

(43) International Publication Date
10 December 2015 (10.12.2015)

(51) International Patent Classification:

C12Q 1/06 (2006.01) *C12Q 1/28* (2006.01)
C12Q 1/26 (2006.01) *G01N 21/23* (2006.01)

(21) International Application Number:

PCT/EP2015/062151

(22) International Filing Date:

1 June 2015 (01.06.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

14170769.5 2 June 2014 (02.06.2014) EP

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strasse 473, CH-8050 Zurich (CH).(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
 BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
 DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
 HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
 KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
 MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
 PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
 SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
 TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

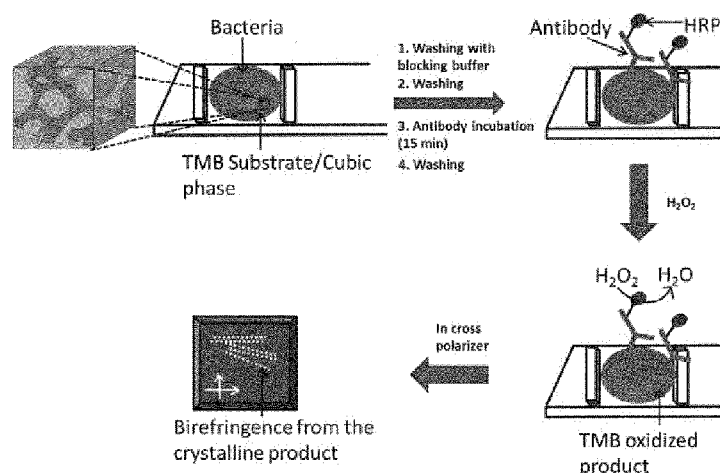
(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
 GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
 TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
 TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
 DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
 LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
 SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
 GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: LIQUID CRYSTAL BIOSENSORS

Fig. 5:



(57) Abstract: The invention relates to a method for visually detecting a reaction from a reactant to a product, and comprises the steps of providing a liquid crystal in bicontinuous cubic mesophase comprising said reactant, further providing conditions in which the reactant is converted into the product in a reaction step, wherein said product is insoluble in the mesophase, and measuring a bi-refrindex value of said mesophase. The invention is particularly suitable for detecting enzymatic conversions of analytes into products. E.g. glucose or cholesterol conversions by means of oxidase/peroxidase systems. The invention further relates to an apparatus (10) for use in a method of the invention, which comprises a volume (11) delimited by transparent optical elements (12) and (13) that are orthogonal to an axis (15) passing through said volume (11), and wherein the axis of polarization (16) and (17) of the optical elements is rotated by 90°, and bicontinuous cubic mesophase is present in said volume.

Liquid crystal biosensors

Description

The diagnostic industry spends every year significant resources on the detection of analytes in biological samples. Conventional detection methods are time consuming, require sophisticated instruments and suitably trained personnel. Enzyme-linked immunosorbent assays (ELISAs) are possibly the most widely used assays due to their relative simplicity, although they typically require several hours for the detection process. ELISA is an example of an assay based on the conversion of a substrate to a product, which is of vital importance in the detection of pathological microorganisms, catalytic antibodies, and screening libraries of potential drugs and inhibitors.

Biosensors generally use a biological receptor compound such as an enzyme, nucleic acid or antibody to produce a physical or physico-chemical change in the environment, which can be used to detect the analytes. They allow the detection of a broad spectrum of analytes in complex sample matrices, and have shown great promise in areas such as food and water analysis, medical diagnostics and bio-threat monitoring.

When mixed with water, lipid-based surfactants self-assemble into different liquid crystalline symmetries such as the lamellar phase, inverted hexagonal phase and the bicontinuous cubic phase.

These liquid crystalline mesophases have been used in controlled release and delivery systems and find diverse applications in food, cosmetics and pharmaceuticals. They also provide an excellent matrix for the entrapment of proteins and enzymes due to their biocompatibility, the presence of both hydrophilic and hydrophobic environments and their thermodynamic stability in excess water.

There is an urgent need for innovative techniques and methods for rapid detection of target analytes using simple devices and at low costs, especially for applications outside the laboratory environment.

The objective of the present invention is to provide a novel method of detecting analytes or microorganisms. This objective is obtained by the subject matter of the independent claims.

The present invention seeks to improve upon the prior art detection methods by providing a novel biosensing platform. This is based on the birefringence evolution from a reaction product within lipid-based lyotropic liquid crystal cubic phases.

Terms and definitions

As used herein, the following terms have the designated definitions, unless the context clearly indicates otherwise.

ABTS is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (CAS number 30931-67-0).

TMB is 3,3',5,5'-tetramethylbenzidine (CAS number: 54827-17-7).

OPD is o-phenylenediamine dihydrochloride (CAS number: 95-54-5).

5 In the context of the present specification, the term *crossed polarizer* is defined as two linear polarizers that are placed in parallel alignment, with their polarizing axes in orthogonal arrangement with respect to each other. The second polarizer is also referred to as analyzer.

In the context of the present specification, the term *chromogenic substrate* refers to a chemical substance capable of conversion into a pigment or dye.

10 In the context of the present specification, the term *lipid based surfactant* refers to an amphiphilic substance, wherein the hydrophobic part comprises a fatty acid and the hydrophilic part comprises glycerol.

Different aspects of the invention

According to a **first aspect of the invention** a method for visually detecting a reaction from a reactant to a product is provided, wherein the method comprises the steps of:

- 15 i. providing a bicontinuous cubic mesophase comprising the reactant and
- ii. providing conditions in which the reactant is (at least partially) converted into the product in a reaction step,

wherein the product is insoluble in the mesophase.

In a measurement step, a birefringence value of the mesophase is measured.

20 The term "insoluble" in the context of the present specification signifies that a compound designated insoluble is present, under conditions of thermodynamical equilibrium, to a significant degree as a solid rather than in solubilized form. While minor amounts of the predominantly solid compound may be present in the liquid phase, in the context of the invention the important aspect is that at least a significant part of the compound is not

25 solubilized.

Without wishing to be bound by theory, it is assumed that the change in birefringence accompanying the reaction is caused by deposition of the insoluble product into the mesophase.

30 In certain embodiments of any aspect of this invention the liquid crystal is in lamellar phase, inverted hexagonal phase or bicontinuous cubic mesophase.

According to a **second aspect of the invention** a method for detecting an analyte is provided, comprising the steps of:

- i. providing the analyte in a bicontinuous cubic mesophase,
- ii. enzymatically converting the analyte into a substrate in a first cascade step,
- iii. converting the substrate with a reactant into a product in a second cascade step,

5 wherein the product is insoluble in the mesophase.

In a measurement step, a birefringence value birefringence of the mesophase is measured. In certain embodiments, the birefringence of the mesophase is measured continuously.

In certain embodiments of any aspect of this invention, the lyotropic liquid crystal in bicontinuous cubic mesophase comprises water and a lipid-based surfactant or phospholipid.

10 In certain embodiments the lyotropic liquid crystal in bicontinuous cubic mesophase contains 10%, 15%, 20%, 25%, 30%, 35%, 40% or 45% of water. In certain embodiments, the lyotropic liquid crystal in bicontinuous cubic mesophase contains from 20% to 35% of water.

In certain embodiments of any aspect of the invention, the lipid-based surfactant is selected from monolinolein and mono-olein.

15 In certain embodiments, the lyotropic liquid crystal in bicontinuous cubic mesophase comprises monolinolein and contains from 20% to 35% of water.

In certain embodiments of any aspect of the invention, the lyotropic liquid crystal in bicontinuous cubic mesophase comprises water and phytantriol (CAS no. 74563-64-7). In some embodiments of any aspect of this invention, the reactant is selected from ABTS, TMB
20 and OPD. In certain embodiments the reactant is a chromogenic substrate characterized in that its horseradish peroxidase (HRP) reaction product has low solubility in the cubic mesophase.

In certain embodiments of any aspect of this invention the product is selected from 3,3',5,5'-tetramethylbenzidine diimine, 2,3-diaminophenazine and the horseradish peroxidase reaction
25 product of ABTS.

In certain embodiments of any aspect of this invention, the reaction step, the first cascade step, and/or the second cascade step is catalysed by an enzyme. In certain embodiments, the enzyme is immobilized or entrapped within the highly viscous mesophase.

In certain embodiments of any aspect of this invention, the enzyme is selected from a
30 peroxidase, glucose oxidase or a cholesterol oxidase.

In certain embodiments of any aspect of this invention, the peroxidase is linked to an antibody. In certain embodiments the peroxidase is horseradish peroxidase.

In certain embodiments of any aspect of this invention, the analyte is by non-limiting example glucose, cholesterol, a microorganism, particularly a prokaryotic microorganism, more particularly a bacterium, or a virus, particularly a retrovirus, more particularly HIV, and said substrate is hydrogen peroxide.

- 5 In certain embodiment of any aspect of this invention, the birefringence is measured during all steps of said method.

In a **third aspect of this invention** an apparatus 10 for use in a method according to the first and second aspect of the invention is provided. Figure 8 is a schematic constructional drawing of the apparatus 10 according to a certain embodiment. As shown the apparatus 10
10 includes a volume 11 that is delimited by a first 12 and a second transparent optical element 13. The first 12 and the second transparent optical element 13 are orthogonal to an axis 15 passing through the volume 11. The first transparent optical element 12 is characterized by a first axis of polarization 16. The second transparent optical element 13 is characterized by a second axis of polarization 17 rotated by 90° around the axis 15 in relation to the first axis of
15 polarization. This aspect of the invention is characterized in that the volume 11 comprises a bicontinuous cubic mesophase.

In other words the first and second transparent optical elements are linear polarizers that are placed in parallel alignment, with their polarizing axes in orthogonal arrangement with respect to each other. This arrangement constitutes crossed polarizers that block light
20 traversing parallel to axis 15. Any content of volume 11 that induces birefringence would allow some light to pass the second polarizer.

In certain embodiments the apparatus comprises a second axis of polarization that is rotated around axis 15 in relation to the first axis of polarization by an angle of 50°, 60°, 70°, 80°, 85°, 90°, 95°, 100°, 110°, 120° or 130°.

