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(54) **Title:** SENSITIVE QUALITATIVE BIOASSAY USING GRAPHENE OXIDE AS ANALYTE REVEALING AGENT

(57) **Abstract:** It is provided a method for detecting an analyte of size comprised from 1 to 10000 nm in a mixture, the method comprising: (i) contacting the analyte with (a) a quantum dot that is attached to a solid support and conjugated to at least one first binding molecule, wherein the first binding molecule has affinity for the analyte, and (b) optionally a spacer particle that is conjugated to at least one second binding molecule, wherein the second binding molecule has affinity for the analyte and may be identical or different to the first binding molecule, and wherein the spacer particle has a size comprised from 20 nm to 10000 nm and is optically inactive, wherein when step (b) is performed steps (a) and (b) are performed in any order; (ii) determining the fluorescence emission intensity of the mixture of step (i) upon exposing the mixture to an electromagnetic radiation capable of exciting the quantum dot; (iii) adding graphene oxide to the mixture of step (ii); and (iv) determining the fluorescence emission intensity of the mixture of step (iii) upon exposing the mixture to the same electromagnetic radiation used in step (ii), wherein a reduction in fluorescence emission intensity of the mixture of step (iv) compared with that of step (i) is indicative of the absence of the analyte; with the proviso that when the analyte has a size comprised from 1 to 19 nm, step (i) (b) must be performed.



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Sensitive qualitative bioassay using graphene oxide as analyte revealing agent

5 The present invention belongs to the field of analytical chemistry. The invention is particularly referred to a biosensing method for detecting analytes, particularly pathogens, by taking advantage of the optical qualities of quantum dots and graphene oxide.

BACKGROUND ART

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Food poisoning and waterborne diseases drastically affect public health worldwide. They lead to countless premature deaths and massive losses in productivity, implying considerable costs to cover healthcare and other consequent expenses. Furthermore, in the long-term they can lead to severe chronic conditions such as reactive arthritis, ocular damage and kidney failure. Hence, being able to detect the pathogens that cause outbreaks of food poisoning or waterborne diseases is fundamental for safeguarding public health. Since the presence of just a few cells of certain pathogens may render food or water unsuitable for human consumption, it is important that the pathogens are rapidly detected at low concentrations.

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Many analytical means have been developed for detecting pathogens in all sorts of samples. Culture and colony counting, polymerase chain reaction (PCR) and immunology-based methods are the most common tools.

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The culturing and colony counting technique is the oldest bacterial detection method. Different selective media are used to detect particular bacterial species. The media often contain inhibitors to stop or delay growth of non-target strains or particular substrates that only the targeted bacteria can degrade. When the colonies have grown, detection is carried out using optical methods, mainly by ocular inspection. Despite remaining the standard method for bacterial detection, culturing and colony counting technique is excessively time consuming (at least 2-9 days are needed for bacterial growth) and usually requires specialised staff and instrumentation. Further, the technique requires that serial dilutions are made when the initial bacterial concentration of the sample is very high, while it is not appropriate to detect very low bacterial numbers.

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PCR is a nucleic acid amplification technique which is also widely used for bacterial detection. It is based on the isolation, amplification and detection of a short DNA sequence that should be specific of the targeted bacteria. PCR-based techniques have become very popular for their versatility, but are also time-consuming. It takes from 5 to 24 hours to produce a detection result, and this does not include any previous enrichment steps that might be necessary. Further, these methods are not devoid of manipulation and need of specialised technical staff.

Immunology based methods provide detection of a wide range of bacterial targets. The most well established immunological based technique for pathogen detection is enzyme-linked immunosorbent assay (ELISA). ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to easily assayed enzymes. Traditional ELISAs are less time consuming than the above techniques - results being obtained in the range from 20 min to 6 hours- but, nevertheless, they require a lot of manipulation. Newer modalities, such as lateral flow immunochromatographic methods, may be quicker and also require little manipulation. However, they lack for sensitivity and often provide false positive or negative results.

More variations of immunological detection assays have been recently developed, among which those operating on the basis of resonance energy transfer (FRET) between a fluorescence donor and a fluorescence acceptor are becoming very popular.

Jung JH, *et al* (*Angew Chem Int Ed Engl.* 2010, vol. 49(33), p. 5708-11) discloses a FRET-based immunobiosensing system for pathogen detection using the fluorescence quenching effect between graphene oxide (GO) and gold nanoparticles that largely resembles an ELISA. The fluorescent GO sheets were deposited on an amino-modified glass surface by electrostatic force, and the carboxylate functional group on the GO surface was used to covalently conjugate rotavirus-antibodies to be linked on the surface. The target rotavirus was then incubated and bound by a specific antigen-antibody interaction. Finally, an engineered gold nanoparticle-labeled antibody probe was attached to the captured target cell whose complexes enable gold

nanoparticles to be close to the GO surface, thereby resulting in the quenching of GO fluorescence signal to identify the pathogen. An increasing number of NPs leads to drastic fluorescence reduction of GO, allowing rotavirus detection with a limit of detection of 10^4 colony forming units/mL (CFU/mL). Despite the innovative approach, this qualitative bioassay does not improve the sensitivity of known methods for rotavirus detection.

Another FRET-based immunoassay has been recently disclosed by Cheng L *et al* (*Analytical Chemistry* 2012, vol. 84(7), p. 3200-3207) for the simultaneous detection of two different virus. The system employs two colored quantum dots (QDs) as fluorescence donors, which are bound to virus-specific antibodies, and graphene oxide (GO) as acceptor. In the absence of the virus, the QD-probes were attached to GO, and the fluorescence of the QDs was quenched by GO because FRET was established among them. In the presence of the virus and interaction of the same with its antibody, the complex detaches from GO and FRET between QDs and GO is interrupted, thus detecting an increase of fluorescence from the QDs. The authors report a rapid detection with detection limits of 0.42 ng/mL for human enterovirus 71 and 0.39 ng/mL for coxsackievirus B3. However, this assay also does not significantly improve the sensitivity of known commercial ELISA kits for virus detection (see http://www.novusbio.com/Enterovirus-71-ELISA-Kit_KA1677.html for enterovirus 71 detection and <http://www.antibodies-online.com/kit/813260/Coxsackie+Virus+and+Adenovirus+Receptor+CXADR+ELISA/> for coxsackievirus B3 detection). Further, despite the possibility of dual detection, this assay would not be able to provide sensitive multiplex detection of a large number of pathogens.

It is therefore desirable to provide alternative rapid and simple methods for pathogen detection with increased sensitivity and versatility which may provide for multiplex detection.

SUMMARY OF THE INVENTION

The inventors have developed a high performance bioassay for detecting the presence of pathogenic microorganisms with surprisingly high sensitivity, while being quick, easy to use, down-scalable and highly specific. The assay

is advantageous for detecting any type of analyte as long as its size is 20 nm or greater and takes advantage of the FRET process that occurs between fluorescent QDs and GO, when the GO is used as pathogen revealing agent.

5 Thus, a first aspect of the invention provides a method for detecting an analyte of size comprised from 20 to 10000 nm in a mixture, the method comprising: (i) contacting the analyte with a quantum dot that is conjugated to at least one binding molecule, wherein the binding molecule has affinity for the analyte which means that the analyte is selectively captured by the
10 binding molecule, (ii) determining the fluorescence intensity of the mixture of step (i) upon exposing the mixture to an electromagnetic radiation capable of exciting the quantum dot, (iii) adding graphene oxide to the mixture of step (ii), and (iv) determining the fluorescence intensity of the mixture of step (iii) upon exposing the mixture to the same electromagnetic radiation used in step
15 (ii), wherein a reduction in fluorescence intensity of the mixture of step (iii) compared with that of step (i) is indicative of the absence of the analyte.

The expression “method for detecting an analyte in a mixture” and the term “qualitative bioassay” has been used indistinctly herein.

20 The method of the invention may detect a pathogenic bacteria which is present in a sample at a concentration as small as 5 CFU/mL. On top of this surprisingly high sensitivity, the method may provide results in a very short time. Due to its convenient configuration, the method is easy to use and
25 versatile. It can be adapted to many types of formats, including solid supports such as microarray plates, microtiter plates or immunochromatographic strips. The method can also proceed in solution. This versatility includes multiplex detection using standard microarray equipment. It is also possible to down-scale the assay to a small device for point of use applications which would
30 not have need of complex laboratory equipment or specially trained staff.

As schematically represented in FIG. 1, The system relies on binding molecule-decorated quantum dots (QD-binding molecule) as analyte attachment mechanism. FIG. 1 represents a particular embodiment of the
35 invention where the binding molecule is an antibody (Ab) specific for *E. coli* . Once the analyte is selectively captured onto the QD- binding molecule probes, which fluoresce when excited with an appropriate electromagnetic

radiation, they are coated with GO platelets that reveal the presence of the pathogen. In the presence of the pathogen (B), the (bound) probes barely interact with the GO; consequently, the GO only minimally quenches their fluorescence (ON state, 1), whereas in the absence of the pathogen (A), the probes are quenched by electrostatic π - π stacking interaction between the probes and the GO (OFF state, 0). In short, the selectively captured analyte does not allow for efficient FRET energy transfer between the QDs and GO, so that minimal variations on the fluorescent intensities of the QDs indicate that the analyte is present in the sample while a sharp reduction of differences on fluorescence intensity of the QDs is indicative of the absence of the analyte.

The limit of detection (LOD) for the method of the invention is defined as the mean of the blank signal, plus three times the standard deviation of the blank signal ($\text{LOD} = mB + 3\delta_B$). The blank signal is the fluorescence intensity of the probes when incubated in the absence of an analyte.

When applied to *E. coli* detection, the method of the invention yielded a limit of detection (LOD) of 5 CFU/mL (see examples below and FIGs. 4 and 5). This means that when more than 5 CFU/mL of *E. coli* cells were present in the sample, the fluorescence intensity of the QDs suffered a minimal, non-significant variation (ON state). On the contrary, when less than 5 CFU/mL of *E. coli* cells were present in the sample, a sharp decrease of the fluorescence intensity of the QDs was observed (OFF state). Results are expressed as the coefficient between final fluorescence intensity (I , after addition of GO) and initial intensity (I_0 , after addition of the sample). It is remarkable that the system's response to the presence of the analyte is digital-like: the system exhibited a sharp transition zone from the OFF state to the ON state at very low concentrations of *E. coli* (FIG. 5). Such a sensitive, digital-like response is surprising, particularly when considering that state of the art commercial tests for *E. coli* detection report a LOD of 3000 CFU/ml (see, for instance, *E. coli* 0157 Antigen ELISA Kit, Catalog Number KA3197, from Abnova).

Further, as also shown in the examples below, the assay has been adapted to a solid-support microarray format and the detection takes place in the solid state using standard microarray instrumentation. Thus, only by modifying the

QD probe (i.e. using a different binding molecule conjugated to the QD) in the different microarray spots, multiplex detection of hundred different analytes is achieved. Alternatively, by using the same probe in the different microarray spots, multiplex detection of the same analyte in tens of different samples is achieved.

