles.

5. Emission spectra of different silica nanoparticles prepared using the protocol 2.

Figure 13 presents the emission spectra of different nanoparticles prepared using protocol 2. These spectra were measured with a Jobin-Yvon Fluoro;max fluorimeter. A wide range of fluorescent nanoparticles can be prepared using the protocol 2, covering the visible and near infrared spectrum. In the emission spectra on figure 13, the FWHM (full-width half-maximum) of the emission signal stays narrow in near IR.

6. Nanoparticles resistance to photobleaching.

Figure 26 presents the photobleaching of dyes prepared using protocol 5, compared to the photobleaching of the equivalent amount of free dye in water.

This experiment was performed by putting 10uL of sample between glass slide and coverslip, and illuminating them continuously on a Nikon Eclipse Ti microscope with a Nikon Intensilight C-HGFI 100W lamp. The emitted light was filtered by a bandpass filter, and pictures were recorded at constant time intervals with Nikon NIS elements software.

The pictures were then analyzed with ImageJ software, and for each, the mean intensity was calculated and plotted as a function of time.

This figure shows that the photobleaching is greatly reduced in the case of rhodamine B ITC, and reduced slightly in the case of FITC. This difference is maybe due to the lower purity of the FITC compared to rhodamine B ITC: there is still free FITC, that photobleached faster.

Example 6: Enhancement of fluorescence of dyes embedded in nanoparticles of the invention

Figures 14 and 15 present the absorption and fluorescence spectra of rhodamine B ITC as a free dye and embedded in a silica nanoparticle according to the invention. The nanoparticles are synthesised according to protocol 2. Encapsulation in silica nanoparticle enhances

Rhodamine B ITC brightness with a ratio of 5.1. The brightness ratio is defined according to

$$\text{the following formula: } \text{brightness} = \frac{\frac{I_{\text{fluo}}^{\text{max}}(\text{SNP})}{I_{\text{fluo}}^{\text{max}}(\text{freedye})}}{\frac{I_{\text{abs}}^{\text{max}}(\text{SNP})}{I_{\text{abs}}^{\text{max}}(\text{freedye})}}.$$

These spectra compare the absorption and emission properties of the dye in its free form, and after encapsulation in the silica nanoparticles. Both spectra are made with an equivalent fluorophore concentration of 50µM.

To compare optical performances of the dye in both states, a “brightness ratio” was defined as above. It means to measure how much more fluorescent encapsulated dyes are, compared to dyes in free form, for an equivalent absorbance maximum.

Figures 16 and 17 present the absorption and fluorescence spectra of Dylight® 680 NHS as a free dye and embedded in a silica nanoparticle according to the invention. The nanoparticles are synthesised according to protocol 2. Encapsulation in silica nanoparticle enhances Dylight® 680 NHS brightness with a ratio of 1.66.

The lower enhancement compared to the case with rhodamine B may be due to the different nature of the reactive moiety (NHS versus ITC), or to the higher molecular weight of the fluorophore (around 1000g/mol, compared to 536g/mol in the case of Rhodamine B ITC).

Example 7: Creation of a 5x5 optical barcode library in droplets

Among the SNPs synthesized in example 1, protocol 2, the FITC and Dylight 680-coated ones were used to create a 5x5 fluorescent barcode library.

First, each of these 2 color SNPs were diluted in series A to E with borate buffer pH 9.2, by mixing both according to the following table:

Sample name	A	B	C	D	E
Dilution factor	1	2	4	8	16
SNP solution volume (uL)	128	64	32	16	8
Borate buffer volume (uL)	0	64	96	112	120

FITC solutions were called A1 to E1, Dylight 680 solutions were called A2 to E2

Then, in a 384 well plate, solutions were mixed according to the following table (20uL of each):

Well	1		2		3		4		5	
A	A1	A2	B1	A2	C1	A2	D1	A2	E1	A2
B	A1	B2	B1	B2	C1	B2	D1	B2	E1	B2
C	A1	C2	B1	C2	C1	C2	D1	C2	E1	C2
D	A1	D2	B1	D2	C1	D2	D1	D2	E1	D2
E	A1	E2	B1	E2	C1	E2	D1	E2	E1	E2

The plate was sealed with aluminium foil and centrifuged at 1200rpm during 10s.

- 5 Then, a droplet library of those barcodes was generated by automatically pipetting some content of each well, generating droplets on a microfluidic chip and collecting the total content in a vial. The vial was shaken to homogenize the droplet content, then the droplets were reinjected on- chip.

The droplets were illuminated by 2 lasers, one at 488nm (Coherent Sapphire 488-20), one at
10 680nm (Newport LQC690-30) and their fluorescence signal was simultaneously detected by 2 PMTs, in front of which bandpass filters (Semrock) were installed to get respective detection windows of $531\pm 20\text{nm}$ (green) and $720\pm 7\text{nm}$.

The signal was processed using a FPGA card and Labview (National Instruments) in-house detection software. This detection was performed 3h and 18h after the droplet library
15 generation. Figures 19 to 24 were plotted using an in-house Labview software.

Figure 19 and 20 show 5 distinct peaks for each color, showing that the pipetting was made accurately according to the previous table. The peaks are not split in subpeaks, indicating that all droplets containing the same amount of one dye have the same intensity of that color, independently of the amount of the other dye.

- 20 Figure 21 and 22 show that the 5 peaks in each color remain very distinct even after 18h.

Figure 23 shows the 25 labels that are distinct and nearly form a square grid, as expected. The slight deviation of the lower line is due to the optical crossover between the dyes, but can be easily circumvented by adjusting concentrations of both colors.

- 25 Finally, figure 24 shows that the code remains nearly completely unchanged, which shows that the size and content of the droplets did not vary overnight.

CLAIMS

1. A method for making a nanoparticle comprising a core and a first label, comprising the steps of:
 - 5 (a) providing a first label bound to a first molecule comprising silane thereby forming a silane functionalized label;
 - (b) providing a first solution comprising free silicon-containing molecules;
 - (c) mixing the silane functionalized label and the first solution to form a first mixed solution;
 - 10 (d) reducing the pH of the first mixed solution thereby allowing conditions for the formation of covalent bonds among the silicon-containing molecules to form silica within which the first label is covalently bound, thereby nucleating the core;
 - (e) allowing sufficient time for the core to grow until stopped.
- 15 2. The method of claim 1, wherein step (d) includes adding an ion-exchange resin to the first mixed solution, thereby to reduce the pH.
3. The method of any one of claims 1-2, wherein the silane comprises APTES, APTMS, MPTMS, and/or MPTES.
- 20 4. The method of any one of claims 1-3, wherein the method further comprises
 - (f) mixing the grown core with a second solution comprising free silicon-containing molecules to form a second mixed solution;
 - (g) reducing the pH of the second mixed solution thereby growing a shell;
 - 25 (h) allowing sufficient time for the shell to grow until stopped.
5. The method of claim 4, wherein the second solution further comprises a second label.
6. The method of claim 4, wherein steps (f) through (h) are repeated one or more times,
30 and wherein the second solution comprises a label different than the first or preceding labels during each repeat.
7. The method of any one of claims 4-6, further comprising the steps of (i) optionally adding a functionalizing agent surrounding the shell to form a functionalized shell, and (j)

tuning one or more properties of the nanoparticle by grafting one or more surface molecules to the shell or, if present to the functionalized shell.

8. The method of claim 7, wherein the functionalizing agent comprises silanes,
5 maleimides, thiols, and/or amines.
9. The method of claim 7, wherein the surface molecules comprise a polymer, protein, antibody, antigen, sugar, PEG, organic molecule, and/or enzyme.
- 10 10. The method of any one of claims 1-9, wherein the free silicon-containing molecules comprise sodium trisilicate, sodium orthosilicate, sodium pyrosilicate, or hydrates thereof.
11. The method of any one of claims 1-10, wherein growth of the core and/or the shell is stopped by depletion of a constituent, addition of a quenching reagent, changing temperature,
15 and/or changing pH.
12. A nanoparticle obtainable by the method of any one of claims 1-11.
13. The nanoparticle of claim 12 having a diameter of 500 nanometers or less, 400
20 nanometers or less, 300 nanometers or less, 200 nanometers or less, 100 nanometers or less, 50 nanometers or less, 25 nanometers or less, 15 nanometers or less, 10 nanometers or less, 5 nanometers or less, 2.5 nanometers or less, or less than 1 nanometer.
14. The nanoparticle of claim 13, wherein the first label comprises an organic molecule,
25 an organic dye, an inorganic dye, an inorganic molecule, a magnetic particle and/or a radioactive compound.
15. The nanoparticle of claim 14, wherein the first label is a fluorescent dye, or phosphorescent dye.
30
16. The nanoparticle of claim 15, wherein the first label is a fluorescent dye.