- 25 In certain embodiments the apparatus comprises a light source 18 being designed to emit visible light along axis 15.

With reference to figure 9, a diagram illustrates the apparatus 9 according to some embodiments. In this example the apparatus 10 includes a diffusor 19, a main 22 and a contact switch 24, a battery 21, a constant current supply 20, a light source 18 and a ventilator 23. The second transparent optical element 13 is integrated into a hinge operated
30 lid 25.

In certain embodiments the bicontinuous cubic mesophase in volume 11 comprises a reactant and a substrate that is converted with the reactant into an insoluble product (insoluble in the liquid phases present in the reaction) in a reaction step.

In certain embodiments the bicontinuous cubic mesophase in volume 11 comprises an analyte and an enzyme converting the analyte into a substrate in a first cascade step and a reactant that is converted with the substrate into an insoluble product (insoluble in the liquid phases present in the reaction) in a second cascade step.

- 5 In a certain embodiment the bicontinuous cubic mesophase comprises water and monolinolein.

In certain embodiments the reactant is selected from ABTS, TMB, and OPD.

In certain embodiments the product is selected from the horseradish peroxidase reaction product of ABTS, 3,3',5,5'-tetramethylbenzidine diimine and 2,3-diaminophenazine.

- 10 In certain embodiments the reaction step, the first cascade step, and/or the second cascade step is catalysed by a peroxidase, a glucose oxidase or a cholesterol oxidase.

In certain embodiments the peroxidase is linked to an antibody.

In certain embodiments the substrate is hydrogen peroxide and the analyte is glucose or cholesterol.

- 15 Wherever alternatives for single features such as, for example, a reactant or substrate are laid out herein as "embodiments", it is to be understood that such alternatives may be combined freely to form discrete embodiments of the invention disclosed herein.

The invention is further illustrated by the following examples and figures, from which further embodiments and advantages can be drawn.

- 20 Brief description of the figures:

Fig. 1: shows a cross-polarized optical microscopy images of the cubic phase before (a) and after 3h of enzymatic reaction using the substrate ABTS (b), TMB (c) and OPD (d). Scale bar corresponds to 50µm. Insets show the visual appearance of each sample.

- 25 Fig. 2: Enzymatic conversion of ABTS into a colored product undergoing crystallization within the cubic phase. (a) Typical kinetic evolution followed by UV-vis absorption. (b) Small and (c) wide angle X-ray scattering spectra of the cubic phase before and after the enzymatic reaction.

- 30 Fig. 3: Illustration of birefringence development in cubic phases immobilizing HRP. (a-j) POM images of the cubic phase taken every two minutes of the enzymatic reaction with 9.118 mM ABTS as substrate. Scale bar corresponds to 50 µm. (k) Plot of the normalized birefringence intensity versus time arising from the conversion of 9.118 mM ABTS. (l) Plot of the normalized birefringence intensity versus time at different ABTS substrate concentrations.

Fig. 4: Illustration of glucose and cholesterol biosensors based on birefringence detection. POM images of the cubic phase after 12min of enzymatic reaction at 37°C with (a) 3, (b) 4, (c) 5, (d) 6, (e) 7 and (f) 8mM glucose. Scale bar corresponds to 50µm and applies to all images. Insets show the visual appearance of each sample. Linear dependence of the birefringence intensity versus (g) glucose and (h) cholesterol concentration.

Fig. 5: Schematic representation of birefringent ELISA for pathogens detection.

Fig. 6: (a) Light microscopy image of immobilized *E.coli* in the cubic phase. Inset shows a fluorescence microscopy image. (b) POM image after the birefringent ELISA at 37°C. Inset shows a POM image of the control sample (no *E.coli*). (c) Photographs of the samples (top two images) and their visualization by the birefringent ELISA in the cross-polarized filter device (bottom two images) at 37°C. (d) Photograph of the portable birefringent ELISA device.

Fig. 7: Polarized optical microscopy image of inverted hexagonal phase before (left image) and after (right image) the enzymatic reaction.

Fig. 8: Schematic diagram of the device. A) Apparatus 10 includes a volume 11 that is delimited by a first 12 and a second transparent optical element 13. The first 12 and the second transparent optical element 13 are orthogonal to an axis 15 passing through the volume 11. B) The first transparent optical element 12 is characterized by a first axis of polarization 16. The second transparent optical element 13 is characterized by a second axis of polarization 17 rotated by 90° around the axis 15 in relation to the first axis of polarization.

Fig. 9: Schematic diagram of the device in accordance with certain embodiments.

Fig. 10: Top view of the device where the birefringence is visible.

Fig. 11: Proposed device for the quantification of analytes.

Fig. 12: Illustration of HIV detection. (a) POM image of p24 antigen contained VLP doped human plasma with in the cubic phase after birefringent ELISA, at 37 °C. Inset shows the POM image of a control sample (cubic phase made of only human plasma). (b) POM image of cubic phase after heat treated at 100 °C for 5 min. Inset shows the POM image of heat treated sample after the birefringent ELISA, at 37 °C. (c) Photographs of samples (bottom images) with different p24 concentration in the plasma and their visualization in the cross-polarized device after birefringent ELISA (top images) with in the cubic phase at 37 °C. (d) Experimental normalized

birefringent intensity values with associated measured uncertainty range arising in the cubic phase at increasing p24 antigen concentration.

Fig. 13: Illustration of *P. falciparum* detection in blood samples. (a) Cross polarized optical microscopy image of a cubic mesophase produced using blood infected with *P. falciparum* parasites. The inset shows the negative control from a mesophase produced with uninfected blood. (b) Photograph of two reservoirs in a 96-well plate, visually observed through the cross-polarized device, containing: (top image) a cubic mesophase produced using infected blood and (bottom image) the control negative sample. (c) Photograph of two reservoirs in a 96-well plate, observed through the cross-polarized device, containing: (top image) a cubic mesophase produced using a buffer with extracted hemozoin and (bottom image) the control negative sample with uncontaminated buffer.

Examples

The concept of encapsulating the enzyme Horseradish peroxidase (HRP) within a bicontinuous cubic phase, while preserving its functionality, is demonstrated herein by the conversion of suitable substrates into colored products. The unique structure of the cubic phase with its two continuous water channels separated by a lipid bilayer, provides a unique platform for the immobilization of the enzyme in its active form, while allowing a fast diffusion of the substrate within the matrix. During the enzymatic reaction within the cubic phase, the converted product induces birefringence in the system due to its crystallization, providing an efficient way to monitor the real-time enzymatic reaction when observed between crossed polarizers. In a further additional step, bi-enzymatic cascade reactions within the cubic phase are performed and the birefringence development is followed to detect glucose and cholesterol in a wide range of concentrations. Finally, it is shown how this novel liquid crystal cubic phase platform based on birefringence detection, birefringent ELISA, can be used for the rapid detection of a model food pathogen, *E.coli.*, in less than one hour. To exemplify the ease of this approach, the design of a portable inexpensive device capable of detecting changes in birefringence intensity is given, enabling quantification of target analytes. This approach is general and readily adaptable to the rapid detection of a variety of analytes without associated complex procedures or equipment, and in such it can serve as an ideal toolbox for identification of pathogens in those contexts where detection time and simplicity have a crucial relevance.

Encapsulation of horse radish peroxidase

The inset of Figure 1a shows a picture of a bicontinuous cubic phase sample just at the beginning of the enzymatic reaction. Over time, the peroxidase enzymatic reaction in presence of H_2O_2 produced typical green (inset of Fig 1b), blue (inset of Fig 1c) and orange

(inset of Fig 1d) colored products within the cubic phase, when run separately with three different organic substrates: 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 3,3',5,5'-tetramethylbenzidine (TMB) and o-phenylenediamine dihydrochloride (OPD), respectively. This shows that immobilized HRP inside liquid crystalline cubic phases retains its activity and can catalyze specific color changes, depending on the appropriate substrate. When analyzed with a cross-polarized optical microscopy (POM), no birefringence was observed at the beginning of the enzymatic reaction (Fig 1a), as expected from the isotropic nature of the cubic phase, whereas a significant amount of birefringence developed after 3h of enzymatic reaction (Fig 1b-1d).

A series of experiments lead us to the confirmation that this birefringence stems from the simultaneous crystallization of the converted, oxidized organic product during the enzymatic reaction in the cubic phase. A typical UV-vis spectrum, taken during the peroxidase enzymatic reaction using ABTS as substrate (Fig 2a), identifies the initial steady-state region of the reaction followed by the characteristic plateau region, which further confirms the enzymatic reaction within the liquid crystalline cubic phase. The effect of the enzymatic reaction on the cubic phase nanostructure can be analyzed using the small angle X-ray scattering (SAXS). The SAXS spectra of the cubic phase before and after the enzymatic reaction (Fig 2b) show that the cubic phase remains essentially unchanged throughout the enzymatic reaction and confirm that the evolving birefringence does not arise from changes in the mesophase. The slightly increased lattice parameter of the cubic phase after the enzymatic reaction (from 12.427 nm to 13.005 nm) may be due to the additional H₂O molecules formed from the conversion of H₂O₂ during the enzymatic reaction, or from the simultaneous decrease in the molecules of substrate dissolved in the water phase, caused by their simultaneous crystallization. After the enzymatic reaction, the sharp peak in the wide angle X-ray (WAXS) spectra at 1.42 Å⁻¹ (Fig 2c) confirms the crystalline nature of the converted ABTS⁺ substrate. An additional control enzymatic reaction run on a cubic phase made of an alternative lipid (Phytantriol), resistant to hydrolysis due to the lack of ester bonds, shows the same evolution of birefringence (data not shown) conclusively confirming that the birefringence arises from the crystallization of the ABTS⁺ converted substrate. Similarly, several alternative control experiments in which either HRP, H₂O₂ or the substrate were missing in the cubic phase to prevent enzymatic reactions, produced no birefringence.