In addition to the diverse advantages described above, the qualitative bioassay of the invention has a pathogenic effect. When the method of the invention is used for pathogenic bacteria detection, interaction of the GO revealing agent with the bacterial cell may kill the bacteria by compromising the integrity of the bacterial membrane, thus comprising a biosensing and microbicidal GO-based system that may both detect bacteria and kill them.

The inventors have also found that detection of analytes may succeed with the same advantages mentioned above by contacting said analyte to a spacer particle, wherein said spacer particle has a size comprised from 20 to 10000 nm and is optically inactive. Use of a spacer particle advantageously enables detection of analytes of size smaller than 20 nm, such as proteins, nucleic acids and viruses.

Thus another aspect of the invention relates to a method for detecting an analyte of size comprised from 1 to 10000 nm in a mixture, the method comprising: (i) contacting the analyte with (a) a quantum dot that is attached to a solid support and conjugated to at least one first binding molecule, wherein the first binding molecule has affinity for the analyte, and (b) optionally a spacer particle that is conjugated to at least one second binding molecule, wherein the second binding molecule has affinity for the analyte and may be identical or different to the first binding molecule, and wherein the spacer particle has a size comprised from 20 nm to 10000 nm and is optically inactive, wherein when step (b) is performed steps (a) and (b) are performed in any order; (ii) determining the fluorescence emission intensity of the mixture of step (i) upon exposing the mixture to an electromagnetic radiation capable of exciting the quantum dot; (iii) adding graphene oxide to the mixture of step (ii); and (iv) determining the fluorescence emission intensity of the mixture of step (iii) upon exposing the mixture to the same electromagnetic radiation used in step (ii), wherein a reduction in fluorescence emission intensity of the mixture of step (iv) compared with that of step (i) is indicative

of the absence of the analyte; with the proviso that when the analyte has a size comprised from 1 to 19 nm, step (i) (b) must be performed.

Figure 6 illustrates the working of the method of the invention when a spacer particle is used. The figure represents a particular embodiment where the binding molecules are antibodies and the spacer particle is a silica bead. Antibody coated QDs (E) are used to capture the analyte (F). In the absence of the target analyte, the probes are quenched by FRET between QDs and GO (A). This state is known as OFF state (0). However, when the analyte is captured (B), detection antibodies conjugated to silica beads are used to avoid FRET. This state is termed ON state (1). The selectively captured analyte and spacer particle do not allow for efficient FRET energy transfer between the QDs and GO, so that minimal variations on the fluorescent intensities of the QDs indicate that the analyte is present in the sample. In contrast, a sharp reduction of differences on fluorescence intensity of the QDs is indicative of the absence of the analyte.

In another aspect, the invention provides a kit for carrying out the method of the invention, which comprises adequate means and instructions for carrying out the method, wherein the means comprise at least QD-binding molecule probes and graphene oxide, wherein said graphene oxide: (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 5, preferably from 1 to 2, (b) contains a lateral size comprised from 5 to 1000 nm, and (c) contains from 1 to 20, preferably 1 to 10, layers. The kit may also include a solid support, spacer particles and other reagents and/or buffers.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: Operational concept of the qualitative bioassay of the invention (illustration not to scale). C: graphene oxide; D: *E. coli*; E: antibody-QD conjugate; G: capture spot; F: chip on glass slide; A: in the absence of analyte; 0: OFF state; B: in the presence of analyte (*E. coli*); 1: ON state.

FIG. 2: Scanning electron microscopy (SEM) images of the explored system. **a.** Ab-QD complexes inside a microspot. **b.** Ab-QD/GO complexes inside a microspot. **c.** Ab-QD/*E. coli* (membrane-bound)/GO complexes inside a microspot. Aminosilane silicon slides were used as substrate.

FIG. 3: **a.** Spot profile of the system's response. The dotted lines denote the original profile of the explored spots. The solid lines indicate the final profile of the observed spots at several concentrations of the target pathogen, *E. coli* ([Ec] in CFU/mL) after GO addition. **b.** In the absence of *E. coli*, the spots, which comprise Ab-QD probes, are quenched by adding GO (blank signal). In the presence of *E. coli* (black bars), the system exhibits a transition zone at [Ec] = 0 to 100 CFU/mL; becomes saturated at [Ec] = 10^7 CFU/mL; and shows a consistent signal at [Ec] = 10^2 to 10^6 CFU/mL, as the probes are scarcely quenched relative to the control signal. The control signal represents the maximum signal that the system can display: this corresponds to the probes being incubated with a blank throughout the assay (i.e. without adding GO). In the presence of a non-target pathogen (*Salmonella*, grey bars), the quenching of the Ab-QD probes barely exceeds the proposed limit of detection (LOD; = mean of the blank signal, plus three times the standard deviation of the blank signal [$mB + 3\delta_B$]). **c.** Behavior of the system for *E. coli* screening in tap water. The error bars were typically obtained from parallel assays of 10 microspots. I/I_0 : final intensity/original intensity. N: normalised intensity.

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FIG. 4: Performance of the Ab-QD pathogen-detection system in the transition zone at *E. coli* concentrations [Ec] of 0 to 100 CFU/mL, in PBS (**b'**) and in tap water (**b''**).

FIG. 5: Response of the qualitative bioassay of the invention (using GO as the pathogen-revealing agent, ●) as compared to an immunosandwich configuration (using Cy3- labelled antibody as the pathogen-revealing agent, x). The error bars were obtained from parallel assays of 10 microspots in each configuration. The dotted line corresponds to the threshold of the LOD. I/I_0 : final intensity/original intensity. N: normalised intensity.

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FIG. 6: Operational concept of the qualitative bioassay of the invention when using a spacer particle (illustration not to scale). C: graphene oxide; D: *E. coli*; E: antibody-QD conjugate; G: capture spot; F: chip on glass slide; A: in the absence of analyte; 0: OFF state; B: in the presence of analyte (*E. coli*); 1: ON state.

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DETAILED DESCRIPTION OF THE INVENTION

The term “quantum dots” (QDs) is understood in the state of the art as photoluminescent semiconductor nanocrystals whose characteristics are closely related to the size and shape of the individual crystal. A QD is capable of emitting electromagnetic radiation or light upon excitation (i.e., the QD is luminescent). Generally, the smaller the size of the crystal, the larger the band gap, the greater the difference in energy between the highest valence band and the lowest the conduction band becomes, therefore more energy is needed to excite the dot, and concurrently, more energy is released when the crystal returns to its resting state. For example, in fluorescent dye applications, this equates to higher frequencies of light emitted after excitation of the dot as the crystal shrinks to smaller sizes, resulting in a color shift from red to blue in the emitted light. Thus a main advantage with QDs is that their photoluminescent properties can be tuned by controlling the growth of the crystal while produced. In addition to such size-tuneable photoluminescence, QDs have broad excitation spectra with narrow emission bandwidths that span the visible spectrum, allowing simultaneous excitation of several particle sizes at a single wavelength. QDs also have exceptional photochemical stability. Altogether, QDs are an advantageous alternative to conventional organic dyes for bioanalytical applications.

A QD is comprised of a first semiconductor material that sometimes can be coated by a second semiconductor material. The coating material will preferably have a bandgap energy that is larger than the bandgap energy of the first material and may be chosen to have an atomic spacing close to that of the first material. The first and/or second semiconductor material can be, but is not limited to carbon, graphene, ZnS, ZnSe, ZnTe, US, CdSe, CdTe, HgS, HgSe, HgTe, MgS, MgSe, MgTe, CaS, CaSe, CaTe, SrS, SrSe, SrTe, BaS, BaSe, BaTe, GaN, GaP, GaAs, GaSb, InN, InP, InAs, InSb, PbSe, or an alloy or mixture thereof. In particular embodiments the QDs are constituted by carbo, graphene, CdSe, CdS, CdSe coated with ZnS (CdSe/ZnS), or by CdS coated with ZnS (CdS/ZnS). Multi-shell QDs are also known in the state of the art.

QD size is usually comprised from about 1 nm to about 50 nm in diameter, sometimes from 2 nm to 20 nm, sometimes from 2 nm to 10 nm. QDs may be

prepared by methods well established in the state of the art.

Graphene oxide (GO) is a sheet-like compound of carbon, oxygen, and hydrogen in variable ratios, obtained by treating graphite with strong oxidizers. Graphene oxide is available at variable C/O ratios (ratio expressed as carbon atoms related to oxygen atoms), usually from 1 to 5, and may be found in the form of single or multiple sheets (multiple in this case usually meaning from 2 to 20 layers). While bearing oxygenated functional groups such as carboxyl (at the lattice edges), ester, hydroxyl or epoxide (on the basal plane of the lattice), GO constitutes an oxygenated lattice of donor/acceptor molecules exposed in a planar surface. Consequently, GO is an excellent optical biosensing platform for detecting biological analytes. In the present invention, the quenching effect of GO when placed in proximity to a fluorescence donor, such as a QD, is exploited.

Fluorescence refers to the emission of light by a substance that has absorbed light or other electromagnetic radiation. It is a form of luminescence. In most cases, the emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation. Many QDs are fluorescent.

"Förster fluorescence resonance energy transfer" or "FRET" refers to a process based on two photoluminescent substances where initially one of the substances (the donor) can transfer energy to the second one (the acceptor). Generally, the efficiency of this energy transfer process is inversely proportional to the sixth power of the distance between donor and acceptor making FRET extremely sensitive to short distances between the two substances. Consequently, FRET is observed when "donor" and "acceptor" are sufficiently close one to another and when there is sufficient spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. When efficient FRET is established between a donor and an acceptor, the acceptor is said to "quench" the donor's emitted radiation. This means that the initial photoluminescent intensity of the donor is reduced because of FRET energy transfer to the acceptor. The acceptor is thus also called "quencher" and is said to have a certain "quenching" efficiency.

"Electromagnetic radiation" is a form of energy emitted and absorbed by charged particles which exhibits wave-like behavior as it travels through

space. Electromagnetic radiation of any wavelength is contemplated by the present invention but, particularly, the invention is referred to visible light and ultraviolet radiation.

5 By "binding molecule" is meant a molecule or molecule segment capable of binding specifically to the target. Non-limitative binding molecules in the sense of the present invention are antibodies, antibody fragments, aptamers, molecular beacons, etc, all of which are binding molecules well known in the state of the art as being useful for bioanalytical applications.

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By "antibody" is meant a whole antibody, including without limitation a chimeric, recombinant, transgenic, humanised, grafted and single chain antibody, and the like, as well as any fusion protein, conjugates, fragments, or derivatives thereof that contain one or more domains that selectively bind the target protein or peptide. An antibody fragment means an Fv, a disulfide linked Fv, scFv, Fab, Fab', or F(ab')₂ fragment, which are well known in the art. There are various advantages for using antibody fragments, rather than whole antibodies. For example, the smaller size of the fragments may lead to an improved penetration to the target site, and effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the fragments.