17. The nanoparticle of any of claims 12-16, wherein (a) the core further comprises a second label bound to silica and/or (b) the shell, if present, further comprises a third label and/or, optionally, a fourth label or no label.

5 18. The nanoparticle of claim 17, wherein the first label, second label, third label, and fourth label each differ from one or more of the other.

19. The nanoparticle of claim 17, wherein any two or more of the first label, second label, third label, and fourth label are the same.

10

20. The nanoparticle of any of claims 12-19, wherein the first label is a fluorescent label having a tunable fluorescence polarization, in particular (a) by controlling a density of, or distances between, the fluorophores; (b) by adding a metal to the core and/or the shell; (c) based on the diameter; and/or (d) by controlling a ratio of the first label and a second label
15 that is bound to the silica in the core, in the shell, and/or on a surface of the shell.

21. The nanoparticle of any one of claims 12-20, wherein the first label is covalently bound to the silica.

20 22. The nanoparticle of claim 21, wherein the first label is covalently bound to the silica via a precursor made by the reaction between an aminopropylsilane and dye-NHS or dye-ITC or a precursor made by the reaction between a mercaptopropylsilane and dye-maleimide.

23. The nanoparticle of any one of claims 12-20, wherein the first label is non-covalently
25 bound to the silica.

24. The nanoparticle of claim 23, wherein the first label is non-covalently bound to the silica via an electrostatic interaction.

30 25. The nanoparticle of any one of claims 12-24, wherein the core, the shell, or both are non-porous.

26. The nanoparticle of any one of claims 12-25, wherein the nanoparticle has a diameter of between 2 nanometers and 15 nanometers.

27. The nanoparticle of any one of claims 12-26, wherein the nanoparticle comprises an additional layer of functionalizing molecules or is grafted with functionalizing molecules.

5 28. The nanoparticle of claim 27, wherein the functionalizing molecules are selected from the group consisting of silanes, maleimides, thiols, amines functions, polymers, proteins, antibodies, antigens, sugars, PEGs, organic molecules, and enzymes.

29. A method, comprising the steps of:

- 10 (a) attaching the nanoparticle of claim 27 or 28 to a biological entity via the surface functionalizing molecules; and
(b) detecting the label.

30. The method of claim 29, wherein the biological entity comprises tissue, tumor,
15 eukaryotic, archaea or prokaryotic cell - such as bacterium - protein, antigen, DNA, RNA, or virus.

31. A method, comprising the steps of:

- (a) providing a microfluidic device;
20 (b) providing at least a first nanoparticle of any one of claims 12-28 having the first label within a first droplet within the device and a second nanoparticle of any one of claims 12-28 having a second label within a second droplet within the device;
(c) detecting the first label and second label within the device.

25 32. The method of claim 31, wherein step (c) is done within the device.

33. The method of claim 31 or 32, wherein the first label and the second label have a different property.

30 34. The method of claim of claim 33, wherein the property comprises intensity; excitation and/or emission wavelength; and/or fluorescence polarization, absorption, and/or fluorescence lifetime.

35. A microfluidic droplet comprising nanoparticles of any one of claims 12-28.

36. The microfluidic droplet of claim 35, wherein the droplet is disposed within a microfluidic device.

5 37. A composition comprising nanoparticles of any one of claims 12-28.

38. Use of a nanoparticle of any one of claims 12-28 or a composition of claim 37 for sample coding (in particular microfluidic droplets coding), for material doping, for labelling a molecular probe or tagging a molecule or compound of interest.

10

39. A nanoparticle, wherein the nanoparticle comprises a core comprising silica and a first label bound to silica and has a diameter of 15 nanometers or less.

15

40. The nanoparticle of claim 39, wherein the nanoparticle further comprises a shell surrounding the core.

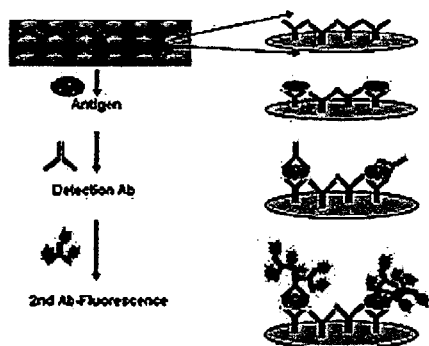
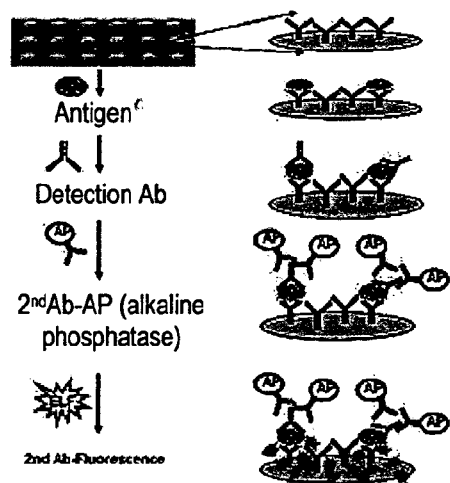
41. The nanoparticle of claim 40, wherein the shell comprises, preferably consists of, silica.

20 42. The nanoparticle of any of claims 39-41, wherein (a) the core further comprises a second label bound to silica and/or (b) the shell, if present, further comprises a third label and/or, optionally, a fourth label or no label.

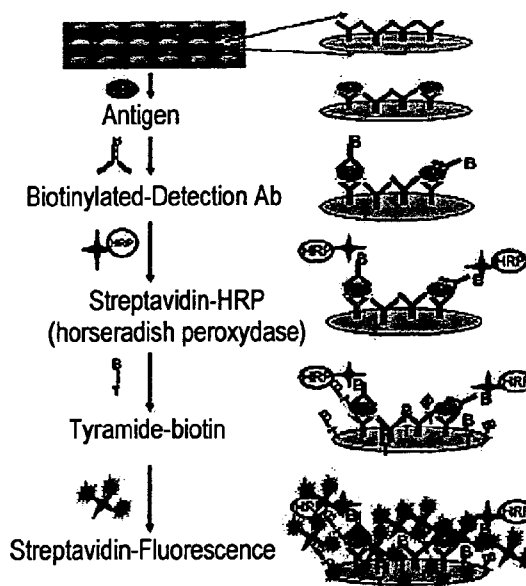
25 43. The nanoparticle of any of claims 39-42, wherein the first label is a fluorescent label having a tunable fluorescence polarization, in particular (a) by controlling a density of, or distances between, the fluorophores; (b) by adding a metal to the core and/or the shell; (c) based on the diameter; and/or (d) by controlling a ratio of the first label and a second label that is bound to the silica in the core, in the shell, and/or on a surface of the shell.

30 44. The nanoparticle of any one of claims 39-43, wherein the core, the shell if present, or both are non-porous.

Fluorescence-linked immunosorbent Assay

Enzyme-linked immunosorbent Assay (ELISA)
Using insoluble fluorescence dye

A few ways to create and detect antibody microarrays

Enzyme-linked immunosorbent assay (ELISA)
Using Tyraside Signal Amplification (TSA) technology

Result



The end result: the fluorescent spot indicates that the antigen has bound to the antibodies at that spot; the identity of the antigen can be inferred from knowledge of the specificity of the antibodies at that spot.