The evolution of birefringence arising from the oxidized ABTS product allows monitoring the enzymatic reaction in real-time by simple observation of the cubic phase under POM (Fig 3a-j). Birefringence started to develop over the sequence of the reaction, increased with time and then slowly reached saturation. The normalized birefringence intensity with time follows the typical enzyme kinetics curve with an initial steady-state region and a characteristic plateau region, in direct analogy with the evolution of UV-vis spectra (Fig 3k

and 2a). The observable lag time during the early stage of the enzymatic reaction, especially at the lowest concentrations of the substrate, is the result of the reduced relative diffusion rate of the substrate and the enzyme in the highly viscous cubic phases, where diffusion is highly hindered and slowed down compared to bulk solutions. Enzymatic reaction rates, and corresponding evolution of birefringence, grow with increasing substrate concentration (Fig 3l), again in close agreement with the UV-vis spectra (data not shown). Thus, an accurate real-time monitoring of the enzymatic activity can be achieved by following the birefringence response (Fig 3k and l). This phenomenon bears similarities with the fluorescence development during enzymatic reactions using appropriate substrates although the latter process requires specific fluorescent probes and given excitation wavelength to be followed whereas birefringence development within cubic phases can be easily followed using cross-polarizers and shows a high degree of generality, being common to substrates such as ABTS and TMB. The same enzymatic reaction with identical concentrations of HRP, H₂O₂ and ABTS was performed in bulk free water where no development of birefringence is detected. This clearly points out the unique nature of the bicontinuous cubic phase in which the confined environment of the hydrophilic/hydrophobic domains triggers the crystallization of the converted product in the cubic phase, which in turn produce the birefringence.

Glucose biosensor

There is a considerable interest on efficient measurement of glucose concentration in the medical industry. For example, close control of glucose concentration in the bloodstream of diabetes mellitus patients is a necessary requirement to avoid severe and fatal long-term health effects. As a consequence, there is a significant ongoing worldwide effort aiming at the development of new non-invasive glucose monitoring methods, and a number of methods have been proposed such as polarimetry, amperometric response and fluorescent probes. By following the same strategy discussed above, but using a bi-enzymatic cascade reaction, the development of birefringence can be followed to detect and quantify glucose concentration. To this end the two enzymes Glucose oxidase (GOD) and HRP were immobilized in the cubic phase. The GOD catalyzes the conversion of β -D-glucose and oxygen to D-glucono-1,5-lactone and hydrogen peroxide. The hydrogen peroxide arising from the first reaction then oxidizes ABTS in the presence of HRP, which in turn results in the formation of birefringence (Fig 4a-f). As can be seen in fig 4g, the overall birefringence intensity generated by the oxidized product after 12 min reaction at 37 °C, shows a linear increase with glucose concentration in the range 3-8 mM.

Cholesterol biosensor

The detection of birefringence from an enzymatic cascade reaction is general enough to be scalable and adaptable to the detection of other molecules: Therefore its generality is

demonstrated by applying the same concept on another model analyte, this time purely hydrophobic in nature: cholesterol. The measurement of cholesterol is of great importance in clinical applications because an abnormal amount of cholesterol in blood can cause clinical disorders such as heart disease, hypertension, arteriosclerosis and coronary artery disease.

5 Cholesterol oxidase (ChOD) and HRP were used to perform the bi-enzymatic cascade reaction and the birefringence developed at 37 °C showed again a perfectly linear correlation with the cholesterol concentration in the range 10-30 mM, as shown in fig 4h. In contrast to the previous case, where glucose was solubilized in the water channels of the cubic phase, in this case cholesterol was solubilized in the hydrophobic part of the cubic phase. The possibility of simultaneous detection of glucose and cholesterol using the same platform, illustrates well how robust and general the present approach is, in which use of lipid-based lyotropic liquid crystals, conveniently provides dual hydrophilic and hydrophobic domains within the same matrix.

Detection of pathogens

15 Since HRP is used extensively in immunoassay applications, the herein disclosed method of following a reaction by means of changes in birefringence an ELISA protocol utilizing this method was established. In a proof of principle experiment the rapid detection of model food pathogens, exemplified here by the detection of *E.coli* was performed. A schematic representation of the birefringent ELISA method for pathogen detection is shown in fig 5. For this task the hydrophobic substrate TMB was used, instead of the hydrophilic substrate ABTS, to avoid release of substrate from the cubic phase during the washing procedure with buffer.

The rapid formation of the cubic phase at room temperature by mixing *E.coli* as suspension in water and the TMB-containing lipid, ensures immobilization of *E.coli* and the solubilization of the substrate TMB in the mesophase. Light and fluorescence microscopy (Fig 6a) images confirm that *E.coli* is homogeneously distributed in the mesophase whereas the absence of any aggregate in light microscopy confirms that the TMB is solubilized in the mesophase. A total weight of 200mg mesophase containing bacteria and TMB, were incubated with HRP-conjugated primary antibody solution, followed by rinsing with buffer to leave in the systems only the antibodies bound to *E.coli*. In the presence of H₂O₂, the antibody-bound HRP starts the enzymatic reaction, giving rise to color changes visible to the naked eye and simultaneously, the converted TMB product in the mesophase starts to crystallize, triggering the development of birefringence in the mesophase (Fig 6b). No color or birefringence changes were observed in the control experiments lacking bacteria (inset of Fig 6b). The rapid detection of food pathogens due to a color change (Fig 6c top images) by visual inspection is particularly appealing for the initial qualitative screening of deadly pathogens

and in resource-lacking environments, but remains limited to a qualitative assessment only. Therefore, a simple, portable device for the detection of pathological microorganisms based on birefringence was developed (Fig 6d): only in samples containing bacteria birefringence can be observed (Fig 6c bottom images). By adding a photodiode allowing a simple quantification of birefringence intensity and via suitable calibration curves, this simple device could become particularly attractive in the medical and food industry to quantitatively screen target analytes and pathogens.

These results show that the new birefringent ELISA technique based on liquid crystal biosensors can detect microorganisms in less than an hour. The successful and general outcome of this technology relies on a combination of some unique properties of lipid-based cubic mesophases: (i) Bacteria can be rapidly immobilized within the cubic phase due to the highly viscous nature of the liquid crystal matrix; (ii) The substrates for the enzymatic reaction can be solubilized in both the hydrophilic/hydrophobic domains of the cubic phase; (iii) The cubic mesophase is at thermodynamic equilibrium with excess water, allowing rinsing with target antibodies buffer solutions; (iv) The isotropic, colorless nature of the cubic phase allows easy monitoring of changes in color/birefringence occurring during enzymatic reaction; (v) Last, but possibly most importantly: the converted, oxidized substrate crystallizes producing birefringence within the cubic phase due to the confinement within the hydrophilic/hydrophobic nanodomains of the cubic phase.

In summary, several substrates were shown to produce birefringence during the peroxidase enzymatic reaction within lipid-based bicontinuous cubic phases. This birefringence development during the enzymatic reaction was used for the real-time monitoring of the enzymatic reaction of HRP. Further bi-enzymatic cascade reactions within the lipid mesophases were performed and the birefringence development followed to detect glucose and cholesterol in a wide range of concentrations. Additionally, peroxidase enzymatic reaction was combined with antibody-mediated molecular recognition to detect pathogenic microorganisms within less than an hour, again, based on the sole detection of birefringence. The methodology proposed here has the potential to be adapted to the detection of virtually any pathological microorganisms, making birefringent ELISA a versatile tool for the rapid, facile and inexpensive detection of analytes and pathogens in a broad range of biotechnological fields.

HIV Detection

The method of the invention can be also utilized for the detection of viruses. Detection of HIV is a representative case of immediate relevance. Rapid detection of p24 antigen can be exploited for the early diagnosis of HIV infection in a cost-effective way, which is of prime importance in poor regions where expensive nucleic acid-based tests cannot be afforded. To

this end, the current inventors designed a birefringent ELISA assay for the detection and quantification of model analyte HIV-1 capsid antigen p24 in human serum, in 1 h.

The aqueous part used to make bicontinuous cubic phase contains human plasma, virus-like particles (VLP) which contains the p24 antigen and lysis buffer. The hydrophobic substrate
5 TMB is solubilized in the lipid surfactant Dimodan at a concentration of 1,25 mg TMB per 100mg of surfactant, and then mixed with the aqueous part to form the cubic phase. This highly viscous cubic phase ensures the physical immobilization of the antigen p24 with in the mesophase and is then transferred into a glass slide with a neoprene spacer of 0.5 mm, which constitutes a microwell of 0.5 mm in height and 16 mm in diameter. This enables the
10 transfer of fixed amount of mesophase in the glass slide. Mouse monoclonal anti-p24 (Aalto Bio Reagents, BC 1071, dilution 1:200) is incubated for 25 min. and then goat anti-mouse IgG conjugated to HRP (KPL, 474-1806, dilution 1:200) is used as the secondary antibody. Introduction of H_2O_2 on the mesophase promoted the start of enzymatic reaction, yielding a simultaneous color change and birefringence (Fig. 12 a) with in the cubic phase. Absence of
15 birefringence in the POM in 5 different control experiments confirms the specificity of the detection (inset of Fig. 12 a).

In the case of clinical detection of HIV using p24 antigen, heat-treated plasma is used to increase the concentration of unbound p24. In HIV infected patients, usually the concentration of unbound p24 antigen is low whereas most of the remaining p24 antigens
20 are bound to the antibody already present in the blood. The concentration of unbound antigen can be drastically increased by heating the HIV infected plasma to 100°C for a short duration. Alternatively, the VLP containing cubic phase may be heated to 100° C for 5 min. in a closed vial and cooled to room temperature. Even though the heat-treated plasma in the bicontinuous cubic phase shows a slight background birefringence signal in the POM (Fig.
25 12 b), the birefringence developed after the ELISA method is significantly higher (inset of Fig. 12 b), which further illustrate the potential of this method in practical application of HIV detection.

This birefringence is dependent on the concentration of p24 antigen (Fig. 12 c) and therefore can be used for the quantification of the analyte using the cross-polarized filter device. The
30 measured birefringent intensity from each concentration (I) of p24 antigen and control sample (I₀) is calculated and the normalized intensity is used for the quantification (Fig. 12 d). The limit of detection in this case using birefringent ELISA method is 2.5 ng/ml. The detection of analytes using this cheap, portable device by imparting a new optical signal generation mechanism to conventional ELISA is anticipated to be of an unprecedented
35 simplicity for the rapid detection of HIV infection in resource-poor regions.