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"Aptamers" are nucleic acid molecules that are designed in such a way that they can bind to desired target molecule and show nuclease resistance. The aptamers can be synthesized through repeated rounds of in vitro partition, selection and amplification, a methodology known in the state of the art as "SELEX". Alternatively, they can be synthesized, for example, by step-wise solid phase. Preparation of any of these binding molecules specific for a particular target analyte is achieved by using methods well known in the state of the art.

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In the sense of the present invention, the term "spacer particle" refers to particle of size comprised from 20 nm to 10000 nm that connects the analyte and the GO revealing agent, providing a larger separation distance between the QD capturing the analyte and the GO. The spacer particle is particularly important for detecting analytes of size smaller than 20 nm with high

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sensitivity. For such small analytes, the presence of a spacer particle determines that the QDs and the GO are separated at a distance that prevents FRET between them. In addition to a size comprised from 20 to 10000 nm, the spacer particles are optically inactive (transparent, so that they do not interfere in the FRET between QDs and GO) and susceptible to biofunctionalization (so that they may be conjugated with binding molecules). Spacer particles for use in the present invention are, for example, silica beads, magnetic silica beads, nanocellulose particles, indium tin oxide particles, ZnS particles, transparent exopolymer particles, titania (titanium oxide) particles, Zinc oxide particles and ceria (cerium oxide) particles.

The inventors have developed a sensitive and versatile qualitative bioassay .

According to the method of the invention, the fluorescence intensity of the QD probes is quenched by GO in the absence of the target analyte (OFF state). Due to its oxygen-containing functional groups, GO can interact non-covalently with the diol, amino and phenyl groups present in the QD-binding molecule probe, for example with the amino groups of an antibody. This interaction brings GO in the proximity of the QDs, thus allowing for FRET energy transfer and efficient quenching of QD emission. In the presence of the target analyte, for instance, a bacteria, the greater affinity of the binding-molecule for its target (bacteria) avoids efficient FRET between the QDs and GO, and consequently, QD emission is not quenched (ON state). Owing to its high density of functional groups, GO can interact with bacteria, resulting in bacterial cell deposition. However, the size of the bacterial analyte precludes quenching of QD emission by the deposited GO.

The method of the invention is not restricted to relatively big analytes, such as bacterial cells. When an analyte of size below 20 nm wants to be determined, it may be done with equally high sensitivity by means of using a spacer particle. The spacer particle, which is conjugated to a binding molecule with affinity for the analyte, is contacted with the analyte prior to adding the GO revealing agent, moving away the GO at a distance that precludes quenching of QD (see figure 6). When the analyte is of size equal or above 20 nm use of the spacer particle is optional.

Any type of analyte may be detected with the bioassay of the invention,

regardless of its chemical composition, as long as its size is comprised from 1 to around 10000 nm. In particular embodiments the size is comprised from 20 to 10000 nm, from 30 to 10000 nm or from 50 to 10000 nm. In other embodiments the size of the analyte is comprised from 1 to 19 nm. For
5 detection of analytes of size comprised from 1 to 19 nm the method of the invention comprises use of a spacer particle. Non-limiting target analytes for the qualitative bioassay of the invention are bacteria, virus, eukaryotic cells, proteins, large organic molecules and heavy metal-containing complexes. The qualitative bioassay of the invention may be of particular interest in the
10 food, environmental and/or medical fields for detecting pathogens (*E. coli*, *Salmonella*, virus, etc), cancer cells, disease-relevant proteins (such as beta amyloid, prostate specific antigen, etc), environmentally or food toxic proteins, biomarker-containing complexes, and environmentally-toxic organic molecules (such as saxitoxin, okadaic acid and other toxin-containing
15 complexes). In a particular embodiment the target analyte is a pathogenic bacteria. In another particular embodiment the target analyte is *E. coli*.

As explained above, the QD probes of the qualitative bioassay of the invention fluoresce when excited by an appropriate electromagnetic radiation.
20 After incubating the sample and adding GO, the intensity of the emitted light greatly differs in the absence or the presence of the analyte, and the fluorescent intensity variation allows for sensitive analyte detection.

The QDs for use in the present invention usually have emission wavelengths
25 comprised from 400 to 750 nm. In certain embodiments, the QDs have emission wavelengths comprised from 500 to 720 nm, or from 600 to 700. In a particular embodiment the emission wavelength of the QDs is 670 nm. In another particular embodiment the QDs are selected from the group consisting of carbon, graphene, CdSe, CdS, or may have a core/shell
30 structure, for example, CdSe coated with ZnS (CdSe/ZnS), CdSe coated with CdS (CdSe/CdS) and CdS coated with ZnS (CdS/ZnS). The coating (shell) of the QDs may be single (only one shell) or multishell.

In order to emit fluorescence, the QDs must be excited by being exposed to
35 an electromagnetic radiation of shorter wavelength than that of the QDs' emission. Usually, the wavelength of the exciting electromagnetic radiation is from 40 to 25 nm, for example 28, 29, 30, 31, 32, 33, 34 or 35 nm, shorter

than the emission wavelength of the QDs. In one embodiment, the electromagnetic radiation capable of exciting the quantum dot has a wavelength comprised from 360 to 725 nm. In other embodiments, the electromagnetic radiation capable of exciting the quantum dot has a wavelength comprised from 450 to 700 nm or from 550 to 680 nm. In a particular embodiment, the electromagnetic radiation capable of exciting the quantum dot has a wavelength of 633 nm. Any source may be employed for producing the excitation electromagnetic radiation, for example, a laser, light-emitting diode (LED) lamp, ultra-violet (UV) lamp.

When excited, the QDs thus emit a read-out signal, which will vary depending on the presence or the absence of the analyte as detailed above. The read-out signal is recorded by a fluorometer, imaging system, or any other device used to measure parameters of fluorescence, by fixing the emission filter at the predetermined emission of the QDs. The fluorometer thus records the emission intensity of the QDs to yield a result (presence or absence of the analyte). Any fluorometer or imaging system may be appropriate for use in the qualitative bioassay of the invention. The type used will depend on the configuration of the assay (in solution, in microtiter plates, in microarray plates, in paper, etc). In a particular embodiment, the fluorometer is a microarray scanner.

According to the qualitative bioassay of the invention, the QDs are conjugated to binding molecules which have high affinity for the target analyte, such that incubating of an analyte-containing sample results in the analyte being selectively captured by the binding molecule. By "conjugation" it is generally meant the joining together of two compounds resulting in the formation of another compound, in this case, the joining together of the QD and the binding molecule. In a particular embodiment the binding molecule is an antibody, an antibody fragment, a molecular beacon or an aptamer, all of which may be obtained by standard techniques.

In order to obtain the QD-binding molecule conjugate, QDs need to be functionalised. First, the QDs need to be water soluble for biological applications. Then, they need to be biofunctionalized in order to meet four key requirements: (1) increased stability in water for long period, (2) presence of sterically accessible functional groups for bioconjugation, (3)

biocompatibility and non-immunogenicity in living systems, and (4) lack of interference with the QD native properties.

5 Solubilisation and biofunctionalisation of QDs is achieved by methods well known in the art. These methods are comprehensively summarised, for example in the following reviews: Medintz I, *et al*, "Quantum dot bioconjugates for imaging, labelling and sensing", *Nature Materials*, 2005, vol. 4, p. 435, and Mazumder S, *et al*, "Biofunctionalized Quantum Dots in Biology and Medicine", *Journal of Nanomaterials*, 2009, vol. 2009. A representative but
10 non-limiting list of solubilisation and biofunctionalisation strategies is shown in table 1 from the above referenced Menditz review.

In a particular embodiment, the QD-binding molecule probes are obtained by the streptavidin-biotin approach, for which streptavidin-covered QDs are
15 contacted with biotinylated binding molecules, for example, biotinylated antibodies (see examples below). Alternatively, the QDs may be capped by a silane shell and functionalised with, for instance, a compound providing amine, sulfhydryl or carboxy functionality which can react with a peptidic or DNA binding molecule.

20 For sensitive detection of analytes of size smaller than 20 nm, the method of the invention requires use of a spacer particle as defined above.

The spacer particles are also conjugated to binding molecules which have
25 high affinity for the target analyte. The binding molecule conjugated to the spacer particle may be the same or different to the binding molecule that is conjugated to the QD. In a particular embodiment the binding molecule is an antibody, an antibody fragment, a molecular beacon or an aptamer, all of which may be obtained by standard techniques.

30 In order to obtain the spacer particle-binding molecule conjugate, spacer particles need to be surface-functionalised. Biofunctionalisation of the surface of particles, particularly nanoparticles such as silica beads or nanocellulose beads, is achieved by methods well known in the art (Froimowicz P., *et al*.
35 "Surface-Functionalized Particles: From their Design and Synthesis to Materials Science and Bio-Applications", *Current Organic Chemistry* 2013, vol. 17, p. 900-912). For instance, the spacer particle may be functionalised

with a compound providing amine, sulfhydryl or carboxy functionality which can react with a peptidic or DNA binding molecule. Surface-functionalised particles can be readily obtained from commercial sources.

5 The spacer particle, when used, may be contacted with the analyte before or after contacting the analyte with the QDs, but always before addition of the GO revealing agent. In one embodiment, the analyte is first incubated with the binding molecule-decorated QDs and then the binding molecule-decorated spacer particles are added to the previously formed QD-analyte complex. In
10 another embodiment, the analyte is first incubated with the binding molecule-decorated spacer particle and then formed spacer particle-analyte complex is incubated with the binding molecule-decorated QD. Examples of the procedure when using a spacer particle in the method of the invention are described in the examples below.

15 In a particular embodiment the QD-binding molecule probe is attached to a solid support. The support may be, among other, glass, plastic, cellulose, nitrocellulose or paper. Attachment of the probe to the solid support may succeed by methods well known in the art (Angenendt P, *Drug Discovery Today*, 2005, vol. 10(7), p. 503). For example, the QD-binding molecule probe may be attached to two-dimensional plain glass slides, which are activated with a variety of coupling chemistries such as aldehyde, epoxy or carboxylic esters. Slides with these surfaces bind biomolecules (for example peptides or nucleic acids) either by electrostatic interactions or through the formation of
25 covalent bonds. Alternatively, the QD-binding molecule probe can be attached to three-dimensional gel or membrane-coated surfaces, such as polyacrylamide, agarose and nitrocellulose. These surfaces bind biomolecules mainly by physical adsorption. Alternatively, the QD-binding molecule probe can be attached to surface coatings, such as dendrimer or
30 avidin slides, which mix both concepts mentioned above. In one embodiment the solid support is amino, epoxy or carboxi-functionalised. In a particular embodiment the support comprises aminosilane glass surface.