FIGURE 1

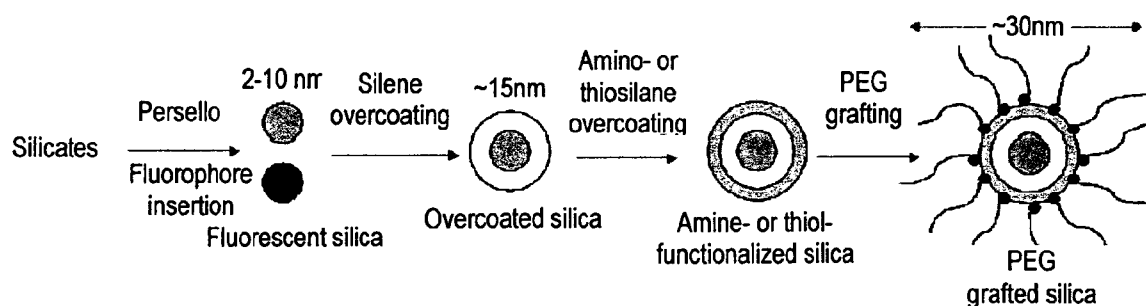


FIGURE 2

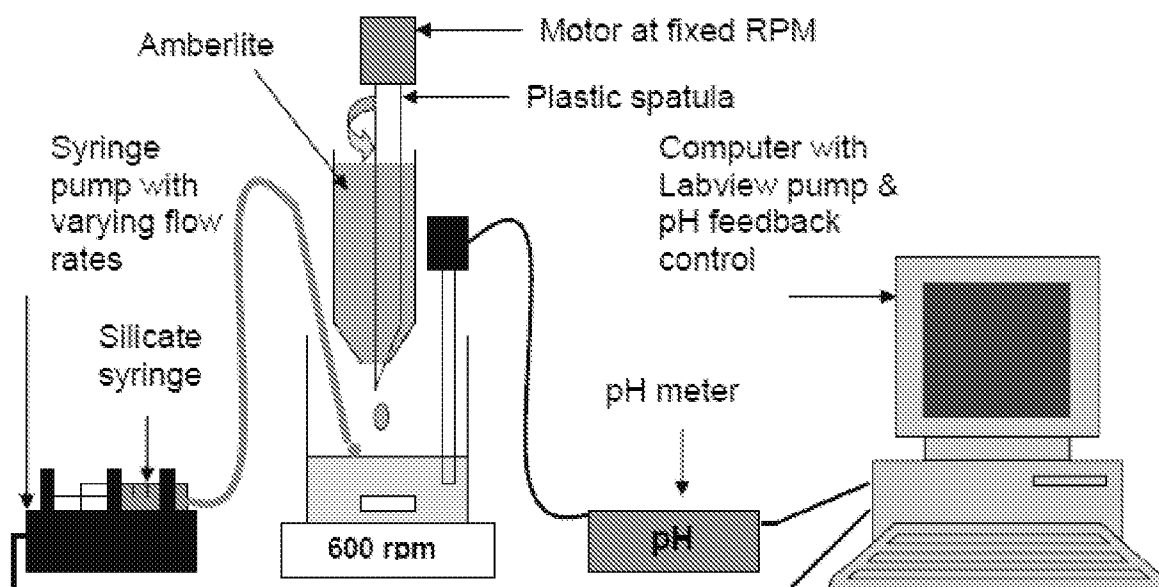


FIGURE 3

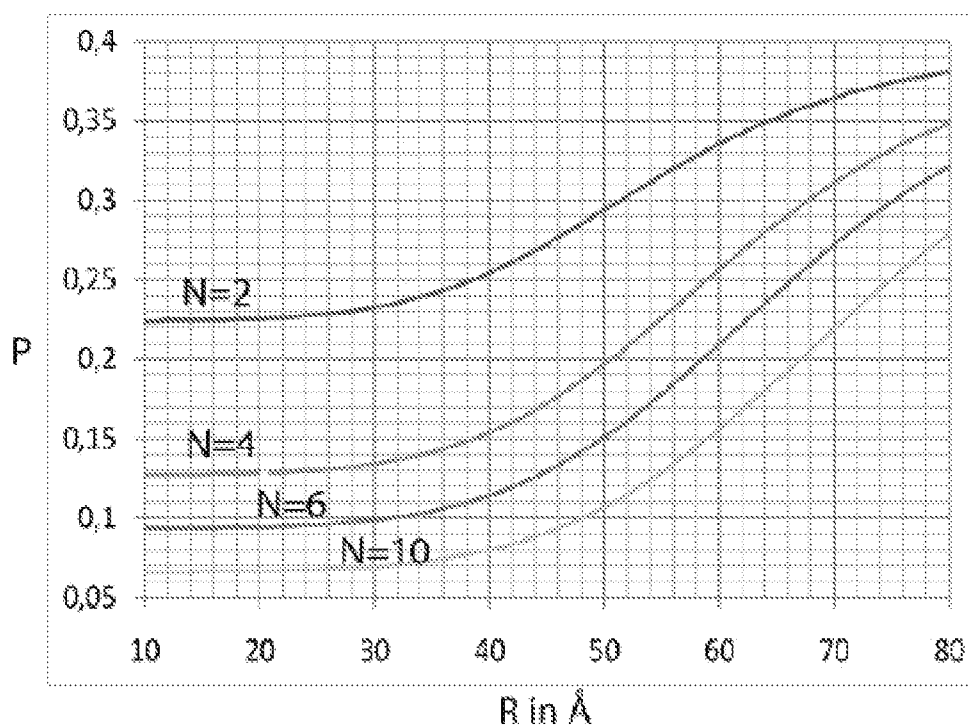


FIGURE 4

particules radius (nm)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
particules volume (nm ³)	4	34	113	268	524	905	1437	2145	3054	4189	5575	7238	9203	11494	14137
Nb of dyes per particule															
estimated mean distance between dyes in nm															
2	1.28	2.56	3.84	5.12	6.40	7.68	8.96	10.24	11.51	12.79	14.07	15.35	16.63	17.91	19.19
3	1.12	2.24	3.36	4.47	5.59	6.71	7.82	8.94	10.06	11.18	12.29	13.41	14.53	15.65	16.77
4	1.02	2.03	3.05	4.06	5.08	6.09	7.11	8.12	9.14	10.15	11.17	12.19	13.20	14.22	15.23
5	0.94	1.89	2.83	3.77	4.71	5.66	6.60	7.54	8.48	9.43	10.37	11.31	12.26	13.20	14.14
6	0.89	1.77	2.66	3.55	4.44	5.32	6.21	7.10	7.98	8.87	9.76	10.65	11.53	12.42	13.31
7	0.84	1.69	2.53	3.37	4.21	5.06	5.90	6.74	7.58	8.43	9.27	10.11	10.95	11.80	12.64
8	0.81	1.61	2.42	3.22	4.03	4.84	5.64	6.45	7.25	8.06	8.87	9.67	10.49	11.29	12.09
9	0.77	1.55	2.32	3.10	3.87	4.65	5.42	6.20	6.97	7.75	8.52	9.30	10.07	10.85	11.62
10	0.75	1.50	2.24	2.99	3.74	4.49	5.24	5.99	6.73	7.48	8.23	8.98	9.73	10.48	11.22
11	0.72	1.45	2.17	2.90	3.62	4.36	5.07	5.80	6.52	7.25	7.97	8.70	9.42	10.15	10.87
12	0.70	1.41	2.11	2.82	3.52	4.22	4.93	5.63	6.34	7.04	7.75	8.45	9.15	9.86	10.56
13	0.69	1.37	2.06	2.74	3.43	4.11	4.80	5.48	6.17	6.86	7.54	8.23	8.91	9.60	10.28
14	0.67	1.34	2.01	2.68	3.34	4.01	4.68	5.35	6.02	6.69	7.36	8.03	8.69	9.36	10.03
15	0.65	1.31	1.96	2.61	3.27	3.92	4.58	5.23	5.88	6.54	7.19	7.84	8.50	9.15	9.80
16	0.64	1.28	1.92	2.56	3.20	3.84	4.48	5.12	5.76	6.40	7.04	7.68	8.32	8.96	9.60
17	0.63	1.26	1.88	2.51	3.13	3.76	4.39	5.02	5.64	6.27	6.90	7.52	8.15	8.78	9.40
18	0.62	1.23	1.85	2.46	3.08	3.69	4.31	4.92	5.54	6.15	6.77	7.38	8.00	8.61	9.23
19	0.60	1.21	1.81	2.42	3.02	3.62	4.23	4.83	5.44	6.04	6.65	7.25	7.85	8.46	9.06
20	0.59	1.19	1.78	2.38	2.97	3.56	4.16	4.75	5.34	5.94	6.53	7.13	7.72	8.31	8.91
21	0.58	1.17	1.75	2.34	2.92	3.51	4.09	4.67	5.26	5.84	6.43	7.01	7.60	8.18	8.76
22	0.58	1.15	1.73	2.30	2.88	3.46	4.03	4.60	5.18	5.75	6.33	6.90	7.48	8.05	8.63
23	0.57	1.13	1.70	2.27	2.83	3.40	3.97	4.53	5.10	5.67	6.24	6.80	7.37	7.94	8.50
24	0.56	1.12	1.68	2.24	2.79	3.36	3.91	4.47	5.03	5.59	6.15	6.71	7.27	7.82	8.38
25	0.55	1.10	1.65	2.21	2.76	3.31	3.86	4.41	4.96	5.51	6.06	6.62	7.17	7.72	8.27
26	0.54	1.09	1.63	2.18	2.72	3.26	3.81	4.35	4.90	5.44	5.99	6.53	7.07	7.62	8.16
27	0.54	1.07	1.61	2.15	2.69	3.22	3.76	4.30	4.84	5.37	5.91	6.45	6.99	7.52	8.06
28	0.53	1.06	1.59	2.12	2.65	3.19	3.72	4.25	4.78	5.31	5.84	6.37	6.90	7.43	7.96
29	0.52	1.05	1.57	2.10	2.62	3.15	3.67	4.20	4.72	5.25	5.77	6.30	6.82	7.35	7.87
30	0.52	1.04	1.56	2.08	2.59	3.11	3.63	4.15	4.67	5.19	5.71	6.23	6.74	7.26	7.78