Detection of protozoan parasites

The device and method disclosed here can also be used for a label-free, naked-eye detection of *Plasmodium falciparum*, the devastating infectious pathogen causing malaria. The protozoan *Plasmodium* parasite invades erythrocytes and digests hemoglobin. The heme component, which is toxic to the parasites is crystallized in the form of a brown birefringent crystal. By detecting hemozoin directly, cross-polarized microscopy may be sufficient to diagnose malaria without any additional marker, although this methodology remains difficult to be implemented in the field, especially in malaria-endemic areas, which often are resource-limited.

The inventors demonstrate that in-meso detection of hemozoin crystals is accessible to naked-eye screening. The birefringence, similar to that observed in infected blood under cross-polarized microscopy, was also accessible in-meso, when cubic phases were formed using blood infected with *P. falciparum* as a hydrophilic component (Figure 13a). As shown by Figure 4b, an intense birefringent signal was visually observed in few microliters of infected blood reconstituted in-meso (Figure 13b top image) as compared to a control sample of uninfected blood treated in the same way (Figure 13b bottom image). The signal could be further enhanced by observing cubic mesophases produced directly with hemozoin suspensions extracted from *P. falciparum* cultures (Figure 13c top image: positive; lower image: negative control).

Methods

Enzymatic reaction in Cubic phase

The cubic phase system consists of an industrial grade of monolinolein (Dimodan U/J; Danisco, Denmark), blended with water. The phase behavior of this system has been extensively investigated in our previous works. HRP (Sigma-Aldrich) stock solution of 0.1mg/ml was prepared in pH7 phosphate buffer (Sigma-Aldrich) and ABTS stock solution of 45.5mM was prepared in pH4.65 (Sigma-Aldrich) acetate buffer. 0.2M H₂O₂ in pH4.65 acetate buffer was prepared from 50% H₂O₂ solution in water (Sigma-Aldrich). A two-syringe (Hamilton) coupled system was used for the preparation of the cubic phase at 37°C. In a typical preparation of the cubic phase, 375mg of monolinolein were loaded together with 5μL of enzyme stock solution in one syringe. In the other syringe 100μL of ABTS stock solution and 20μL of H₂O₂ solution were loaded. The mixing of the solutions from the two syringes was carried out for 3 min through a narrow connecting coupled needle. This ensured the formation of a completely transparent cubic phase with homogenous distribution of the reactants. Immediately after mixing, a green color starts to develop homogenously within the mesophase. The enzymatic reaction was run with varying ABTS concentration for POM and UV-vis measurements. At high concentrations of substrate or enzyme, the color development

was not uniform or started even before the highly viscous cubic phase was formed. Therefore the amount of enzyme and substrate was optimized to get a slow and homogenous color development in the cubic phase. The initial cubic phase was always prepared with a surfactant-to-water ratio of 75:25. This secured a starting Ia3d symmetry; however, for the experiment with bacteria, a Ia3d-Pn3m order-order transition takes place during the rinsing with excess buffer, which however does not affect the birefringence development, nor the main detection mechanism. For the assay with the substrate OPD (Sigma-Aldrich) the cubic phase was prepared in the same way using the OPD stock solution (55mM). Since the TMB (Sigma-Aldrich) substrate is not soluble in water TMB (20mM) was solubilised in the lipid-loaded syringe, whereas the HRP and H₂O₂ were loaded in the other syringe. The cubic phase was then prepared as described above. Attempts to reproduce the enzymatic reaction with other substrates such as pyrogallol (Sigma-Aldrich) and o-dianisidine (Sigma-Aldrich) were made but the higher solubility of the converted product did not trigger any crystallization/birefringence.

Polarized light microscopy

A small amount of the cubic phase was transferred on a clean glass slide, closed with a cover glass and analysed under cross-polarized light using a Zeiss Axioskop 2 mot microscope, at 37°C.

UV-vis measurement

Cubic phase samples from the syringe were directly transferred into a demountable UV cell (Starna, Type 20/C/Q/1) and UV-vis measurements were carried out on a CARY-100 Bio UV-visible spectrophotometer, at 37°C.

SAXS and WAXS measurements

SAXS and WAXS measurements were performed using a microfocused Rigaku X-ray source of wavelength $\lambda=1.54 \text{ \AA}$ operating at 45 kV and 88 mA. The diffracted x-rays signal was collected either by a two-dimensional argon-filled detector (for SAXS) or with the help of a Fuji Film BAS-MS 2025 imaging plate system: 15.2 × 15.2 cm, 50 μm resolution (for WAXS). For all measurements the samples were placed inside a Linkam HFS91 hot stage and measured at 37°C. Data were collected and averaged azimuthally to yield one-dimensional intensity versus scattering vector q .

Enzymatic cascade reaction for glucose detection

The desired concentrations of β -D-glucose (Sigma-Aldrich) and ABTS (45.5 mM) were prepared separately in pH 4.65 buffers. GOD (Sigma-Aldrich) stock solution of 0.1 mg/ml and HRP stock solution of 1 mg/ml were prepared in pH 7 phosphate buffer. 100 μL of β -D-glucose and 130 μL of ABTS were collected in one syringe while 750 μL of monolinolein

together with 10 μ L of GOD and 10 μ L of HRP were taken in another syringe and mixed through a needle connecting the two syringes as mentioned above. In the control sample the β -D-glucose solution was replaced by 100 μ L of buffer.

Enzymatic cascade reaction for cholesterol detection

- 5 The required concentration of cholesterol (Sigma-Aldrich) was directly solubilised in the monolinolein. Cholesterol oxidase (ChOD, Sigma-Aldrich) stock solution of 0.2 mg/ml and HRP stock solution of 1 mg/ml were prepared in pH 7 phosphate buffer. 100 μ L of buffer, 130 μ L of ABTS, 10 μ L of ChOD stock solution and 10 μ L of HRP stock solution were loaded in one syringe while 750 mg of dimodan together with the required concentration of
- 10 cholesterol were loaded in the other syringe and mixed as described above. The control sample without cholesterol was prepared in the same way.

Bacteria

- E.coli* (K-12 MG 1655) were cultured overnight on nutrient agar at 37°C. Then single bacterial colonies were selected from LB agar and transferred into 5 ml LB media. After
- 15 5 hours, 20 ml additional media were added and incubated overnight at 37°C with shaking at 225 rpm. The following day, cells were pelleted (2500 g, 10 mins) and washed with PBS and pelleted again. Bacteria were suspended as 10^7 ml⁻¹ in pH 4.65 acetate buffer and used within 1 h of preparation.

Immobilization of bacteria in the cubic phase

- 20 For the immobilization of *E.coli* in the cubic phase one syringe was filled with 50 μ L of *E.coli* suspension in acetate buffer (5×10^4 *E.coli*) and the other syringe with 200 mg of monolinolein together with 2 mg of TMB. Mixing and preparation of the cubic phase was carried out via the double connected syringes as mentioned before. The control samples were prepared without *E.coli*.

Observation of *E. coli* in wide field fluorescence microscopy

E.coli in the cubic phase was stained with 1 μ g/ml DAPI (4', 6-diamidino-2-phenylindole) and imaging was carried out with a 100x oil immersion lens (NA 1.4) with an excitation wavelength of 365 nm and a 420 nm long pass filter for the emission.

Birefringent ELISA

- 30 An O-ring (1 mm thickness and 8 mm inner diameter) was filled with the mesophase mixed with bacteria and TMB and placed on a glass slide. The sample was washed with blocking buffer (5% BSA solution) followed by rinsing with the PBS buffer. HRP-conjugated primary antibody solution (Abcam ab68450, 1mg/ml, dilution 1:100) was added on the mesophase and incubated for 15 min. After washing the mesophase three times with buffer, 0.5 mol/l

H₂O₂ were added to start the peroxidase reaction. A control sample was prepared in the same way without adding *E.coli*.

P. falciparum culture

5 The *P. falciparum* 3D7 strain was cultured in vitro in human 0+ erythrocytes at 5 % haematocrit using RPMI medium containing 0.5 % Albumax. Parasites were synchronized with 5 % sorbitol. Cultures were harvested by centrifugation and lysed hypotonically in H₂O. 100 µL of this lysed culture is then added on top of 50 mg of phytantriol lipid, allowing 3 minutes to form the cubic mesophases in a 96 well plate.

Hemozoin crystallites from *P. falciparum* culture

10 30 ml *Plasmodium falciparum* culture (7 % late stage parasitaemia) was pelleted and lysed in 5 ml ddH₂O at room temperature for 5 minutes. The lysate was centrifuged at 3500 g for 10 minutes. The pellet was dissolved in 2 % SDS and vortexed vigorously. The lysate was centrifuged for 10 minutes at 21000 g. The resulting hemozoin pellet was twice-washed in ddH₂O and stored in 500 µl ddH₂O at room temperature. 100 µL of this ddH₂O suspension
15 were then added on top of 50 mg of phytantriol lipid, allowing 3 minutes to form the cubic mesophases in a 96 well plate. The control sample contained 500 µl uninfected human erythrocytes lysed in 1,5 ml ddH₂O.