In one particular embodiment the solid support has microarray format,
35 meaning that QD probes are deposited or immobilised onto the solid support following a microarray design, i.e., the probes are densely spotted onto the support on a few square microns such that large number of samples may be

analysed simultaneously. Different immobilisation strategies known in the art may be employed to this end, including, among others, immobilisation of antibodies by DNA-directed immobilisation (DDI), direct spotting, and streptavidin-biotin attachment (Wacker, R. *et al.* "Performance of antibody
5 microarrays fabricated by either DNA-directed immobilization, direct spotting, or streptavidin-biotin attachment: a comparative study". *Anal. Biochem.* 2004, vol. 330, p. 281–287). Usually, such microarray format comprises a glass surface.

10 In another embodiment the solid support has lateral flow format. Lateral flow tests, also known as lateral flow immunochromatographic assays, are simple devices intended to detect the presence (or absence) of a target analyte in a sample (matrix) without the need for specialized and costly equipment, though many lab based applications exist that are supported by reading equipment.
15 These lateral flow tests are well known in the art and appropriate for point of use applications. Often they comprise a series of capillary beds, each of which has the capacity to transport fluid. The sample thus flows through the different capillary beds contacting the reagents so that a detection result is finally observed. In a particular embodiment the solid support with lateral flow
20 format comprises a nitrocellulose membrane to which the QD-binding molecule label is attached.

In other embodiments the solid support has microtiter plate format, such as those used for ELISA assays.

25 According to the method of the invention, GO is added at the final stage of the assay as revealing agent. Thus, addition of GO allows for differentiation between the presence or absence of the analyte in the sample. In one embodiment of the method of the invention, the GO contains an atom carbon
30 to oxygen ratio (C/O) comprised from around 1 to around 5. In a particular embodiment the C/O ratio is comprised from 1 to 2, or from 1 to 1.5, or from 1 to 1.25. In other particular embodiments the C/O ratio is 1. Further, the GO in the sense of the present invention may comprise from 1 to 20 layers. In other embodiments, the GO comprises from 1 to 15, or from 1 to 10 or from 2 to 10
35 or from 1 to 5 or from 2 to 5 layers. Further, the lateral length of GO sheets may be comprised from 5 to 1000 nm. In one embodiment, the lateral length of GO sheets is comprised from 100 to 1000 nm. In one embodiment, the

lateral length of GO sheets is comprised from 5 to 500 nm. In one embodiment, the lateral length of GO sheets is comprised from 50 to 500 nm. In one embodiment, the lateral length of GO sheets is comprised from 250 to 1000 nm. In another embodiment, the lateral length of GO sheets is
5 comprised from 300 to 900 nm, or from 300 to 800 nm, or from 400 to 700 nm. In another embodiment, the lateral length of GO sheets is around 50 nm.

In one embodiment, GO is added to the assay (specifically, step (iii) of the method of the first aspect of the invention as defined above) at a final
10 concentration comprised between 0.01 to 400 $\mu\text{g/mL}$. In particular embodiments GO is added at a final concentration comprised from 0.1 to 400 $\mu\text{g/mL}$, or from 1 to 400 $\mu\text{g/mL}$ or from 1 to 250 $\mu\text{g/mL}$, or from 5 to 200 $\mu\text{g/mL}$, or from 10 to 175 $\mu\text{g/mL}$, or from 20 to 150 $\mu\text{g/mL}$, or from 25 to 125 $\mu\text{g/mL}$, or from 50 to 100 $\mu\text{g/mL}$, or from 60 to 80 $\mu\text{g/mL}$. In another particular
15 embodiment GO is added at a final concentration of around 70 $\mu\text{g/mL}$.

In a particular embodiment, the GO added in step (iii) of the method defined by the first aspect of the invention: (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 5, (b) contains a lateral size comprised from 5
20 to 1000 nm, (c) contains from 1 to 20 layers, and (d) is added at a final concentration comprised from 0.1 to 400 $\mu\text{g/mL}$. In another particular embodiment, the GO added in step (iii) of the method defined by the first aspect of the invention: (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 2, (b) contains a lateral size comprised from 5 to 1000
25 nm, (c) contains from 1 to 10 layers, and (d) is added at a final concentration comprised from 0.1 to 400 $\mu\text{g/mL}$. In another particular embodiment, GO in step (iii): (a) contains an atomic carbon to oxygen ratio (C/O) comprised from around 1.5 to around 2, (b) contains a lateral size comprised from 5 to 750 nm, (c) contains from 1 to 5 layers, and (d) is added at a final concentration
30 comprised from 0.01 to 100 $\mu\text{g/mL}$. In another particular embodiment, GO in step (iii): (a) contains an atomic carbon to oxygen ratio (C/O) comprised from around 1 to around 1.5, (b) contains a lateral size comprised from 5 to 750 nm, (c) contains from 1 to 15 layers, and (d) is added at a final concentration comprised from 0.1 to 200 $\mu\text{g/mL}$. In another particular embodiment, GO in
35 step (iii): (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 1.25, (b) contains a lateral size comprised from 5 to 700 nm, (c) contains from 1 to 10 layers, and (d) is added at a final concentration comprised from 1

to 400 µg /mL. In another particular embodiment, GO in step (iii): (a) contains an atomic carbon to oxygen ratio (C/O) of 1, (b) contains a lateral size of 500 nm, (c) contains around 2 layers, and (d) is added at a final concentration of around 70 µg/mL.

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The analyte containing sample must be placed in contact (or incubated) with the QD probes enough time for selective binding to take place between the sample and the binding molecule. The incubation time depends on the analyte and the binding molecule. In one particular embodiment, incubating time of the sample with the probe is from 5 min to 180 min. In other embodiments, the incubating time is from 15 to 180 min, or from 30 to 160 min, or from 45 to 120 min, or from 60 to 120 min. Further, as described above, detection of the analyte succeeds after addition of the GO (which acts as analyte-revealing agent). In some embodiments the time comprised between addition of GO and determining the fluorescence intensity to yield a detection result (GO incubation) may range between 10 and 120 min. In particular embodiments, GO incubation is comprised from 45 to 90 min, or from 60 to 80 min, or from 70 to 80 min. In another particular embodiment GO incubation takes place during 75 min.

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The qualitative bioassay of the invention may take place in solution/suspension, i.e., the fluorescence intensity may be recorded from a solution/suspension containing the reagents. Alternatively, the qualitative bioassay may proceed in the solid state, i.e. steps (ii) and (iv) of the method as defined in the first aspect of the invention (determining the fluorescence intensity) may take place in dry state. In order to determine the fluorescence of the reagents in the dry state the samples may be previously dried, for example by centrifugation.

25

A second aspect of the invention provides a kit for carrying out the method of the invention. The kit comprises adequate means for detecting an analyte of size comprised from 1 to 10000 nm by following the steps of the method of the invention. The means may include readily conjugated QD-binding molecule probes. In some embodiments the probes are more than one type of QD-binding molecule probe designed for more than one different analytes. The means may also include a solid support, which in certain embodiments may be readily functionalised for direct attachment of the QD-binding

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molecule probes. In some embodiments the solid support has a format selected from microarray format, microtiter plate format and lateral flow format. In a particular embodiment the kit of the invention comprises a solid support to which the QD-binding molecule probes are already attached. In particular embodiments, in addition to the pre-attached probes, the solid support is also blocked for direct use, i.e., ready for addition of the analyte. In another particular embodiment, the solid support pre-loaded with QD-binding molecule probes has a microarray or lateral flow format. In other embodiments the solid support with microarray format contains more than one type of QD-binding molecule probe designed for more than one different analyte. In other embodiments the solid support with microarray format contains more than one QD-binding molecule probe designed for the same analyte. Alternatively, in some embodiments the kit comprises QDs and binding molecules, such that the user may perform the conjugation of the QD-binding molecule probe. In particular embodiments both the QDs and the binding molecules are adequately functionalised for direct conjugation.

The means may also include spacer particles. In one embodiment the spacer particles are readily conjugated to at least one binding molecule. In other embodiments spacer particles and binding molecules are provided separately, such that the user may perform the conjugation of the spacer particle-binding molecule. In particular embodiments both the spacer particles and the binding molecules are adequately functionalised for direct conjugation. In some embodiments the kit provides more than one type of spacer particle-binding molecule probe designed for more than one different analyte.

The kit may also comprise adequate buffers and solvents to perform the method of the invention, for example, binding buffer, washing buffer, blocking buffer. When the kit comprises QDs, binding-molecule, spacer particles and/or solid support intended for previous conjugation and, if appropriate, attachment to solid support, the kit may also comprise the reagents required for appropriate functionalisation of the QDs, binding-molecules, spacer particles and/or solid support.

In a particular embodiment the kit comprises a device for point of use fluorescence detection in addition to the probes and reagents for performing

the method of the invention. Such point of use fluorometers have reduced dimensions and are known in the state of the art for analyte detection. One example of the point of use/personal devices is commercialised by Promega (see Quantus fluorometer). In another particular embodiment the kit
5 comprises a device for point of use fluorescence detection and a solid support which comprises a nitrocellulose membrane to which the QD-binding molecule probes are attached, and wherein the assay has lateral-flow format.

Throughout the description and claims the word "comprise" and variations of
10 the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word "comprise" encompasses the case of "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following
15 examples and drawings are provided by way of illustration, and they are not intended to be limiting of the present invention. Reference signs related to drawings and placed in parentheses in a claim, are solely for attempting to increase the intelligibility of the claim, and shall not be construed as limiting the scope of the claim. Furthermore, the present invention covers all possible
20 combinations of particular and preferred embodiments described herein.

EXAMPLES

25 1. Detection of bacteria

Materials and methods

Reagents. Glycerol, PBS, Tween 20, milk powder, (3-
30 aminopropyl)triethoxysilane and absolute ethanol were obtained from Sigma-Aldrich (Taufkirchen, Germany). Graphene oxide (GO) of C/O ratio around 1, lateral size around 500 nm, monolayer, was from Angstrom Materials (Ohio, USA). NH₂-functionalized aminosilane glass slides (Cat No. 40006 GAPS II, Corning Incorporated, New York, USA) and silicon slides from Silicon Inc.
35 (Idaho, USA). Anti-*E. coli* antibodies were from Abcam (ab68451, Cambridge, UK). Streptavidin–Cy3 and streptavidin–Quantum dot 655 (Cat No. Q10121MP) were obtained from Life Technologies (California, USA). PBS

with 2% (v/v) glycerol was used as spotting buffer. PBS supplemented with 5% (w/v) of milk powder and 0.005% (v/v) of Tween 20 was prepared as blocking buffer. PBS supplemented with Tween 20 at 0.05% (v/v) (PBST) was used as washing buffer. PBS with 0.5% (v/v) Tween 20 containing 1% of BSA fraction V (w/v) was employed as immunobuffer. All aqueous solutions were prepared in Milli-Q water. *Escherichia coli* O157:H7 (CECT 4783) and *Salmonella typhimurium* (CECT 722T) strains were obtained from the Colección Española de Cultivos Tipo (CECT; Spain). All reagents were handled according to the material safety data sheets provided by the respective suppliers.