FIGURE 5

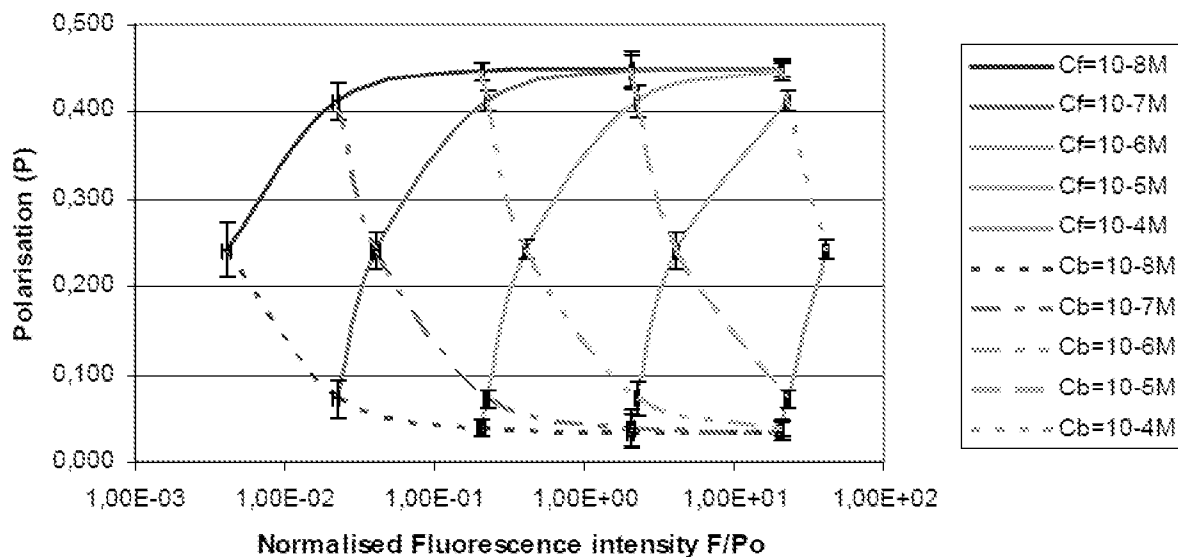


FIGURE 6

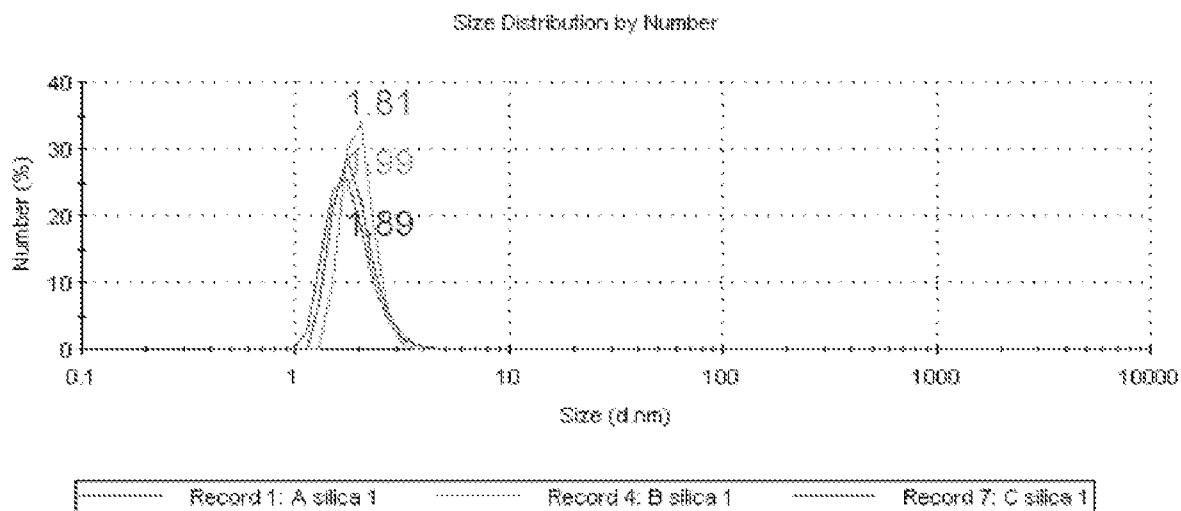


FIGURE 7

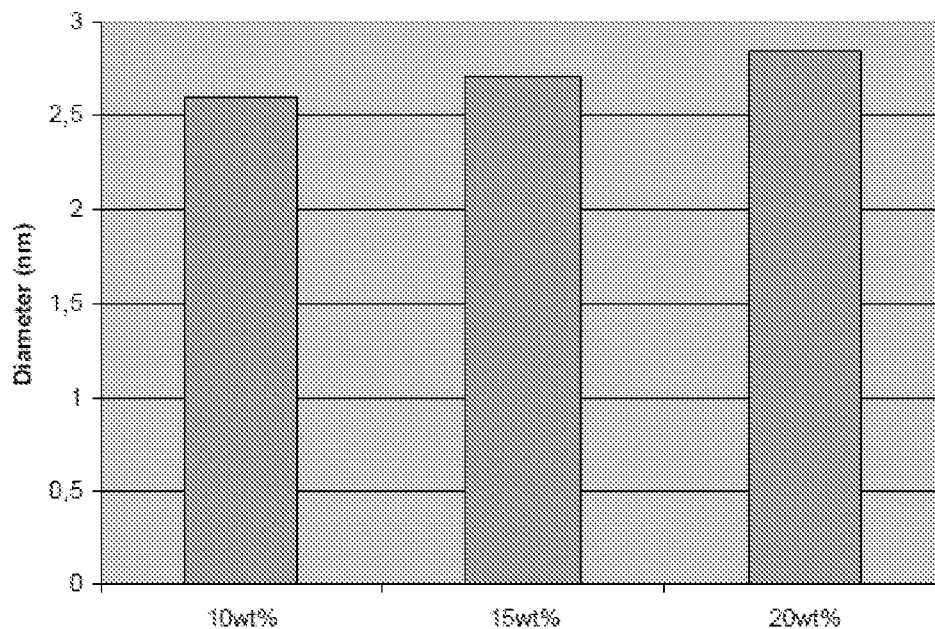


FIGURE 8

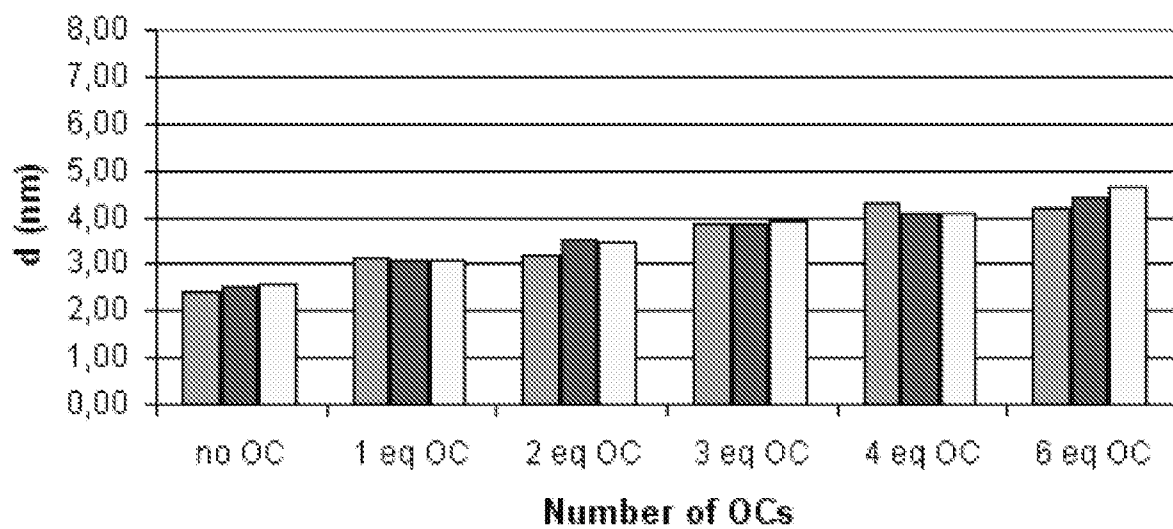


FIGURE 9

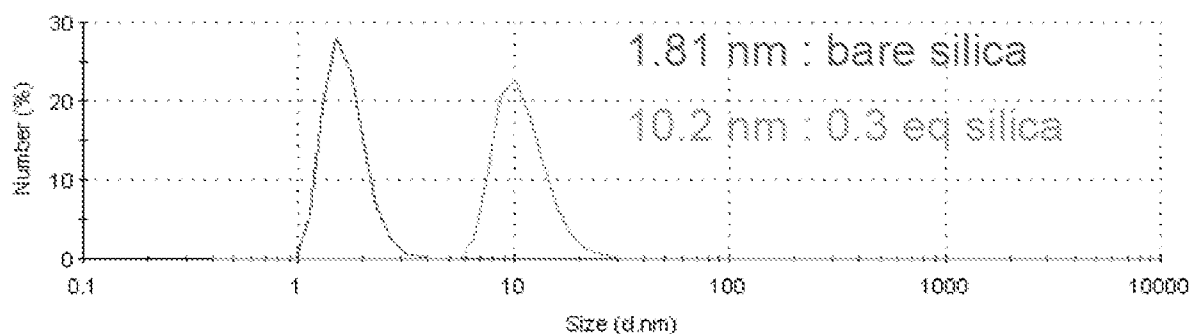


FIGURE 10