List of reference numerals:

- | | |
|----|--|
| 10 | apparatus according to claim 11 |
| 20 | 11 volume |
| | 12 first transparent optical element |
| | 13 second transparent optical element |
| | 14 detector |
| | 15 axis passing through said volume 11 |
| 25 | 16 first axis of polarization |
| | 17 second axis of polarization rotated by 90° around axis 15 in relation to first axis of polarization |
| | 18 light source emitting light along axis 15 |
| | 19 diffusor |
| 30 | 20 constant current supply |
| | 21 rechargeable battery |
| | 22 main power switch |

- 23 ventilator
- 24 contact switch
- 25 second transparent optical element 13 integrated into a hinge operated lid
- 26 display
- 5 27 controller/amplifier

Claims:

1. A method for visually detecting a reaction from a reactant to a product, wherein said method comprises the steps of:
 - i. providing a liquid crystal in bicontinuous cubic mesophase comprising said reactant,
 - ii. providing conditions in which said reactant is converted into said product in a reaction step,wherein said product is insoluble in said mesophase, and
 - iii. measuring a birefringence value of said mesophase.
2. A method for detecting an analyte, comprising the steps of
 - i. providing said analyte in a bicontinuous cubic mesophase,
 - ii. enzymatically converting said analyte into a substrate in a first cascade step,
 - iii. converting the substrate with a reactant into a product in a second cascade step,wherein said product is insoluble in said mesophase, and
 - iv. measuring a birefringence value of said mesophase.
3. The method according to claim 1 or 2, wherein the bicontinuous cubic mesophase comprises water and monolinolein or an equivalent lipid.
4. The method according to any one of the previous claims, wherein the liquid crystal is in lamellar phase or inverted hexagonal phase.
5. The method according to any one of the previous claims, wherein the reactant is selected from
 - a. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS),
 - b. 3,3',5,5'-tetramethylbenzidine, and
 - c. o-phenylenediamine dihydrochloride.
6. The method according to any one of the previous claims, wherein the product is selected from
 - a. the horseradish peroxidase reaction product of ABTS;
 - b. 3,3',5,5'-tetramethylbenzidine diimine, and
 - c. 2,3-diaminophenazine.

7. The method according to any one of the previous claims, wherein said reaction step, said first cascade step, and/or said second cascade step is catalysed by an enzyme.
8. The method of claim 7, wherein said enzyme is selected from a peroxidase, a glucose oxidase or a cholesterol oxidase.
- 5 9. The method according to claim 8, wherein the peroxidase is linked to an antibody.
10. The method according to claims 2, 3, 4, 5, 6, 7, 8 or 9, wherein:
- a. said analyte is glucose, or
 - b. said analyte is cholesterol, or
 - c. a microorganism, particularly a eukaryotic microorganism such as plasmodium falciparum, or a prokaryotic microorganism, more particularly a bacterium, or
 - 10 d. a virus, particularly a member of the families of filoviridae or retroviridae, more particularly HIV or Ebolavirus,
- and said substrate is hydrogen peroxide.
11. The method according to any one of the preceding claims, wherein the birefringence
- 15 is measured during all steps of said method.
12. An apparatus 10 for use in a method according to any one of the preceding claims, comprising
- a volume 11 delimited by a first transparent optical element 12 and a second transparent optical element 13, wherein said first transparent optical element 12 and said second transparent optical element 13 are orthogonal to an axis 15 passing through said volume 11, and wherein
 - 20 - said first transparent optical element 12 is characterized by a first axis of polarization 16, and
 - said second transparent optical element 13 is characterized by a second axis of polarization 17 rotated by 90° around said axis 15 in relation to said first axis of polarization.
- 25 characterized in that
- said volume comprises a bicontinuous cubic mesophase.
13. The apparatus according to claim 12, wherein the second axis of polarization is
- 30 rotated around said axis 15 in relation to said first axis of polarization by an angle of 50°, 60°, 70°, 80°, 85°, 90°, 95°, 100°, 110°, 120° or 130°.

14. The apparatus according to claim 12 or 13, wherein the volume 11 comprises a lamellar phase or inverted hexagonal phase.

15. The apparatus according to claim 12, 13 or 14, comprising a light source 18 being designed to emit visible light along axis 15.

5 16. The apparatus according to claims 12, 13, 14 or 15 wherein said bicontinuous cubic mesophase comprises

i. a reactant, and

ii. a substrate that is converted with said reactant into a product in a reaction step,

10 characterized in that

said product is insoluble.

17. The apparatus according to claims 12, 13, 14, 15 or 16 wherein said bicontinuous cubic mesophase comprises

i. an analyte

15 ii. an enzyme converting said analyte into a substrate in a first cascade step, and

iii. a reactant that is converted with said substrate into a product in a second cascade step,

characterized in that

said product is insoluble and birefringent.

20 18. The apparatus according to claims 12, 13, 15, 16 or 17, wherein the bicontinuous cubic mesophase comprises water and monolinolein.

19. The apparatus according to claims 12, 13, 14, 15, 16, 17 or 18, wherein the reactant is selected from

25 a. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS),

b. 3,3',5,5'-tetramethylbenzidine, and

c. o-phenylenediamine dihydrochloride.

20. The apparatus according to claims 12, 13, 14, 15, 16, 17, 18 or 19, wherein the product is selected from

30 a. the horseradish peroxidase reaction product of ABTS;

b. 3,3',5,5'-tetramethylbenzidine diimine, and

c. 2,3-diaminophenazine.

21. The apparatus according to claims 12, 13, 14, 15, 16, 17, 18, 19 or 20 wherein said reaction step, said first cascade step, and/or said second cascade step is catalysed by an enzyme, selected from a peroxidase, a glucose oxidase or a cholesterol oxidase.

22. The apparatus according to claims 12, 13, 14, 15, 16, 17, 18, 19, 20 or 21, wherein the peroxidase is linked to an antibody.

23. The apparatus according to claims 12, 13, 14, 15, 16, 17, 18, 19, 20, 21 or 22, wherein said analyte is

a. glucose, or

b. cholesterol, or

c. a microorganism, particularly a eukaryotic microorganism such as plasmodium falciparum, or a prokaryotic microorganism, more particularly a bacterium, or

d. a virus, particularly a member of the families of filoviridae or retroviridae, more particularly HIV or Ebolavirus,

and said substrate is hydrogen peroxide.

Fig. 1:

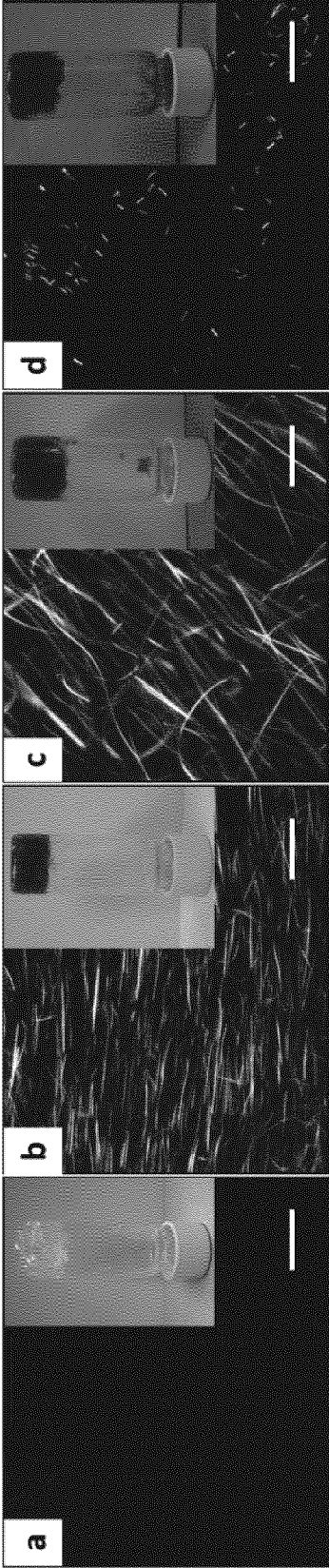


Fig. 2:

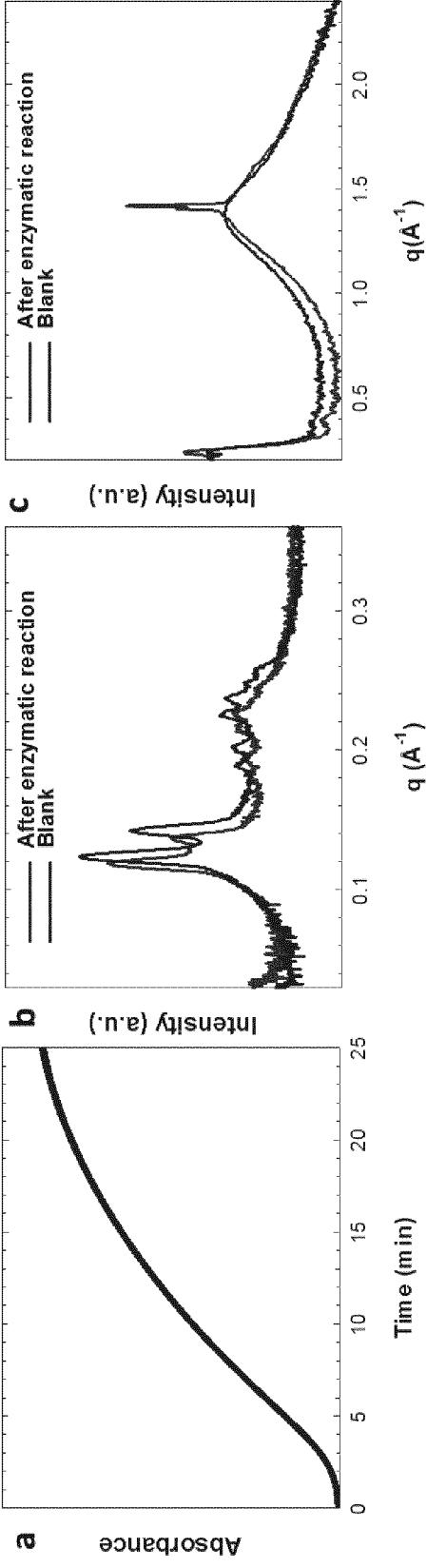


Fig. 3:

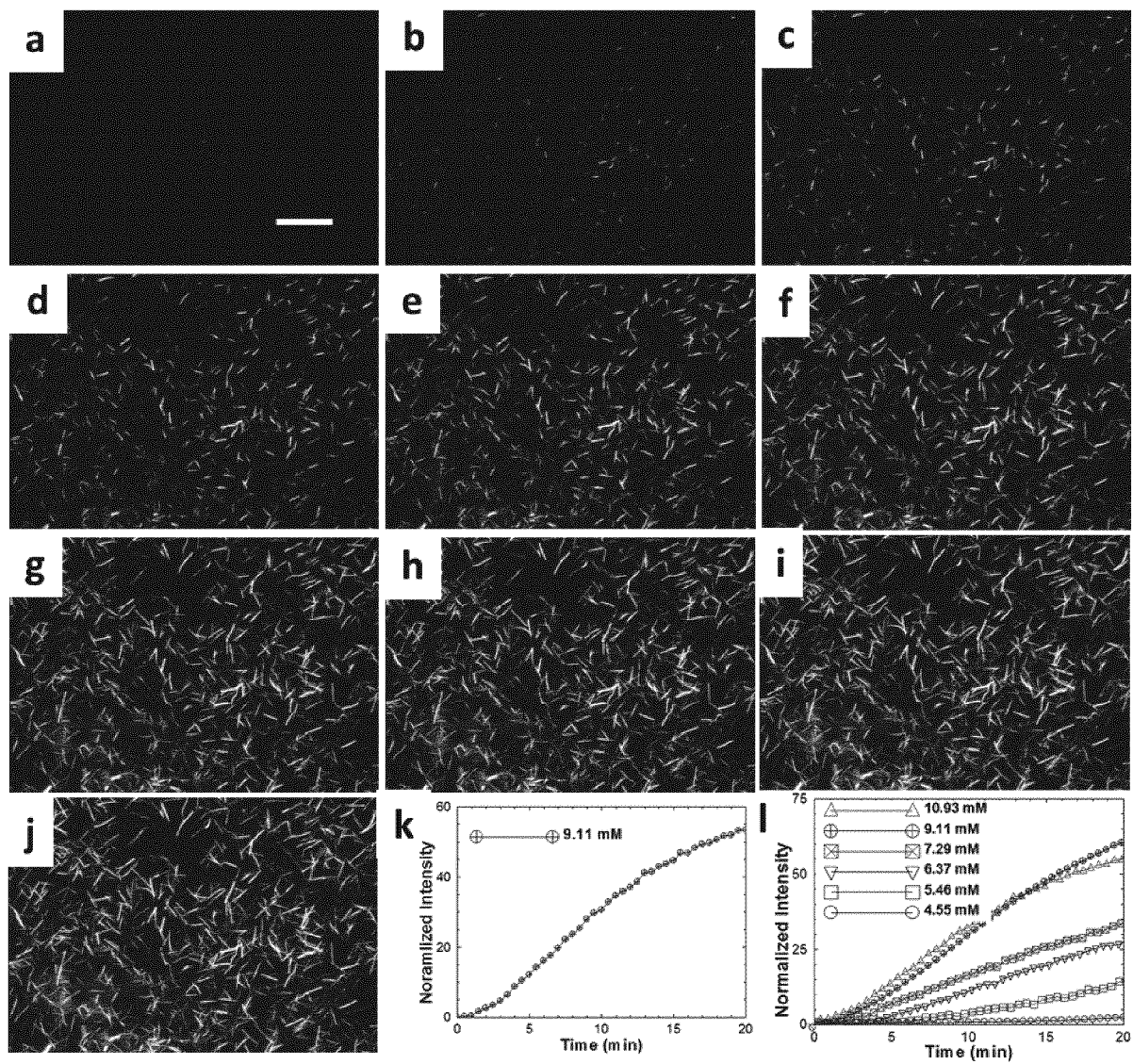


Fig. 4:

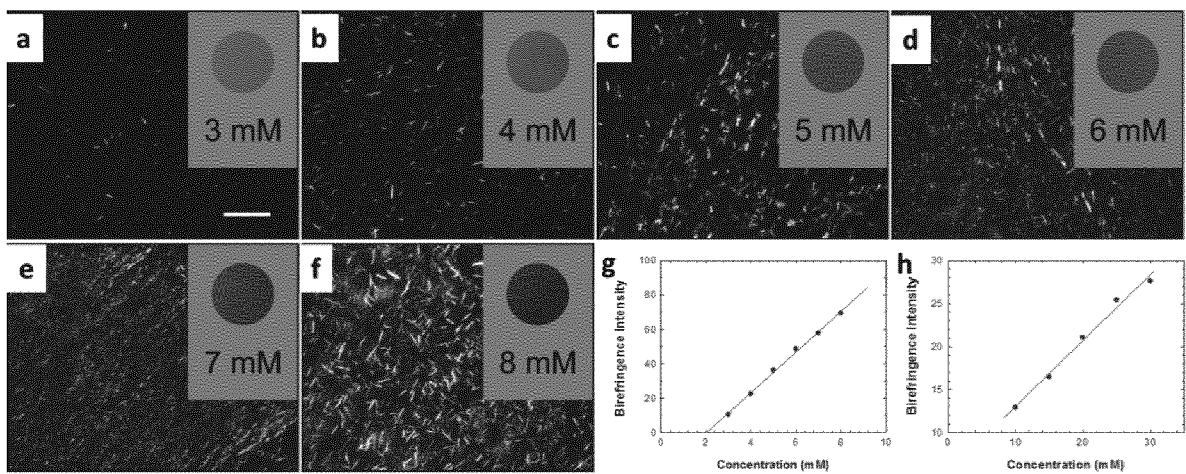


Fig. 5:

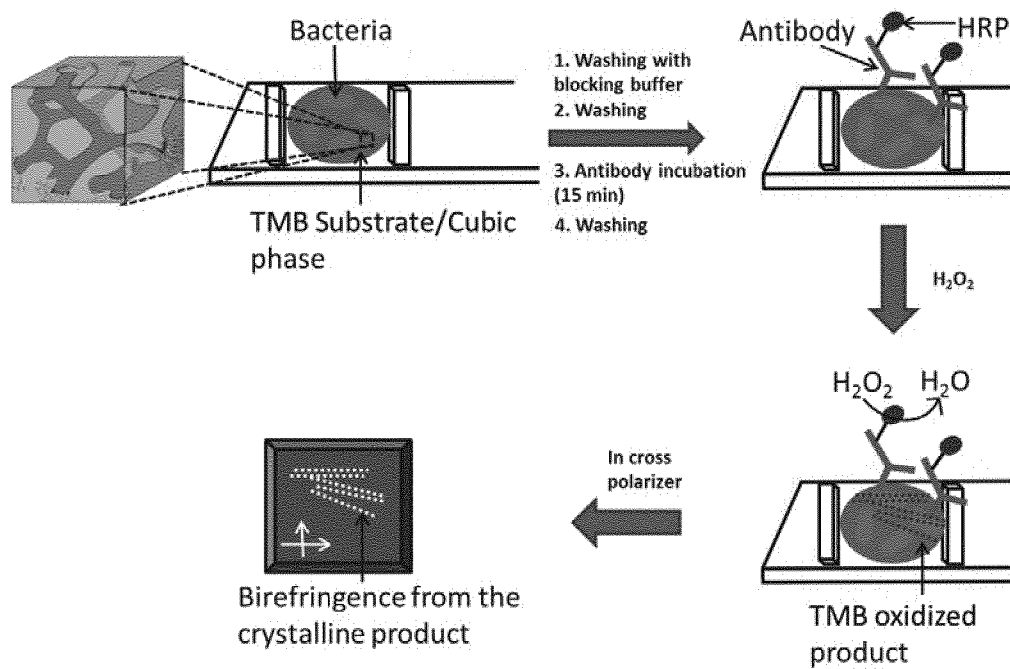


Fig. 6:

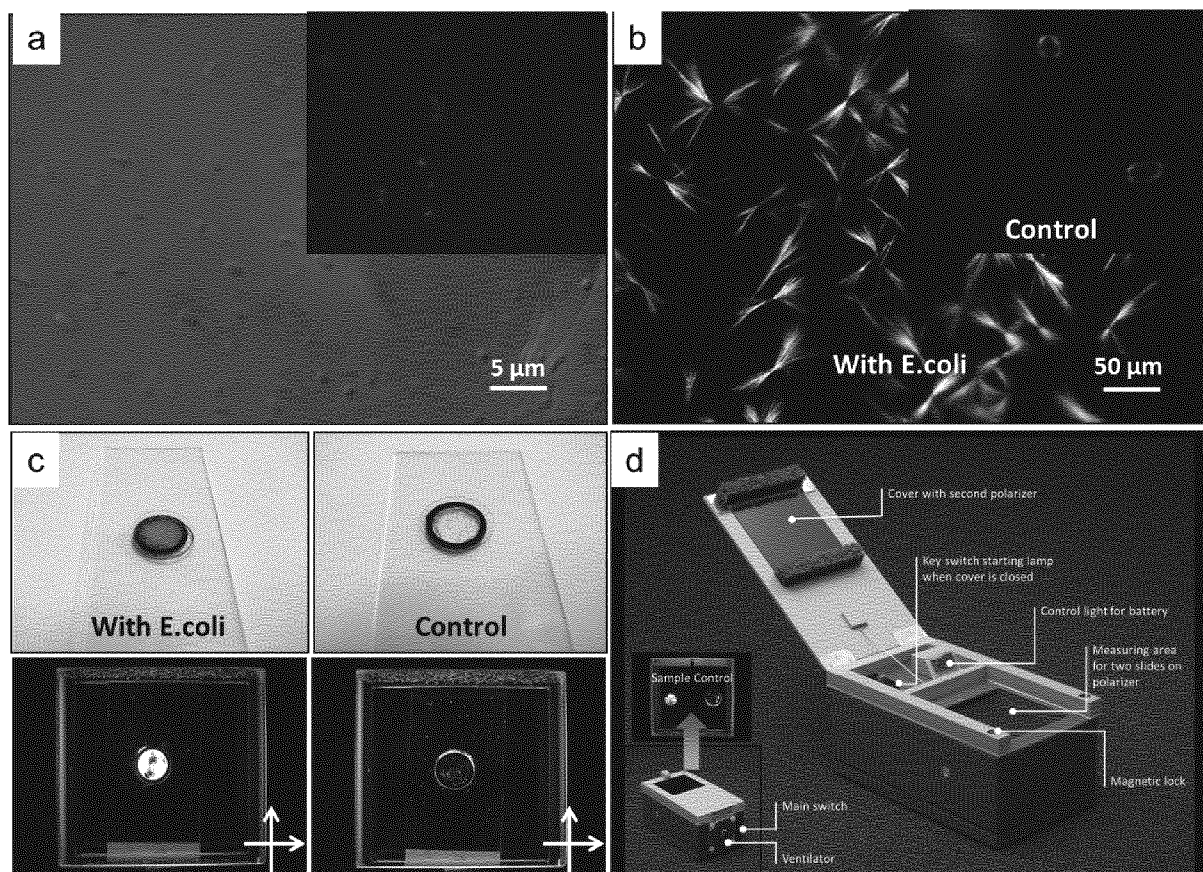


Fig. 7:

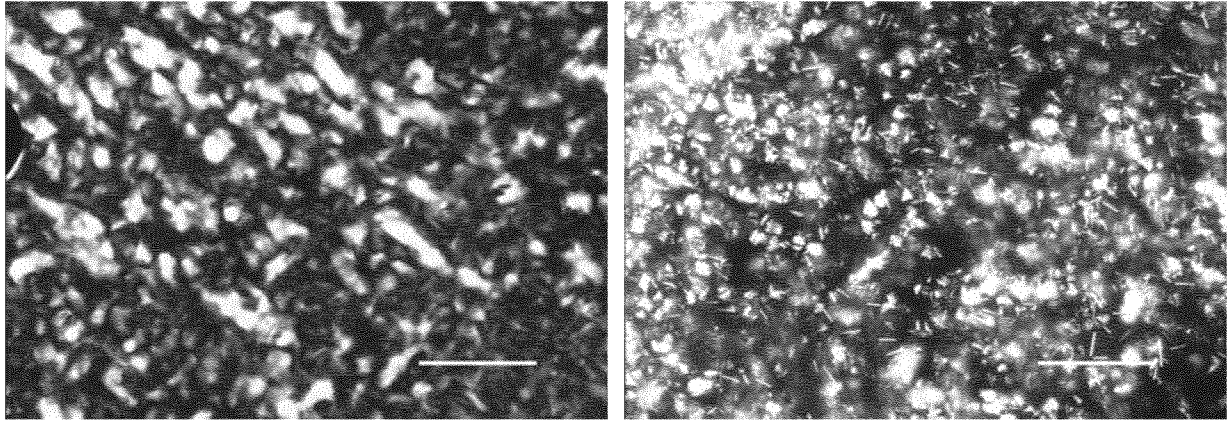


Fig. 8:

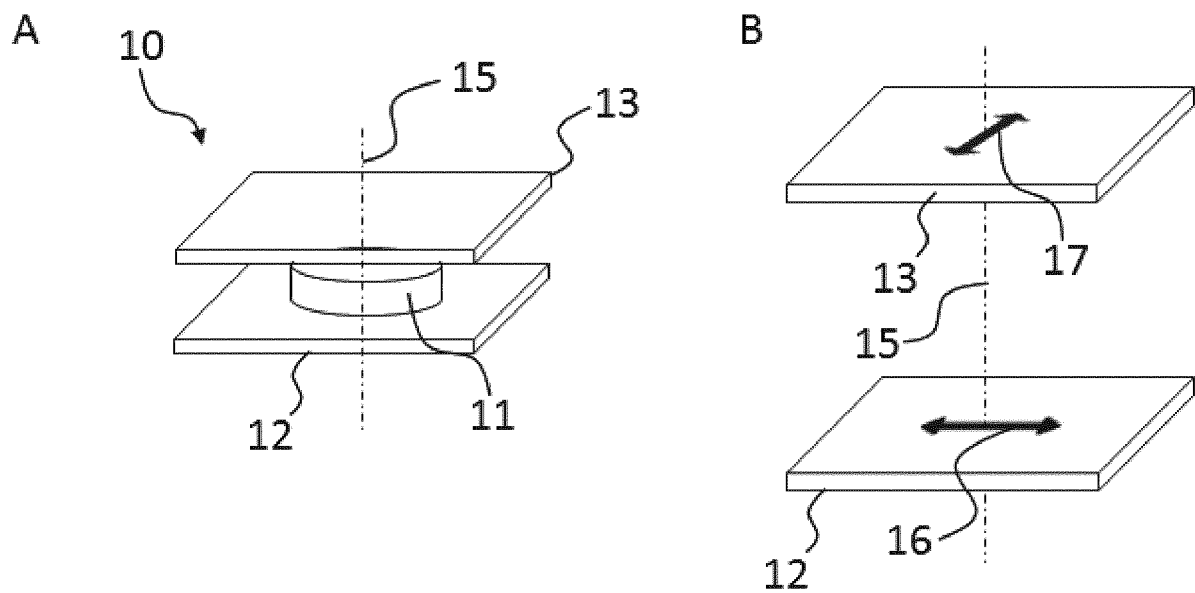


Fig. 9:

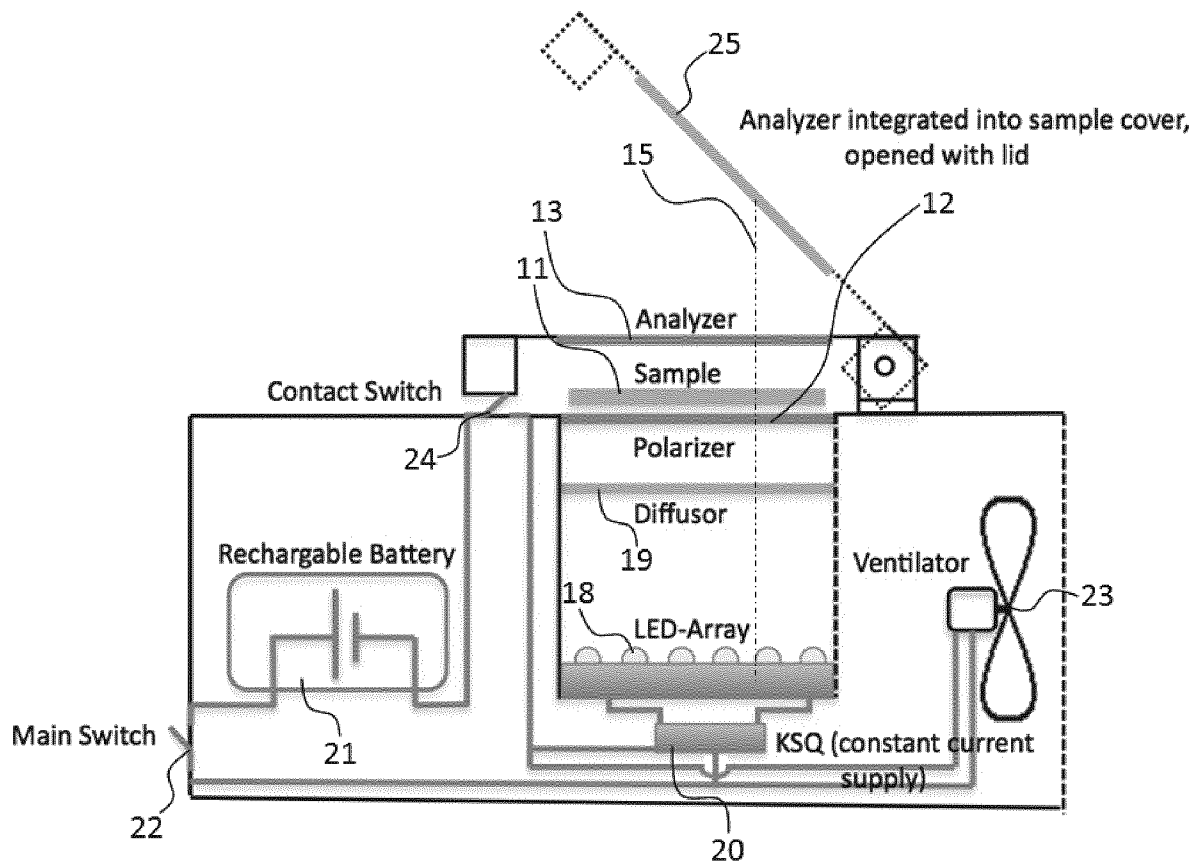


Fig. 10:

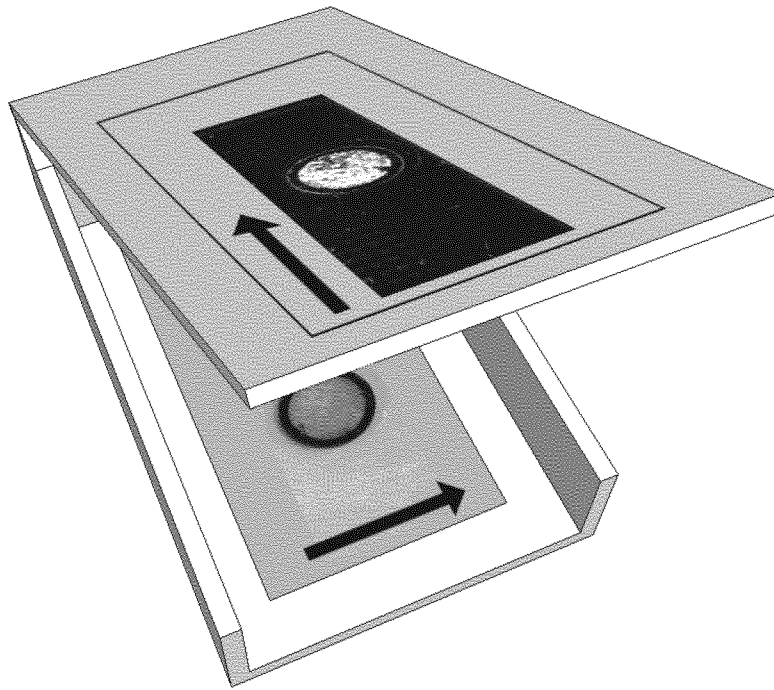


Fig. 11:

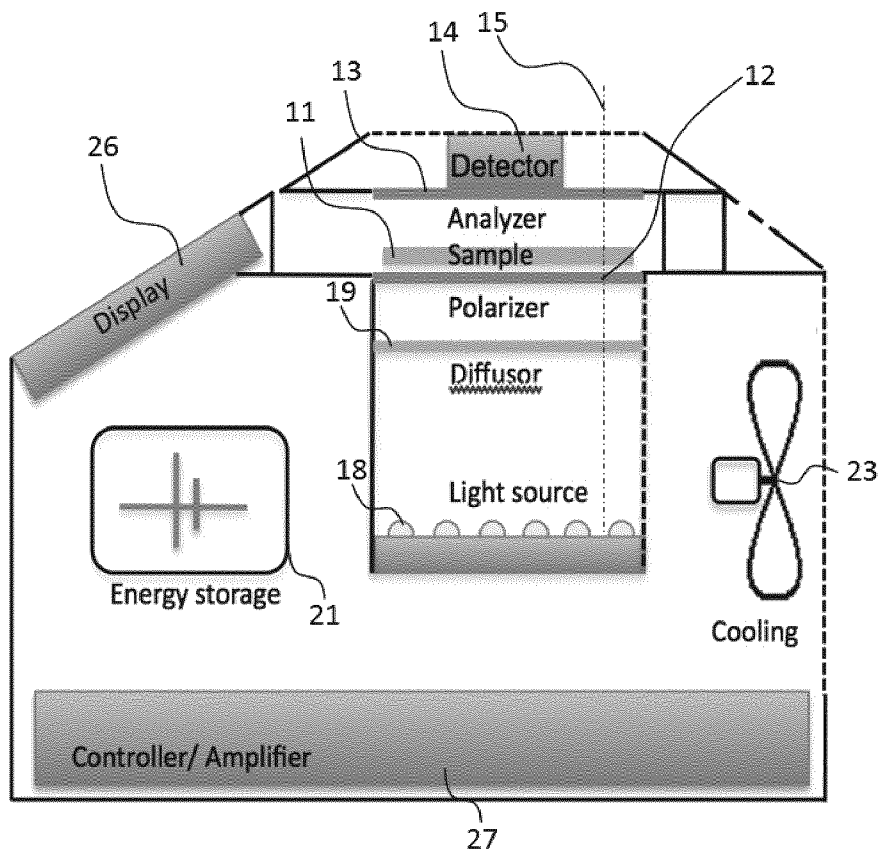


Fig. 12

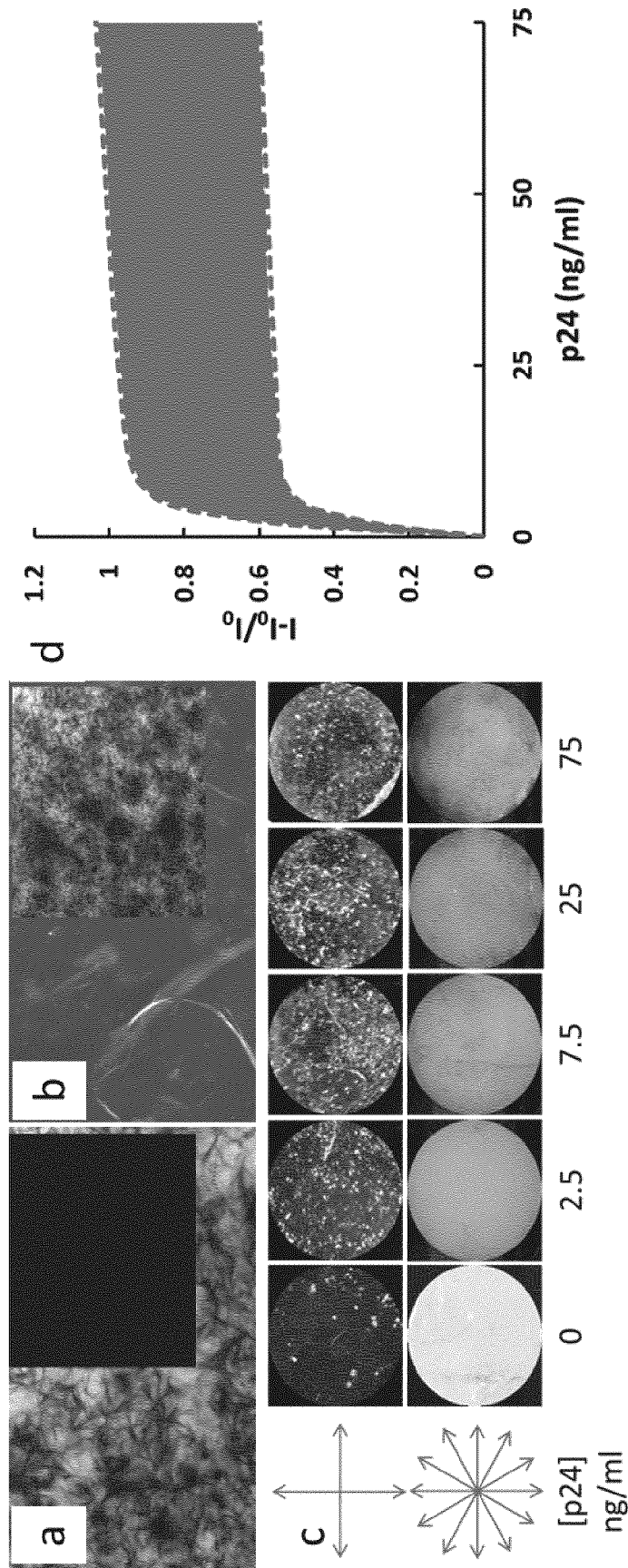
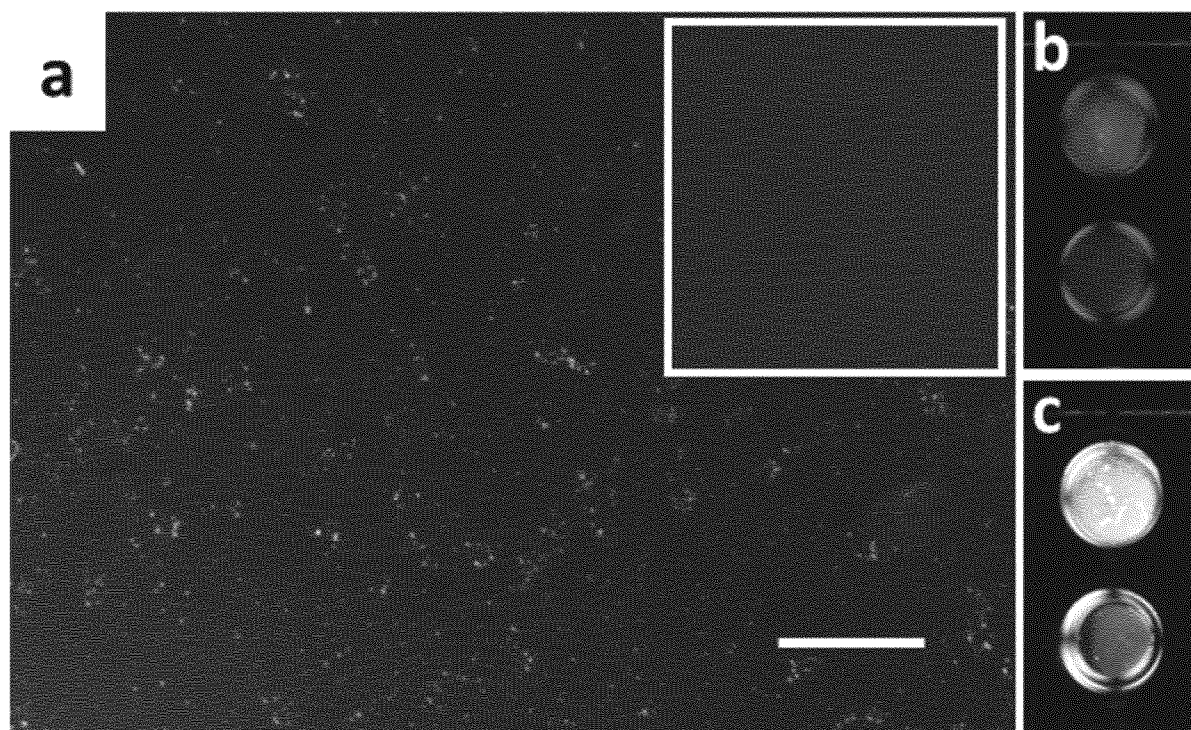


Fig. 13



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/062151

A. CLASSIFICATION OF SUBJECT MATTER INV. C12Q1/06 C12Q1/26 C12Q1/28 G01N21/23 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) C12Q 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, WPI Data, BIOSIS, COMPENDEX, EMBASE, INSPEC		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOSSENA G A ET AL: "A novel cubic phase of medium chain lipid origin for the delivery of poorly water soluble drugs", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 99, no. 2, 30 September 2004 (2004-09-30), pages 217-229, XP004569543, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2004.06.013	12-15,18
A	the whole document in particular: page 219, paragraph 2.3 - page 220	1-11,16, 17,19-23
X	US 2008/177164 A1 (HELLER ADAM [US] ET AL) 24 July 2008 (2008-07-24)	12-15,18
A	example 7 claims 1,11,16	1-11,16, 17,19-23
	----- -/-	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 30 July 2015		Date of mailing of the international search report 17/08/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Tuytman, Antonin

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/062151

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WENJIE SUN ET AL: "Controlling enzymatic activity and kinetics in swollen mesophases by physical nano-confinement", NANOSCALE, vol. 6, no. 12, 1 January 2014 (2014-01-01), page 6853, XP055204953, ISSN: 2040-3364, DOI: 10.1039/c4nr01394h the whole document -----	1-23
A	BILALOV AZAT ET AL: "Embedding DNA in surfactant mesophases: The phase diagram of the ternary system dodecyltrimethylammonium-DNA/monoolein/water in comparison to the DNA-free analogue", JOURNAL OF COLLOID AND INTERFACE SCIENCE, ACADEMIC PRESS, NEW YORK, NY, US, vol. 394, 28 December 2012 (2012-12-28), pages 360-367, XP028972193, ISSN: 0021-9797, DOI: 10.1016/J.JCIS.2012.11.067 the whole document -----	1-23
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A	WO 92/10758 A1 (BIOCIRCUITS CORP [US]) 25 June 1992 (1992-06-25) claim 1 -----	1-23

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International application No

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