Bacterial strains and inoculum preparation. Freeze-dried cultures of *E. coli* O157:H7 and *Salmonella typhimurium* were revived in Tryptone Soy Broth (TSB, Oxoid Ltd.; Cambridge, UK), incubated at 37 °C for 24 h, and then transferred onto Tryptone Soy Agar plates (TSA, Oxoid Ltd.; Cambridge, UK). Stock cultures of each strain were prepared on Tryptone Soy Agar slopes, incubated at 37 °C for 24 h, and then stored at 4 °C for up to 8 weeks. The stock cultures were sub-cultured into 10 mL of TSB, and then incubated at 37 °C for 20 h. A loopful of broth was spread onto TSA plates using a disposable plastic loop, and the plates were incubated at 37 °C for 20–24 h. Subsequently, cell suspensions were prepared directly in 5 mL sterile PBST or tap water, using the bacterial colonies to obtain a bacterial load of 1.5×10^8 CFU/mL using Densimat apparatus (bioMérieux, Lyon, France). Tubes with the assayed samples were placed into a boiling water bath (100 °C) for 15 min to stop pathogen replication. The tubes were then cooled at room temperature, and finally, refrigerated (at 4 °C) until used.

Amino-functionalization of silicon slides. Silicon slides were submerged in a bath of piranha solution for 20 minutes, washed with Milli-Q water, and dried then dried under an N₂ stream. The washed slides were then activated via plasma treatment for 2 minutes, submerged in a bath of (3-aminopropyl)triethoxysilane solution at 2% (in absolute ethanol) for 60 minutes, washed with absolute ethanol, dried under an N₂ stream and finally, placed in an oven (J.P. Selecta 200210; Barcelona, Spain) at 120 °C for 120 minutes. The dried slides were stored in a dessicator at room temperature until used.

Fabrication of antibody-QD (Ab-QD) microarrays. In a typical experiment, a mixture of streptavidin-QD 655 (80 nM) and biotinylated antibodies (1 mg/mL) in spotting buffer was spotted onto aminosilane glass slides, using a Microgrid II microarrayer (Digilab; Massachusetts, USA), at room temperature and 40-60% humidity. The average diameter of the printed spots was 140 ± 10 μ m. After spotting, the slides were incubated at 4 °C overnight in a dessicator. The microarray slides were masked, and then divided into wells through a microarray cassette (Arrayit; California, USA). Each well was incubated with 100 μ L immunobuffer for 15 minutes, and then washed with PBST (3 x 150 μ L). Each well was blocked with 100 μ L blocking buffer for an incubation time of 30 minutes.

Study of the transduction system. Once blocked, the masked slides were washed with PBST (5 x 100 μ L per well) and with Milli-Q water (2 x), unmasked, and then dried by centrifugation (1500 rpm x 1 min). The resulting microarray slides were scanned (excitation: 633 nm; emission filter: 670 nm) in a ScanArray 4000 microarray scanner (Perkin Elmer; Massachusetts, USA), and the observed intensity of the probes was taken as the starting intensity. The slides were re-masked, and then incubated with GO (diluted in Milli-Q water at different concentrations from 50 to ~350 μ g/mL) at two different incubation times (30 or 75 minutes), and then, washed again with Milli-Q water (3 x 150 μ L per well). Finally, the slides were unmasked, and then dried by centrifugation (1500 rpm x 1 min). The slides were scanned again (excitation: 633 nm; emission filter: 670 nm). The fluorescence intensity of the arrays was quantified using GenePix software (Molecular Devices; California, USA). The fluorescence intensity values were estimated by first measuring the mean intensities of the assayed spots, and then subtracting the local background. The intensity values were normalized by dividing each one by the maximum value of the respective screening.

Selective pathogen attachment and screening using GO. Once blocked, the masked slides were washed with PBST (5 x 100 μ L per well) and the microarrays were incubated with the target pathogen (at different concentrations) in the matrix (PBST or tap water; 100 μ L per well) for 2 hours. The masked slides were washed with PBST (5 x 150 μ L per well) and Milli-Q water (x 2), unmasked, and then dried by centrifugation (1500 rpm x 1 min). The resulting microarray slides were scanned (excitation: 633 nm; emission

filter: 670 nm), and the observed intensity of the probes was taken as the starting intensity (I_0). The microarray slides were remasked, incubated with GO (diluted in Milli-Q water at $\sim 70 \mu\text{g mL}^{-1}$) for 75 minutes, and then washed with Milli-Q water ($3 \times 150 \mu\text{L}$ per well). Finally, the slides were unmasked, and then dried by centrifugation ($1500 \text{ rpm} \times 1 \text{ min}$). The slides were scanned and the fluorescence intensity of the arrays was quantified as described above and taken as the final intensity (I). The fluorescence intensities were estimated as described above. The intensity values were normalized as described above. The limit of detection (LOD) of the respective monitoring was taken as the sum of the mean intensity among spots incubated with a blank, and three times the standard deviation of the mean ($mB + 3 \delta_B$).

***E. coli* detection using an immunosandwich configuration.** Once blocked, the masked slides were washed with PBST ($5 \times 100 \mu\text{L}$ per well) and the microarrays were incubated for 2 hours with *E. coli* in PBST (at different concentrations; $100 \mu\text{L}$ per well), washed with PBST ($\times 3$), incubated with Cy3-labelled anti-*E. coli* antibodies ($3 \mu\text{g/mL}$) for 1 hour, and then re-washed with PBST ($\times 3$) and Milli-Q water ($\times 2$). Finally, the slides were unmasked, and then dried by centrifugation ($1500 \text{ rpm} \times 1 \text{ min}$). The slides were scanned again (excitation: 543 nm; emission filter: 570 nm). The fluorescence intensity of the arrays was quantified using GenePix software (Molecular Devices; California, USA). The fluorescence intensity values were estimated by first measuring the mean intensities of the assayed spots, and then subtracting the local background. The intensity values were normalized by dividing each one by the maximum value of the respective screening. The limit of detection (LOD) of the respective monitoring was taken as the sum of the mean intensity among spots incubated with a blank, and three times the standard deviation of the mean ($mB + 3\delta_B$).

SEM imaging. Aminosilane silicon slides were subjected to the assay steps described above, and then analyzed by scanning electron microscopy (SEM) in Merlin FE-SEM (Zeiss, Oberkochen, Germany) and Magellan 400L SEM (FEI, Oregon, USA) microscopes.

Results

The performance of the GO platelets as FRET acceptors of photons donated

by the Ab-QDs in solid phase was ascertained. Ab-QD complexes were printed in microspots of ca. $140 \pm 10 \mu\text{m}$, and then quenching of their fluorescence by GO at different concentrations (from 50 to 350 $\mu\text{g/mL}$) was assayed. It was observed that an incubation time of 75 minutes gave a quenching efficiency of $\sim 96\%$. Blank incubations exhibited a quenching efficiency of $\sim 20\%$; this effect was likely due to microenvironment changes in the assay (e.g. changes in pH, humidity, ionic strength, etc. due to the washing and drying steps).

In the assayed system, the hexagonal carbon rings in GO likely interact (non-covalently) with the aromatic amino acids in the antibody used. Moreover, due to its oxygen-containing functional groups, GO can interact noncovalently with the diol, amino and phenyl groups in biomolecules. In fact, Π - Π stacking interactions between GO and nanoparticle-antibody complexes has been reported in colloidal systems (liquid phase). In the present system (solid phase), strong interactions between the spotted Ab-QD probes and the GO were observed, which were characterized by Scanning Electron Microscopy (SEM). FIG 2a shows a SEM image of Ab-QD complexes inside a microspot, and FIG. 2b, a SEM image of Ab-QDs/GO complexes inside a microspot.

Ab-QD probes were again printed in microspots of ca. $140 \pm 10 \mu\text{m}$, and then monitored their interaction with *E. coli* O157:H7 as target pathogen, at different concentrations of *E. coli* (from 5 to 10^7 CFU/mL), as described in the materials and methods section. Owing to its high density of functional groups, GO can interact with bacteria, resulting in bacterial cell deposition. The pathogen samples were assayed in phosphate-buffered saline (PBS) supplemented with Tween 20 (0.05 %, v/v). The Ab-QD spots were incubated with GO, and subsequently became coated with GO—likely due to electrostatic/ Π - Π stacking interactions. FIG. 2c shows SEM images of *E. coli* cells (and cell fragments) that were pre-attached (via their membranes) to Ab-QD probes, and then covered with GO platelets. It was observed that under the assayed conditions only a small amount of analyte (around 5 CFU/mL in the case of a bacterial pathogen such as *E. coli*) below a light/disperse coating is sufficient for preventing the energy transfer between the fluorescent spots and the GO platelets.

At the assayed conditions (i.e. incubation at GO concentration of 70 $\mu\text{g/mL}$

during of 75 minutes), the system exhibited a sharp transition at an *E. coli* concentration of ca. 10 CFU/mL and became saturated at an *E. coli* concentration of ca. 10^7 CFU/mL (see FIGs. 3 and 5). However, in the *E. coli* concentration range of 10^2 to 10^6 CFU/mL, the probes were scarcely quenched relative to the control signal (i.e. the probes only, incubated with blanks throughout the assay) (see FIG. 3 and FIG. 5). As a threshold value for the limit of detection (LOD), the following was proposed: the mean of the blank signal, plus three times the standard deviation of the blank signal ($mB + 3\delta_B$).

To explore the behavior of our system in a matrix other than PBS, the target pathogen was analysed at several concentrations (from 3 to 10^7 CFU/mL) in tap water samples. Interestingly, the system exhibited different quenching levels when PBS was used as matrix. This was probably due to the influence of changes in the microenvironment, which typically affects the fluorescence quantum yield and fluorescence decay behavior of QDs. Important aspects of the microenvironment include the polarity and hydrogen bonding capability of the matrix, and the local viscosity, pH and ionic strength. The influence of the matrix was evidenced in the blank signal: the quenching in tap water was ~ 0.33 units, and in PBS, ~ 0.46 units. Later, in the presence of the target pathogen (*E. coli*) the quenching in tap water was typically ~ 0.7 units, and in PBS, ~ 0.8 units (see FIG. 3). However, despite the effects of any microenvironmental change, our system still enabled novel, qualitative pathogen detection in both matrices.

In order to compare the method of the invention with an immunosandwich assay, Cy3-labeled-antibodies, rather than GO, was used as the pathogen-revealing agent. In the configuration according to the invention (i.e. using GO), the system gives a digital-like response: OFF indicates the absence of the pathogen (*E. coli*); and ON, the presence of it (FIG. 5). Thus, the system exhibited a sharp transition zone from the OFF state to the ON state at very low concentrations of *E. coli* (~ 10 CFU/mL). Since we observed a similar response in the presence of *E. coli* regardless of its concentration (from ca. 100 to 10^7 CFU/mL), we considered this configuration to be qualitative (FIG. 3). Interestingly, in the immunosandwich configuration (i.e. using Cy3-labelled antibodies instead of GO), the system gives an analog-like response: the signal (Cy3 fluorescence) gradually strengthening in function of increasing

pathogen concentration, until the system becomes saturated. However, this configuration did not give a clearly observable (strong) signal in the *E. coli* concentration range of 10 CFU/mL to 100 CFU/mL (FIG. 5). In this context, the proposed GO configuration can reveal the bound analytes that are barely
5 observable in the immunosandwich configuration, thanks to the sensitive FRET phenomenon that the former is exploiting. The LOD of the immunosandwich configuration is $\sim 3.8 \times 10^3$ CFU/mL; thus, the GO configuration is advantageous in terms of sensitivity. Altogether, it is shown that the method of the invention significantly out-performs alternative assays
10 for pathogenic bacterial detection.