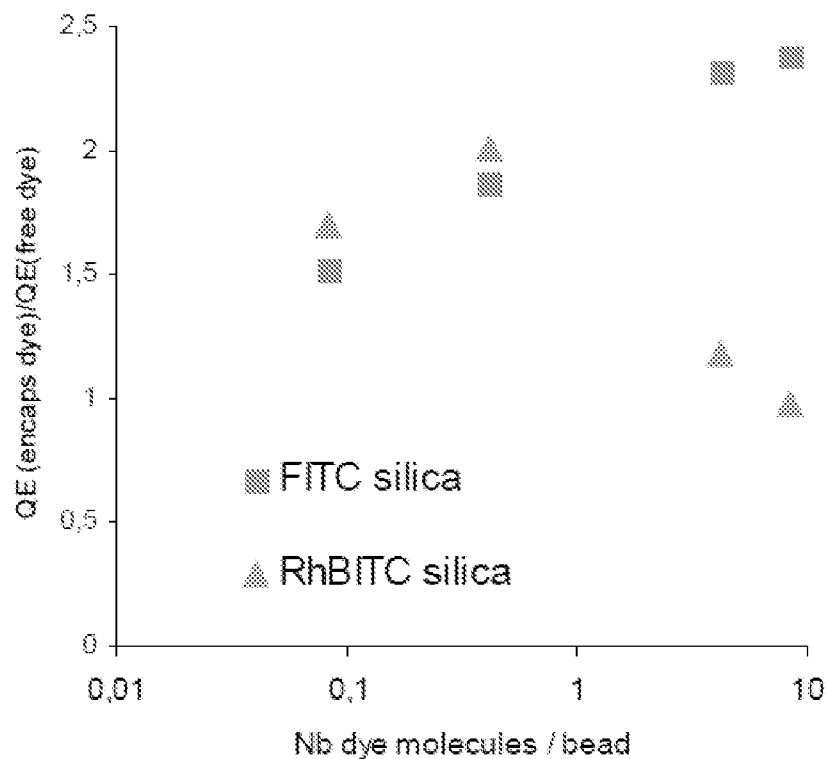


FIGURE 11

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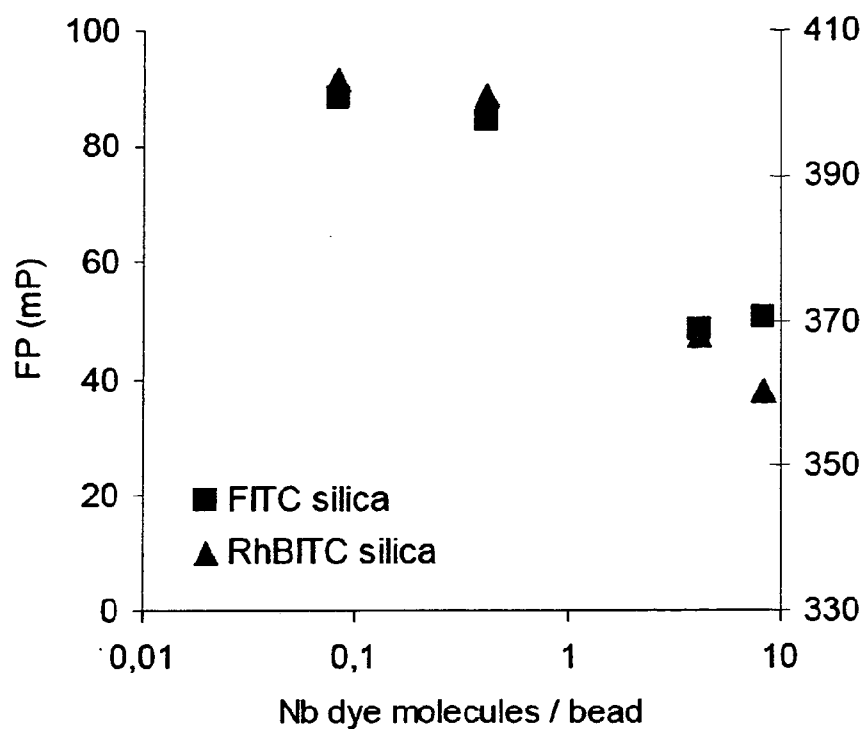


FIGURE 12

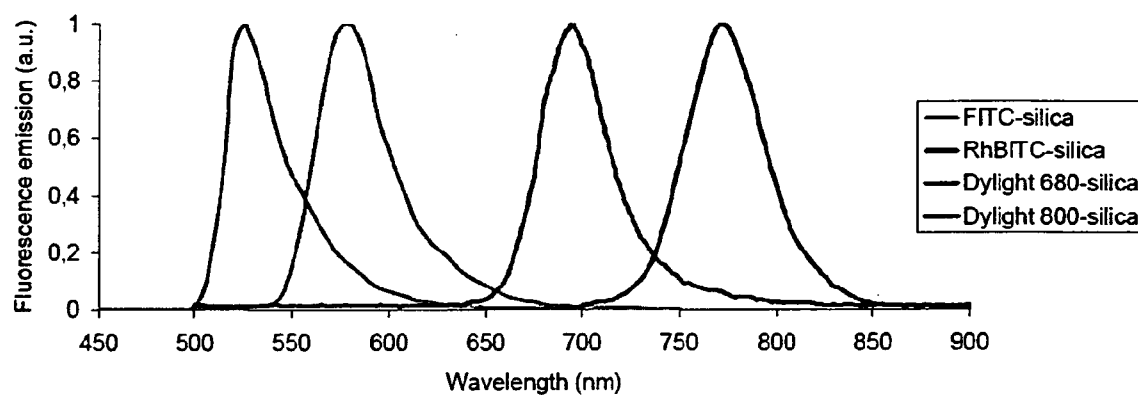


FIGURE 13

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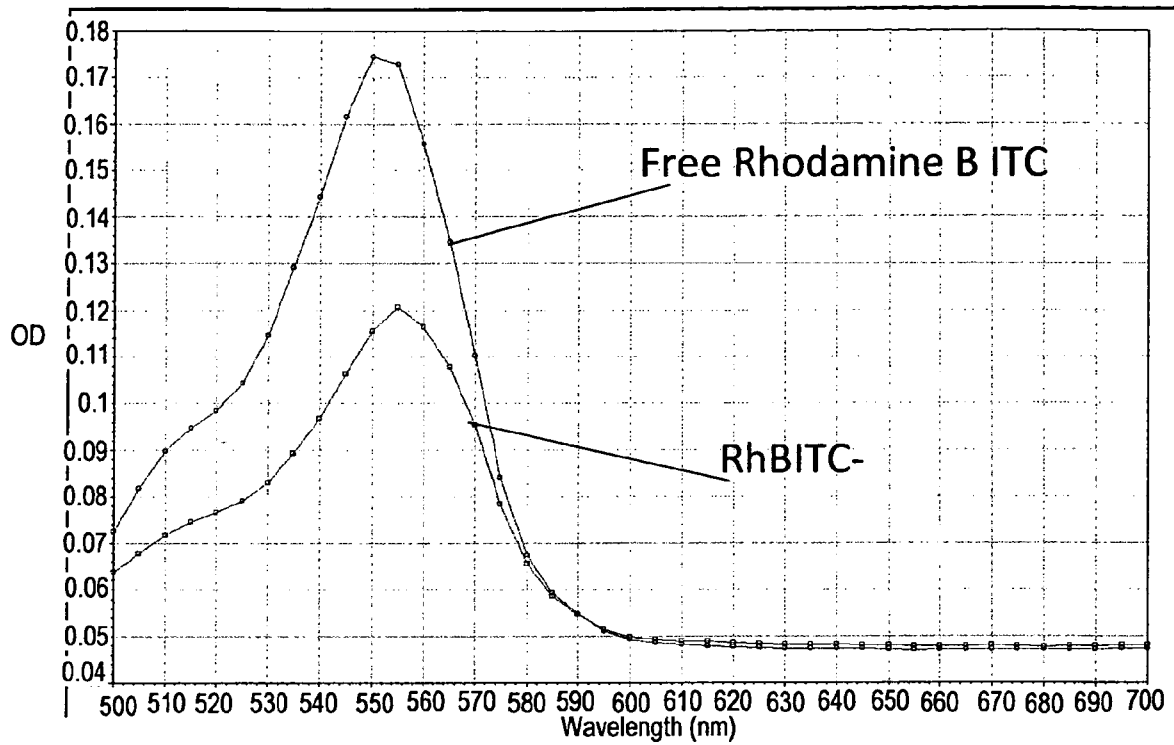


