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(54) **A DEVICE FOR VISUAL DETECTION OF BILIRUBIN**

VORRICHTUNG ZUR VISUELLEN DETEKTION VON BILIRUBIN

DISPOSITIF POUR LA DÉTECTION VISUELLE DE LA BILIRUBINE

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(73) Proprietor: **Indian Institute of Technology, Guwahati**
**Guwahati, Assam 781039 (IN)**

(72) Inventors:
- **CHATTOPADHYAY, Arun**
  **Guwahati**
  **Assam 781039 (IN)**
- **PAUL, Anumita**
  **Guwahati**
  **Assam 781039 (IN)**
- **BASU, Srestha**
  **Guwahati**
  **Assam 781039 (IN)**
- **SAHOO, Amaresh Kumar**
  **Guwahati**
  **Assam 781039 (IN)**

(74) Representative: **Petraz, Gilberto Luigi et al**
**GLP S.r.l.**
**Viale Europa Unita, 171**
**33100 Udine (IT)**

(56) References cited:
**WO-A2-2006/138698** **US-A1- 2004 002 089**
**US-A1- 2012 065 614** **US-B2- 6 882 873**

- **MALLESH SANTHOSH ET AL: "Selective and sensitive detection of free bilirubin in blood serum using human serum albumin stabilized gold nanoclusters as fluorometric and colorimetric probe", BIOSENSORS AND BIOELECTRONICS, vol. 59, 8 April 2014 (2014-04-08), pages 370-376, XP055527261, AMSTERDAM, NL ISSN: 0956-5663, DOI: 10.1016/j.bios.2014.04.003**



EP 3 302 449 B1

## Description

### FIELD OF THE INVENTION:

**[0001]** The present invention relates to visual detection of bilirubin. In particular, the present invention is directed to develop a device or more appropriately a diagnostic kit for ready and easy visual detection of bilirubin and/or Hyperbilirubinemia condition in a person by using blood serum and/or thumb impression of said person.

### BACKGROUND ART.:

**[0002]** Molecular level understanding of diseases far outweighs traditional symptom perception in the current era of medical practices. This is based on advancement of optical, electrical and electronic probes for measuring changes in concentrations of key molecular species in the body, which are responsible for the onset of a large number of medical conditions.

**[0003]** Constant improvement in the versatility, sensitivity and speed of probes based on the newest technological developments provide much needed boost in the diagnostics. For example, pathological laboratory tests performed using blood, stool, urine or saliva sample provide a wealth of information about the health of the patient. Further, modern techniques like ultra-sonography, magnetic resonance imaging and X-ray computed tomography are also widely used to visualize internal body organs and damages therein. These techniques, though accurate, often impose high expenses and tedious diagnostic period; however, they are deemed necessary for accurate prognosis of several diseases.

**[0004]** Recently, the process of diagnosis has been made easier and faster in case of pregnancy (Ref. Gnoth, C.; S. Johnson. Geburtshilfe und Frauenheilkunde: 2014, 661-669.), diabetes (Ref. Azad launches indigenously developed Diabetes Screening System, Test Strips. India Medical Times, dated January 13, 2014. Accessed from http://www.indiamedical-times.com/2014/01/13/azad-launches-indigenous-technologies-for-detection-of-diabetes) and a few other abnormalities.

**[0005]** A pathogen detection kit enabling detection of five pathogens namely salmonella, staphylococcus aureus, listeria, vibrio cholera and vibrio parahaemolyticus, has been recently developed for easy detection of the said pathogens. In an analogous attempt, an indigenous kit allowing measurement of serum ferritin in blood or iron content has been designed, thereby indicating the state of anemia which is closely associated with the deficiency of iron content (Ref. Affordable pathogen detection kit launched. The Hindu, New Delhi edition, dated February 21, 2013; http://www.thehindu.com/todays-paper/tp-national/affordable-pathogen-detection-kit-launched/article5711927.ece).

**[0006]** In these cases, simple analyses can be done at home based on colorimetric assay or an electrochemical device. More importantly, these kits are available to a large members of the populace because of ease of operation and observation and because they are rather inexpensive.

**[0007]** In line with the above kit based diagnostic techniques, recent rapid development in the field of nanoscale science and technology promises much greater opportunity in terms of speed, versatility, sensitivity and ease of making molecular marker based health diagnosis. For example, there are a large number of biochemical tests which have been developed to probe diseases based on the surface plasmon resonance properties of gold nanoparticles and photoluminescence properties of quantum dots (QDots). Some of these have been developed as markers of DNA specific to a particular disease. In addition, detection of viruses, bacteria, assays of proteins have been possible with the changes in optical properties of gold nanoparticles, nanorods and quantum dots. Some exemplary illustration of nano technology based diagnostic techniques can be found in Deka, J et al (Ref. Deka, J.; Paul, A.; Chattopadhyay, A. The Journal of Physical Chemistry C 2009, 113,6936); Mirkin, C.A. et al (Ref. Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff, J. J. Nature 1996, 382, 607.) and Storhoff et al (Ref. Storhoff, J. J.; Lucas, A. D.; Garimella, V.; Bao, Y. P.; Muller, U. R. Nat Biotech 2004, 22, 883.).