Qualitative sensors might prove highly utile for diagnosis and other analytical applications. When used with GO, the pathogen- detection system according to the invention is highly sensitive, exhibiting an LOD of ~ 5 CFU/mL for *E. coli*
15 in PBS and in tap water. This configuration might be extended to detect other types of analyte (e.g. cancer cells), perform other tasks (e.g. molecular logic operations) or be applied to other nano-biosystems.

2. Detection of analytes with spacer particle.

20

2.1. Option 1.

Materials and methods

25 **Reagents:** First antibody (Ab1) (biotinylated antibody), working solution diluted in immunobuffer. Second antibody (Ab2), working solutions diluted in immunobuffer. Analyte (respective antigen), working solutions prepared in immunobuffer. NH₂-functionalized Magnetic Silica Beads (MSB) (diameter of 1000/600 nm, Mobitec GmbH). NH₂-functionalized aminosilane glass slides
30 (Cat No. 40006 GAPS II, Corning Incorporated, New York, USA). Bovine serum albumin fraction V (BSA) (Sigma 85040C). Tween-20. (i.e. Sigma P2287). PBS (P4417, Sigma). PBST (PBS + Tween 20 at 0,05%). Immuno-Buffer: PBS + BSA fraction V (1%, w/v) + Tween 20 (0,5 % v/v). Blocking buffer: PBS + Milk powder (5%, w/v) + Tween 20 (0,005% v/v). Spotting
35 buffer: PBS + glycerol (2%, v/v). Gluteraldehyde (G5882, Sigma Aldrich) at 7 %. Graphene oxide (GO), C/O ratio around 1, lateral size around 500 nm, monolayer (Angstrom Materials, Ohio, USA). Streptavidin–Quantum dot 655

(Cat No. Q10121MP) were obtained from Life Technologies (California, USA)

Instrumentation: Biomagnetic processing platform (i.e. Dynal MPC-S®).
ScanArray 4000 microarray scanner (Perkin Elmer; Massachusetts, USA).

5

Functionalization of QD with Ab1: Mix in an Eppendorf tube: 1.6 μL of QD from a stock solution of $[\text{QD}] = 1 \mu\text{M}$, 18.4 μL of Ab1 diluted in spotting buffer (the Ab1 concentration has to be previously optimized, e.g. by spotting just the Ab1, performing a complete sandwich immunocomplex with a constant concentration of the analyte, reporting such immunocomplex using a fluorophore and choosing the spots with the best signal to noise ratio as the optimal Ab1 concentration) Ab1-QD conjugates are formed in this step.

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MSB pre-treatment: 1- Add 150 μL of MSB (stock solution $[\text{MSB}] = 1 \text{ mg/mL}$) into an Eppendorf tube (of 0.5 mL), extract beads using a magnetic field and wash twice with 150 μL of PBST and re-suspend in 150 μL of Gluteraldehyde at 7 %. 2- Shake for 30 min at 650 rpm (at room temperature). 3- Extract beads using a magnetic field and wash twice with 150 μL PBST. 5- extract beads and wash once with 150 μL PBS.

20

Functionalization of MSB with Ab2: 1- Extract pre-treated beads obtained above in PBS and re-suspend in 150 μL of Ab2 (e.g. at $[\text{Ab2}] = 100 \mu\text{g/mL}$) in PBS. 2- Shake for 30 min at 650 rpm (at room temperature). 3- Extract the formed Ab decorated MSB from the incubation solution by using a magnetic field and wash twice with 150 μL of PBST + once with PBS buffer. 4- Re-suspend the Ab2-MSB in 150 μL of blocking buffer and shake for 30 min at 650 rpm (at room temperature). 5- Wash 5 times with 150 μL PBST. 6- Extract beads and resuspend in 150 μL of immunobuffer. 7- Extract and re-suspend the antigen solutions in 150 μL of Immunobuffer. 8- Dilute 1:8 in immunobuffer. Ab2-MSB conjugates are obtained in immunobuffer.

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Analyte (respective antigen) detection using an immunosandwich

configuration with spacer particle: the detection of the analyte (antigen) is performed on a solid support (NH₂-functionalized aminosilane glass slides), following a procedure similar to that described in example 1 but incorporating the extra step of adding antibody-decorated MSBs as spacer particles. The procedure is summarised in the following table:

35

Table 1. Procedure for detection of analyte when using spacer particle, option 1.

Step	Reagents	Buffer	Conditions	Time
1. attachment to slide	20 μ l/ well of Ab1-QD	Spotting Buffer	4°C desiccated environment	Overnight
2. Wash	150 μ l/ well	PBST	RT	3 times
3. Block	100 μ l/ well	Blocking buffer	RT Agitation: 300 rpm	30 min
4. Wash	150 μ l/well	PBST	RT	5 times
5. Analyte incubation	100 μ l/ well of antigen (at different concentrations) or blank	Immuno-Buffer	RT Agitation: 300 rpm	2 hr
6. Wash	150 μ l/well	PBST	RT	3 times
7. Add spacer	100 μ l/ well Ab2-MSB	Immuno-Buffer	RT Agitation: 300 rpm	1 hr
8. Wash	150 μ l/well 150 μ l/well	PBST Milli-Q	RT	3 times 2 times
9. Dry			centrifugation (350 rpm)	
10. Scan			excitation: 633 nm emission: 670 nm	
11. Revealing agent	GO, 100 μ l/ well	Milli-Q	RT	75 min
12. Wash	150 μ l/well	Milli-Q	RT	3 times
13. Dry			centrifugation (350 rpm)	
14. Scan			excitation: 633 nm emission: 670 nm	
15. Measure	Estimate the photoluminescent intensity of the explored spots with GenePix software			

5 RT: room temperature

In the above protocol the analyte is first incubated with the antibody-decorated QDs that are attached to a solid support and then contacted with the spacer particle. However, the same results may be achieved by first incubating the analyte with the spacer and then contacting the analyte-spacer conjugate with the antibody-decorated QDs that are attached to a solid

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support. The latter option is further described below.

2.2. Option 2

- 5 Reagents, instrumentation, functionalization of QD with Ab1 and MSB pre-treatment are the same as above. Functionalization of MSB with Ab2 is also the same up to step 7. Differently from the procedure above, however, the Ab2-MSB conjugates are not diluted. Instead, they are extracted by means of a magnetic field and added to 150 μ L of the analyte (antigen) working solutions (at different concentrations) or blank solution (only immunobuffer).
 10 The mixtures are shaken for 30 min, 650 rpm, room temperature, forming by this way the immunocomplex Analyte/Ab2-MSB (Antigen/Ab2-MSB). The immunocomplex is washed twice with 150 μ L PBST, extracted and re-suspended in 100 μ L of Immunobuffer. This immunocomplex is used for the
 15 assay as described in table 2:

Table 2. Procedure for detection of analyte when using spacer particle, option 2.

Step	Reagents	Buffer	Conditions	Time
1. attachment to slide	20 μ L/ well of QD-Ab1	Spotting Buffer	4°C desiccated environment	Overnight
2. Wash	150 μ L/ well	PBST	RT	3 times
3. Block	100 μ L/ well	Blocking buffer	RT Agitation: 300 rpm	30 min
4. Wash	150 μ L/well	PBST	RT	5 times
5. Analyte incubation (with spacer)	100 μ L/ well of Antigen/Ab2-MSB or blank/Ab2-MSB	Immuno-Buffer	RT Agitation: 300 rpm	2 hr
6. Wash	150 μ L/well	PBST	RT	3 times
7. Wash	150 μ L/well 150 μ L/well	PBST Milli-Q	RT	3 times 2 times
8. Dry			centrifugation (350 rpm)	
9. Scan			excitation: 633 nm emission: 670 nm	
10. Revealing agent	GO, 100 μ L/ well	Milli-Q	RT	75 min
11. Wash	150 μ L/well	Milli-Q	RT	3 times

12. Dry			centrifugation (350 rpm)	
13. Scan			excitation: 633 nm emission: 670 nm	
14. Measure	Estimate the photoluminescent intensity of the explored spots with GenePix software			

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CLAUSES

1. A method for detecting an analyte of size comprised from 20 to 10000 nm in a mixture, which comprises:

5 (i) contacting the analyte with a quantum dot that is conjugated to at least one binding molecule, wherein the binding molecule has affinity for the analyte,

(ii) determining the fluorescence emission intensity of the mixture of step (i) upon exposing the mixture to an electromagnetic radiation capable of exciting the quantum dot,

(iii) adding graphene oxide to the mixture of step (ii), and

(iv) determining the fluorescence emission intensity of the mixture of step (iii) upon exposing the mixture to the same electromagnetic radiation used in step (ii),

wherein a reduction in fluorescence emission intensity of the mixture of step (iii) compared with that of step (i) is indicative of the absence of the analyte.

2. The method according to clause 1, wherein the fluorescent emission wavelength of the QDs is comprised from 400 to 750 nm.

3. The method according to any of the clauses 1-2, wherein the electromagnetic radiation capable of exciting the quantum dot has a wavelength comprised from 360 to 725 nm.

4. The method according to any of the clauses 1-3, wherein the QDs are selected from the group consisting of carbon, graphene, CdSe, CdS, CdSe coated with ZnS (CdSe/ZnS) and CdS coated with ZnS (CdS/ZnS).

5. The method according to any of the clauses 1-4, wherein the graphene oxide of step (iii):

(a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 2,
(b) contains a lateral size comprised from 5 to 1000 nm,
(c) contains from 1 to 20 layers, and wherein

(d) the final concentration of graphene oxide in the mixture is comprised from 0.01 to 400 µg /mL.

5 6. The method according to any of the clauses 1-5, wherein the quantum dot is attached to a solid support.

7. The method according to clause 6, wherein the solid support is selected from the group consisting of glass, plastic, nitrocellulose and paper.

10 8. The method according to any of the clauses 6-7, wherein the solid support has microarray format.

9. The method according to any of the clauses 6-7, wherein the solid support comprises a nitrocellulose membrane and has lateral flow format.

15 10. The method according to any of the clauses 1-9, wherein the binding molecule is an antibody or an aptamer.

20 11. The method according to ny of the clauses 1-10, wherein the binding molecule is biotinylated and the QD is streptavidin-covered.