FIGURE 14

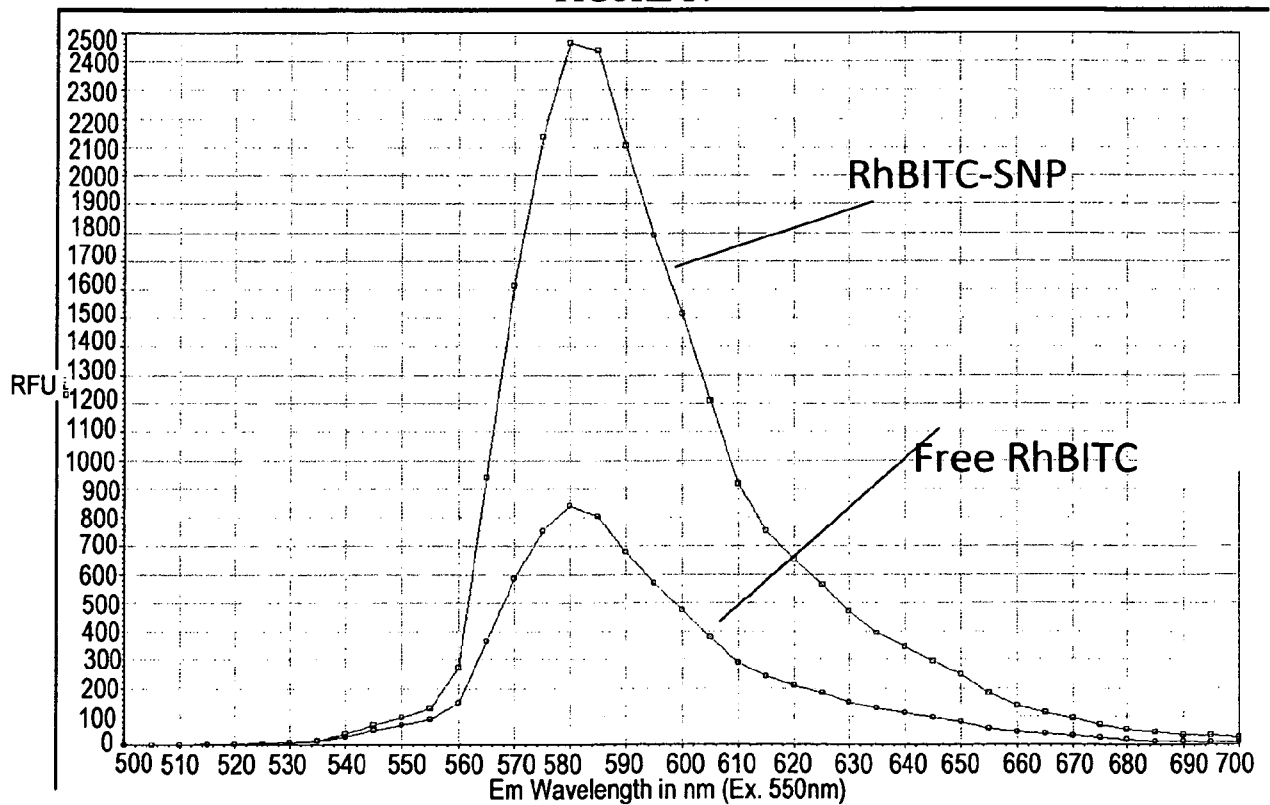


FIGURE 15

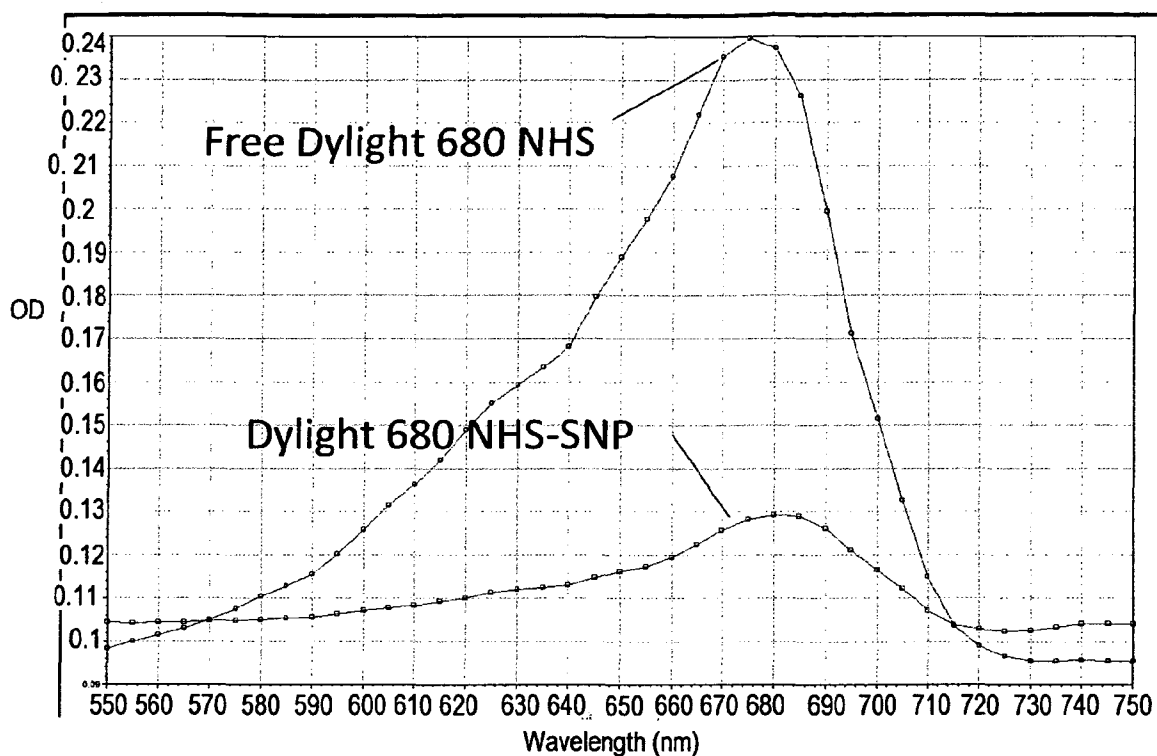


FIGURE 16

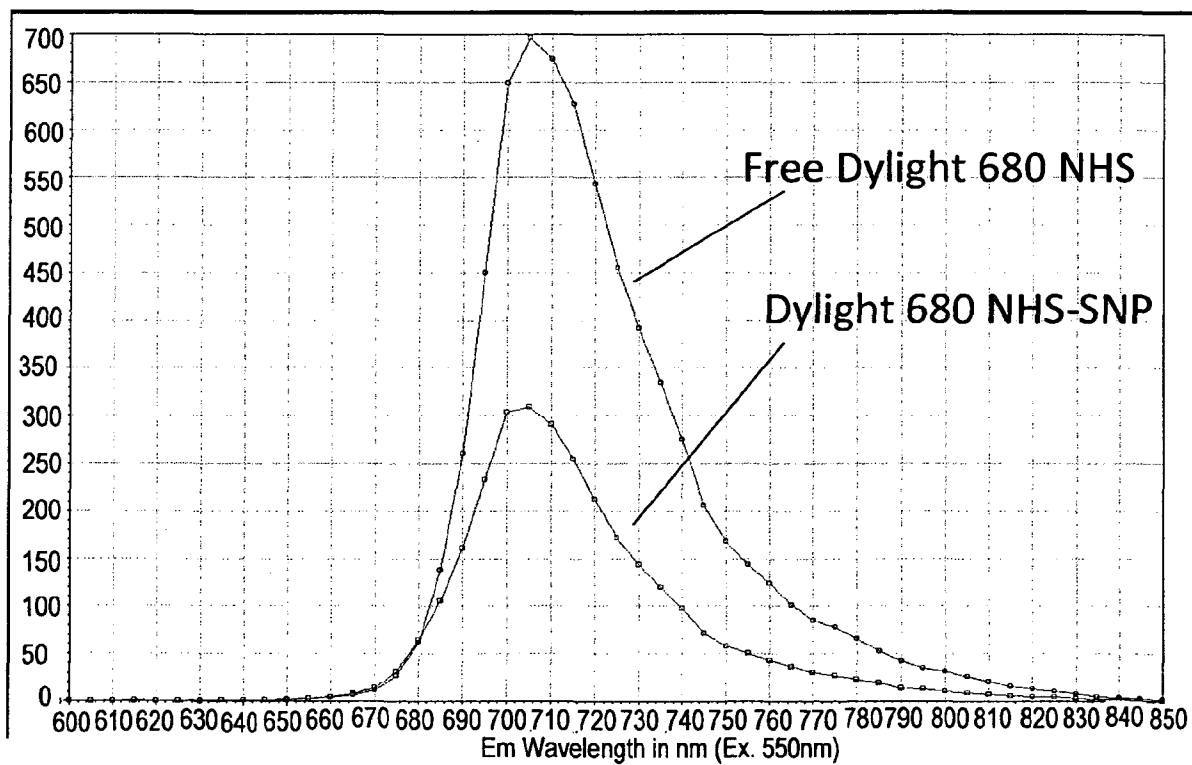
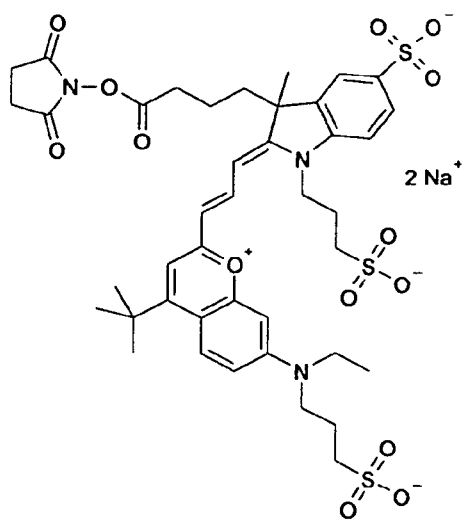
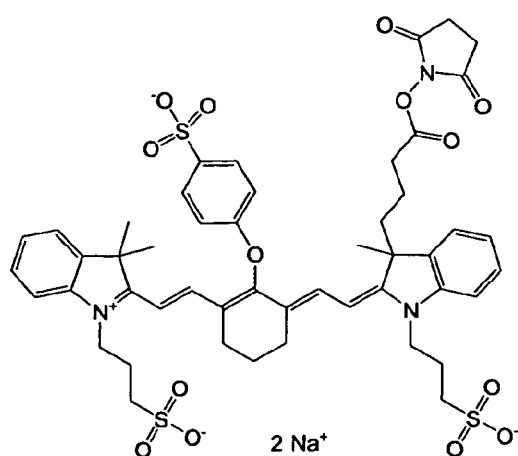


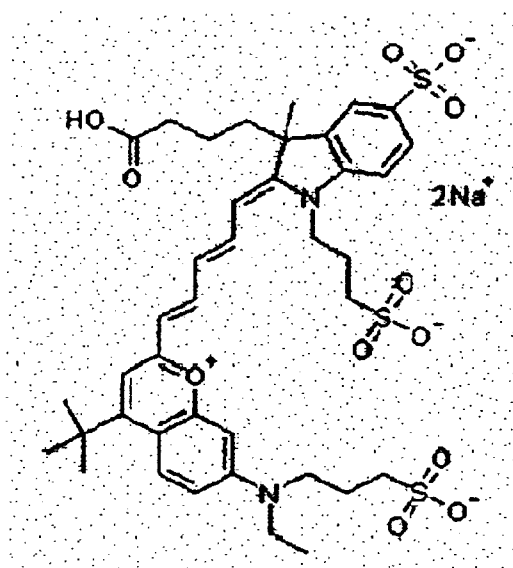
FIGURE 17



Dylight® 680-NHS



Dylight® 800-NHS



DY-782

FIGURE 18

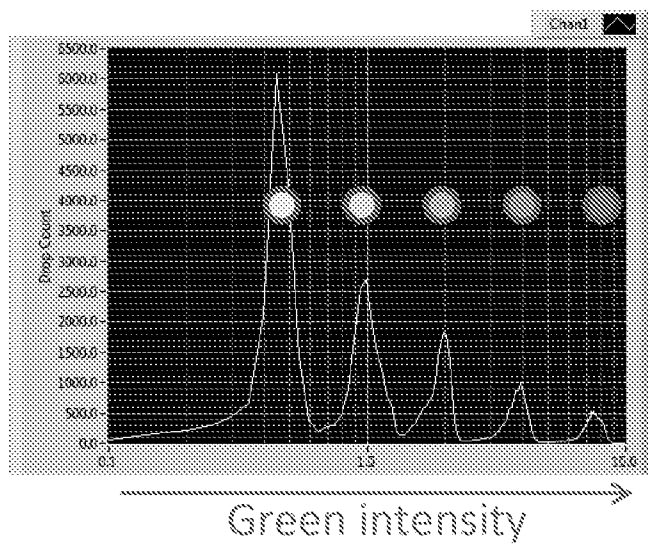


FIGURE 19

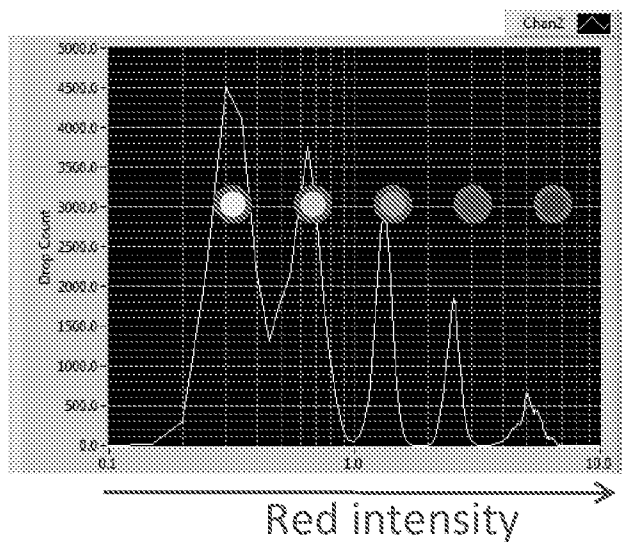


FIGURE 20

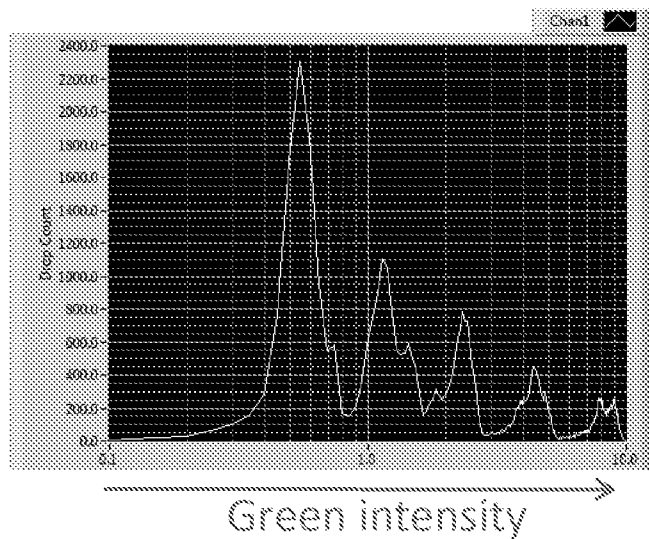


FIGURE 21

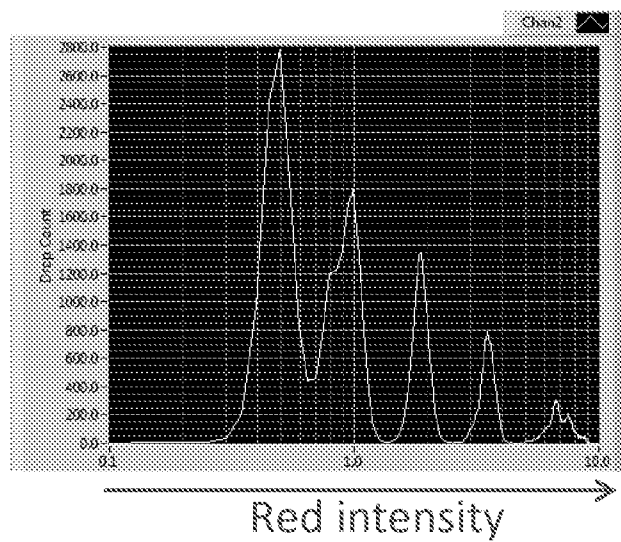


FIGURE 22

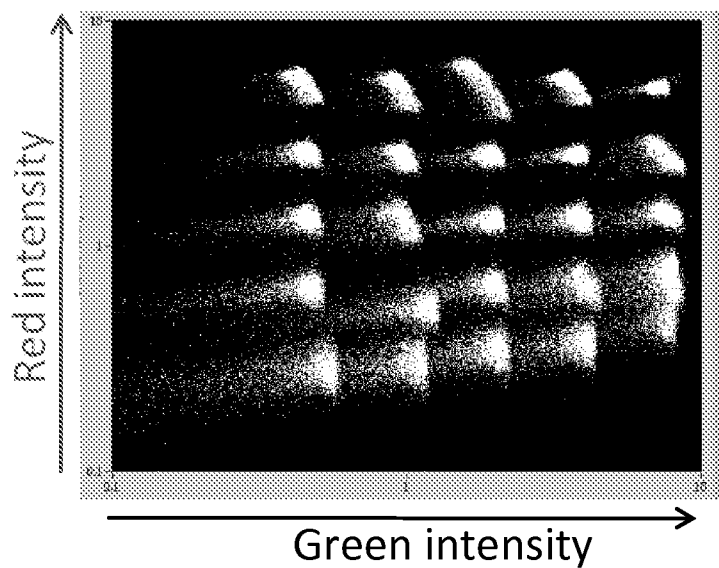


FIGURE 23

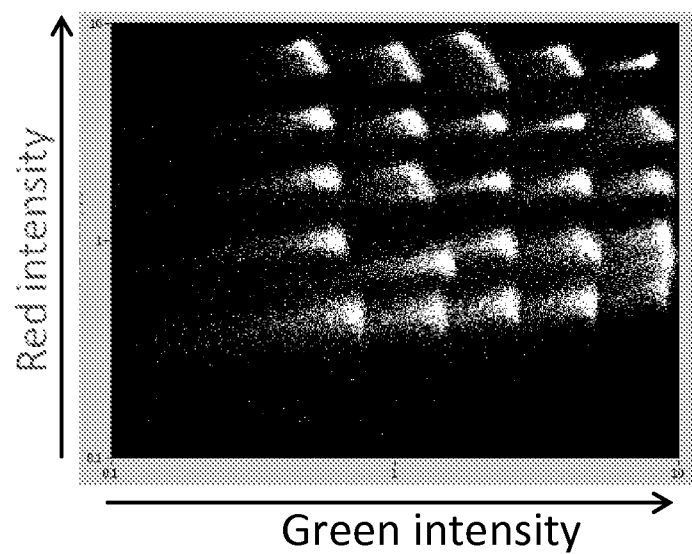


FIGURE 24

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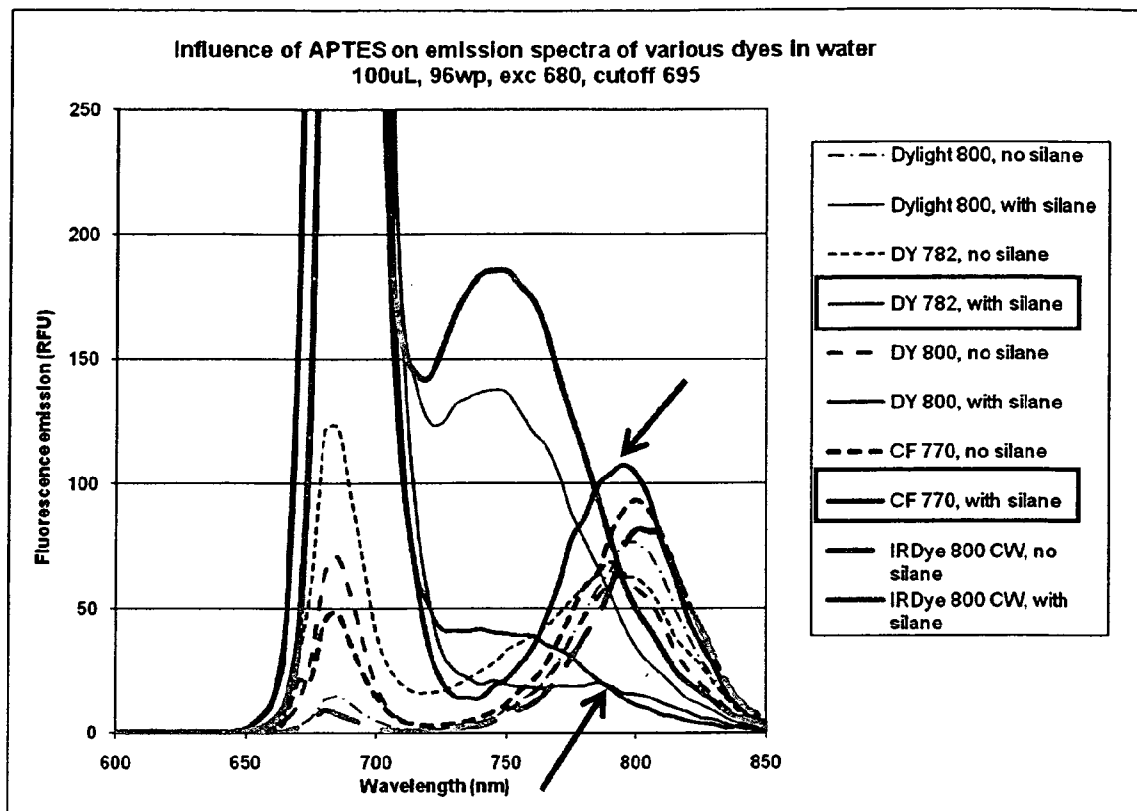


FIGURE 25

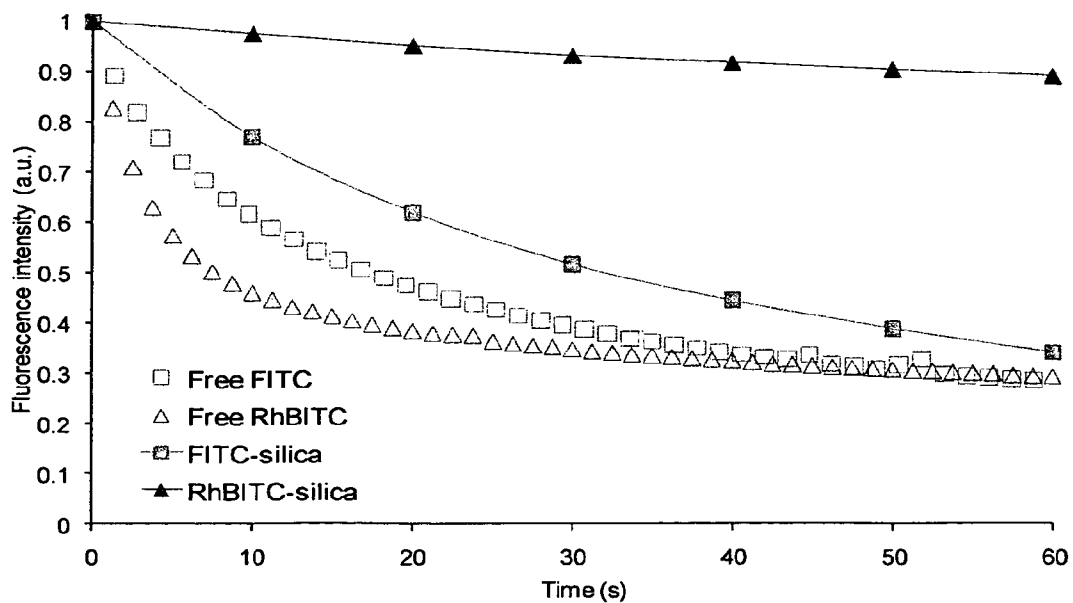


FIGURE 26

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/065188

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/543 G01N33/58 G01N33/552
ADD.

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)

G01N

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03/099843 A2 (DOW CORNING; GENENCOR INT) 4 December 2003 (2003-12-04) page 11 - page 12; examples 17-26 -----	1-44
A	TLEUGABULOVA D ET AL: "Evaluating formation and growth mechanisms of silica particles using fluorescence anisotropy decay analysis", LANGMUIR, vol. 20, no. 14, 2004, pages 5924-5932, XP002615333, page 5925, right-hand column -----	1-44
A	WO 2004/074504 A2 (CORNELL RES FOUNDATION INC) 2 September 2004 (2004-09-02) claims 1-13 ----- -/--	1-44

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

28 December 2010

Date of mailing of the international search report

12/01/2011

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Authorized officer

Gunster, Marco

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/065188

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LU ET AL: "Robust fluorescein-doped silica nanoparticles via dense-liquid treatment", COLLOIDS AND SURFACES. A, PHYSICACHEMICAL AND ENGINEERING ASPECTS, vol. 303, no. 3, 2007, pages 207-210, XP022097835, paragraph 2.2</p> <p style="text-align: center;">-----</p>	1-44
A	<p>SANTRA S ET AL: "Fluorescence lifetime measurements to determine the core-shell nanostructure of FITC-doped silica nanoparticles: An optical approach to evaluate nanoparticle photostability", JOURNAL OF LUMINESCENCE, vol. 117, no. 1, 2006, pages 75-82, XP025185759, [retrieved on 2006-03-01] paragraph 2.3</p> <p style="text-align: center;">-----</p>	1-44

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/065188

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			US 2010035365 A1	11-02-2010
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