12. The method according to any of the clauses 1-11, wherein the analyte is selected from the group consisting of a prokaryotic cell, an eukaryotic cell and a virus.

25 13. The method according to clause 12, wherein the analyte is a bacteria.

14. The method according to clause 13, wherein the analyte is *Escherichia coli* and the binding molecule is an *E. coli* -specific antibody.

30 15. A kit for carrying out a method according to any of the clauses 1-14, which comprises adequate means and instructions for carrying out the method, wherein the means comprise at least QD-binding molecule probes and graphene oxide, wherein said graphene oxide:

35 (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 2,
(b) contains a lateral size comprised from 5 to 1000 nm, and
(c) contains from 1 to 20 layers.

CLAIMS

1. A method for detecting an analyte of size comprised from 1 to 10000 nm in a mixture, the method comprising:

5

(i) contacting the analyte with

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a) a quantum dot that is attached to a solid support and conjugated to at least one first binding molecule, wherein the first binding molecule has affinity for the analyte, and

b) optionally a spacer particle that is conjugated to at least one second binding molecule, wherein the second binding molecule has affinity for the analyte and may be identical or different to the first binding molecule, and wherein the spacer particle has a size

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wherein when step b) is performed steps a) and b) are performed in any order;

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(ii) determining the fluorescence emission intensity of the mixture of step (i) upon exposing the mixture to an electromagnetic radiation capable of exciting the quantum dot,

(iii) adding graphene oxide to the mixture of step (ii), and

25

(iv) determining the fluorescence emission intensity of the mixture of step (iii) upon exposing the mixture to the same electromagnetic radiation used in step (ii),

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wherein a reduction in fluorescence emission intensity of the mixture of step (iv) compared with that of step (i) is indicative of the absence of the analyte;

with the proviso that when the analyte has a size comprised from 1 to 19 nm, step (i) b) must be performed.

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2. The method according to claim 1, wherein the analyte has a size comprised from 20 to 10000 nm

3. The method according to claim 2 that is performed in the absence of a spacer particle.
- 5 4. The method according to claim 1, wherein the analyte has a size comprised from 1 to 19 nm and the method is performed in the presence of a spacer particle.
- 10 5. The method according to any of the claims 1-4, wherein the fluorescent emission wavelength of the QDs is comprised from 400 to 750 nm.
- 15 6. The method according to any of the claims 1-5, wherein the electromagnetic radiation capable of exciting the quantum dot has a wavelength comprised from 360 to 725 nm.
- 20 7. The method according to any of the claims 1-6, wherein the QDs are selected from the group consisting of carbon, graphene, CdSe, CdS, CdSe coated with ZnS (CdSe/ZnS) and CdS coated with ZnS (CdS/ZnS).
- 25 8. The method according to any of the claims 1-7, wherein the graphene oxide of step (iii):
- (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 5,
(b) contains a lateral size comprised from 5 to 1000 nm,
(c) contains from 1 to 10 layers, and wherein
(d) the final concentration of graphene oxide in the mixture is comprised from 0.01 to 400 µg /mL.
- 30 9. The method according to any of the claims 1-8, wherein the solid support is selected from the group consisting of glass, plastic, nitrocellulose and paper.
10. The method according to any of the claims 1-9, wherein the solid support has microarray format.
- 35 11. The method according to any of the claims 9-10, wherein the solid support comprises a nitrocellulose membrane and has lateral flow format.

12. The method according to any of the claims 1-11, wherein the binding molecules are antibodies or aptamers.
13. The method according to any of the claims 1-12, wherein the binding molecules are biotinylated and the QD is streptavidin-covered.
14. The method according to any of the claims 1-2 and 4-15, wherein the spacer particle, when present, is a silica bead.
15. The method according to any of the claims 1-14, wherein the analyte is selected from the group consisting of a protein, a prokaryotic cell, an eukaryotic cell and a virus.
16. The method according to claim 15, wherein the analyte is a bacteria.
17. The method according to claim 16, wherein the analyte is *Escherichia coli* and the binding molecule is an *E. coli*-specific antibody.
18. The method according to claim 15, wherein the analyte is a protein.
19. A kit for carrying out a method according to any of the claims 1-18, which comprises adequate means and instructions for carrying out the method, wherein the means comprise at least QD-binding molecule probes, a solid support and graphene oxide, wherein said graphene oxide:
- (a) contains an atomic carbon to oxygen ratio (C/O) comprised from 1 to 5,
 - (b) contains a lateral size comprised from 5 to 1000 nm, and
 - (c) contains from 1 to 20 layers.

1/5

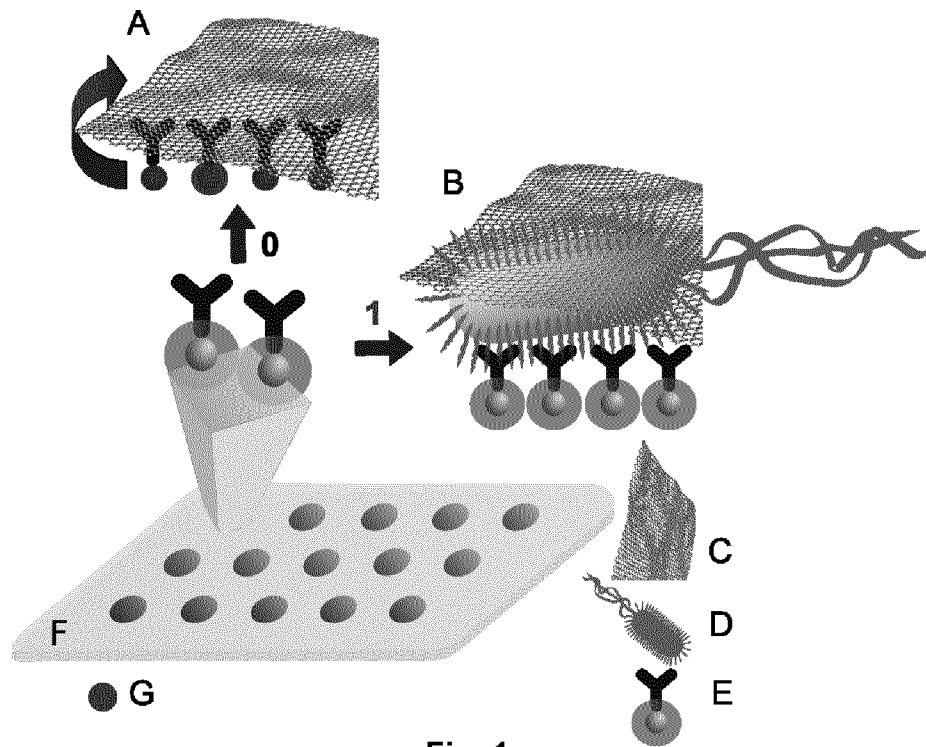


Fig. 1

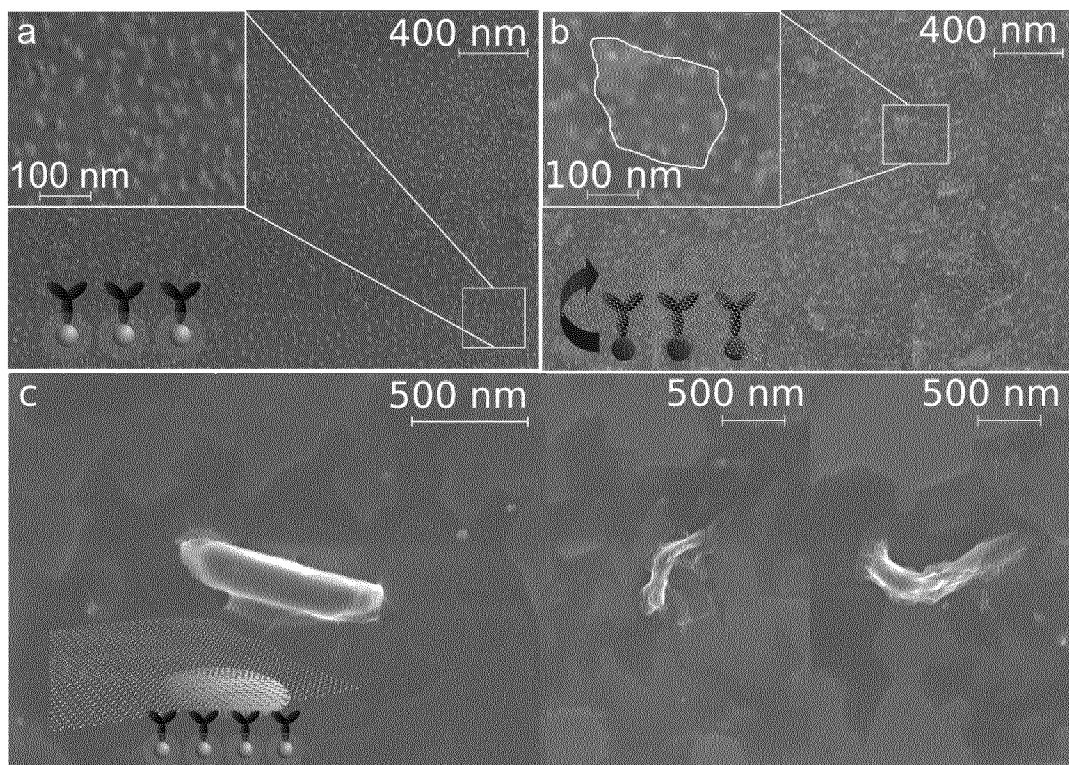


Fig. 2

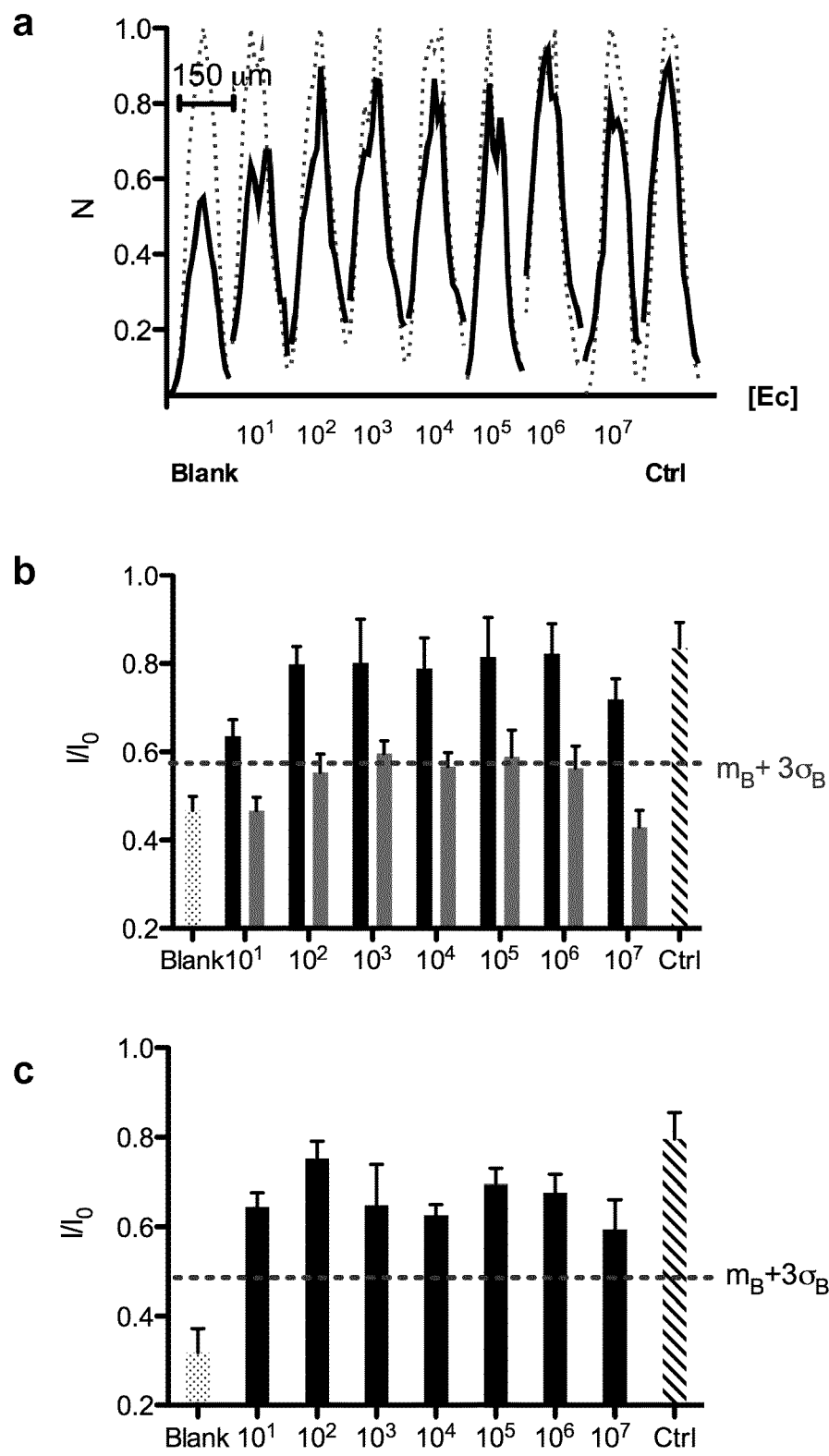


Fig. 3

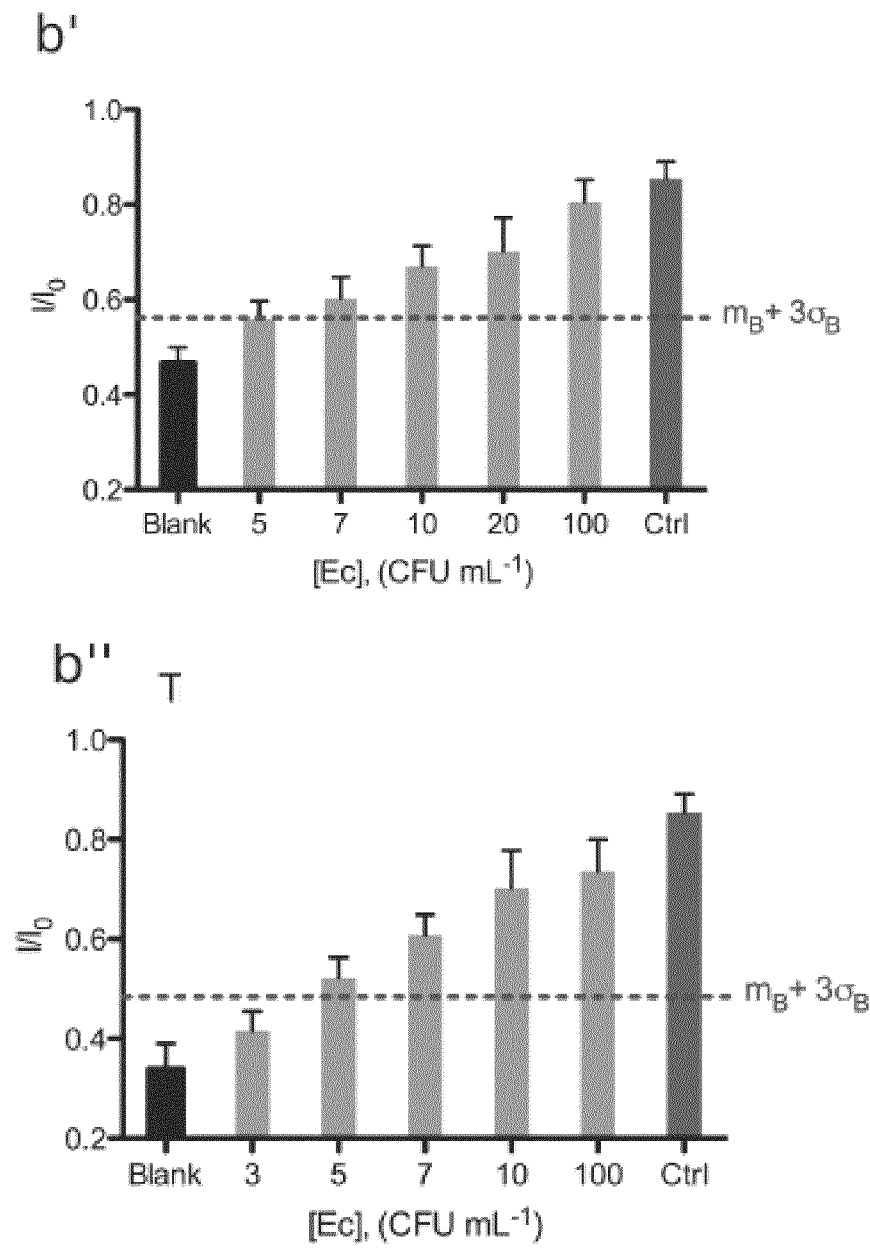


Fig. 4

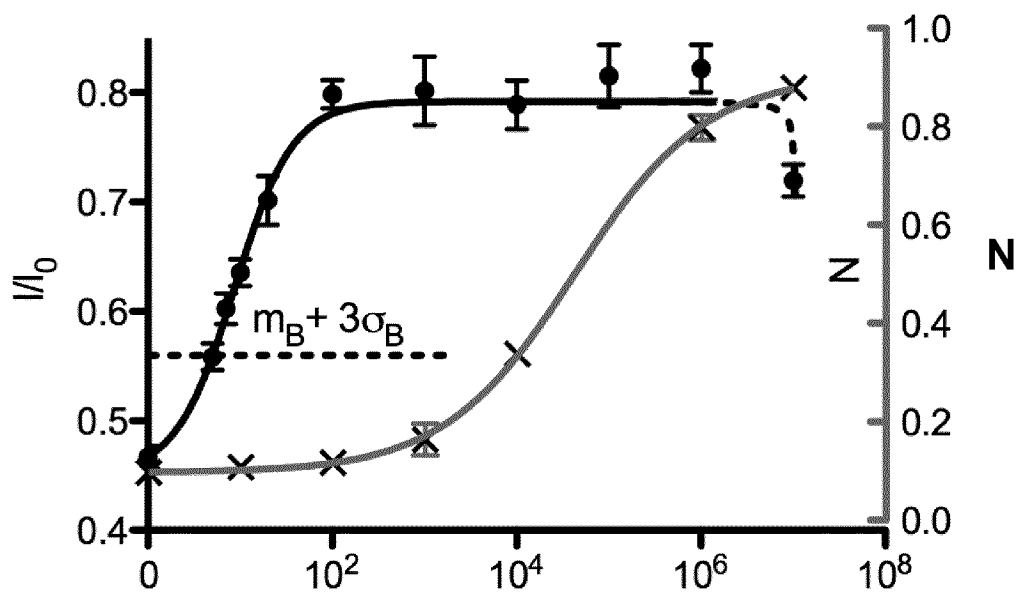


Fig. 5

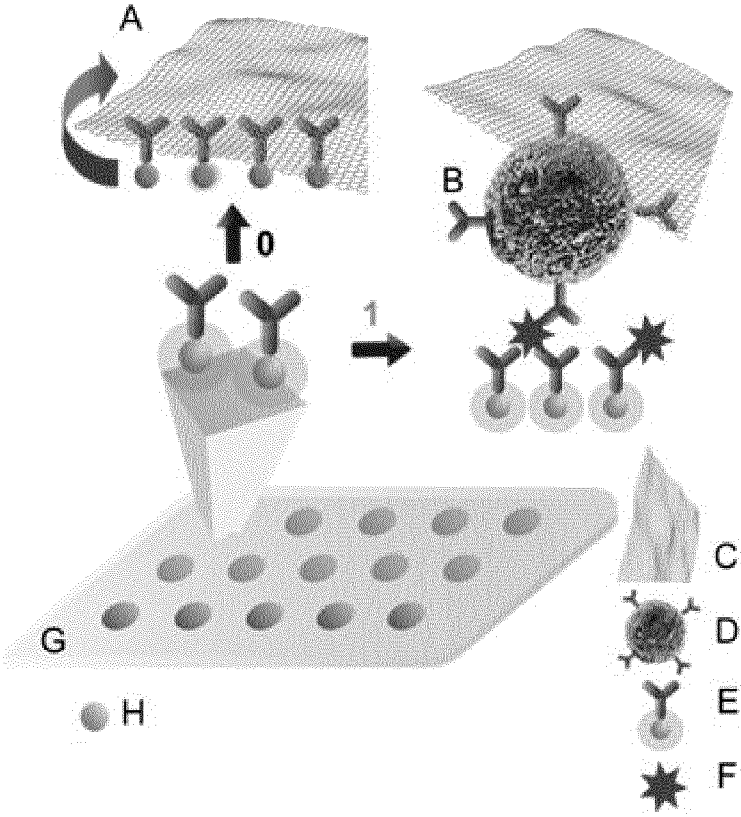


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/072100

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/569 G01N33/58

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 practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	MORALES-NARVAEZ ET AL: "Graphene oxide as a pathogen revealing agent: Sensing with a digital-like response.", ANGEWANDTE CHEMIE, vol. 52, 16 December 2013 (2013-12-16), pages 13779-13783, XP002720312, DOI: 10.1002/anie.201307740 the whole document	1-19
A	----- CN 102 608 090 B (UNIV WUHAN) 9 October 2013 (2013-10-09) the whole document ----- -/--	1-19

☒ 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

8 December 2014

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16/12/2014

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/072100

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DONG ET AL: "Fluorescence resonance energy transfer between quantum dots and graphene oxide for sensing biomolecules", ANALYTICAL CHEMISTRY, vol. 82, 1 July 2010 (2010-07-01), pages 5511-5517, XP002720313, abstract Scheme 1 page 5517 -----	1-19
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/072100

Patent document
cited in search report

Publication
date

Patent family
member(s)

Publication
date

CN 102608090 B 09-10-2013 NONE