**[0008]** The latest entrant (Ref. Hemmateenejad, B.; Shakerizadeh-shirazi, F.; Samari, F. Sensors and Actuators B: Chemical 2014, 199, 42.) in the field of nanoscale science and technology is the luminescent atomic clusters especially of those of noble metals (as mentioned above). For example, the red luminescence of few atom gold clusters is highly sensitive to presence of molecular species which quench its photoluminescence. This is potentially forming the basis of biosensing.

**[0009]** Fluorescence based detection of biologically relevant molecules such as bilirubin has recently been done using noble metal nanoclusters. (Ref. Santhosh, M.; Chinnadayyala, S. R.; Kakoti, A.; Goswami, P. Biosensors and Bioelectronics 2014, 59, 370.) The fluorescence intensity of HSA stabilized gold nanoclusters was effectively quenched in presence of bilirubin at a pH range of 6 to 9, thereby allowing sensitive detection free bilirubin in blood serum.

**[0010]** In the technique adapted by Santhosh, et al, bilirubin has been used as quencher of gold nanoclusters luminescence intensity. As a matter of fact, various chemical and/or biochemical agents can also serve as a quencher of luminescent nanoclusters (Ref.: S. K. Sailapu, A. K. Sahoo, S. S. Ghosh, A. Chattopadhyay, Hierarchical Logic Structures Based on Responsive Fluorescent Gold Nanoclusters. Small 10, 4067-4071 (2014)10.1002/smll.201401421 and C. Banerjee, J. Kuchlyan, D. Banik, N. Kundu, A. Roy, S. Ghosh, N. Sarkar, Interaction of gold nanoclusters with IR light emitting cyanine dyes: a systematic fluorescence quenching study. Physical Chemistry Chemical Physics 16, 17272-17283 (2014)10.1039/C4CP02563F). Thus, as

per the technique adapted by Santhosh quenching of intensity or fluorescence of gold nanoclusters by any material is not directed to any confirmatory indication of the presence of bilirubin in said material. Further, the method employed by Santhosh et al. does not offer the feasibility of a device especially using thumb impression or upon contact with skin.

**[0011]** WO2006138698A2 discloses a detection system for determining unbound bilirubin from serum wherein the system comprises : a metallic material deposited on a substrate and a polymeric film positioned on the metallic particles/film, wherein at least the surface of the polymeric film is impregnated with Human Serum Albumin in an amount sufficient to capture unbound bilirubin, a source of electromagnetic energy and a detector for measuring fluorescence emission of the bound bilirubin in the polymeric material wherein the polymeric layer is of sufficient thickness . The thickness of the polymeric layer is from about 40nm to about 300nm. The intrinsic fluorescence is enhanced by positioning the polymeric material near the metallic material from about 4nm to 100nm. The said method requires coating of the polymeric film by a fluorescent substance such as HAS and teaches detection of bilirubin from serum that is liquid phase only and not from solid phased sample

**[0012]** It is thus there has been a need for advancements in luminescence based techniques for confirmatory detection of presence and amount of bilirubin in samples such as blood serum or skins such as to enable prominent visualization contrast to sense bilirubin, thereby providing for methods facile in terms of detection and indentify the condition of hyperbilirubinemia / jaundice by probing excess bilirubin (BR) deposition on skin in early stages of the disease.

## OBJECTIVE OF INVENTION:

**[0013]** The basic objective of the present invention is to develop a device for visual detection of bilirubin level in human body, using blood serum and/or thumb impression.

**[0014]** Another objective of the present invention is to develop a bilirubin presence indicator which would be adapted to incorporate in any diagnostic kit platform and facilitate non-invasive detection of bilirubin level in human body.

**[0015]** Yet another objective of the present invention is to develop a diagnostic kit which would be adapted to detect Hyperbilirubinemia condition in a person based on thumb impression of said person.

**[0016]** A still further objective of the present invention is to develop a device for visual detection of bilirubin level which would be adapted to detect presence of bilirubin in both solid and liquid phase based on the same principle.

## SUMMARY OF THE INVENTION:

**[0017]** Thus according to basic aspect of the present invention, there is provided a diagnostic device or kit for visual detection of bilirubin according to claims 1-10.

**[0018]** In accordance with another aspect of the present invention there is provided a process for manufacturing diagnostic device or kit according to claims 11-14.

## BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES:

**[0019]**

Figure 1 is a photographic illustration of gold nanocluster coated polyvinylidene difluoride (PVDF) as visualized under UV lamp with excitation at 254 nm, in accordance with the present invention.

Figure 2 is a photographic illustration of the gold nanocluster coated PVDF membrane as observed using 254 nm UV light, following addition of copper sulphate solution, in accordance with the present invention.

Figure 3 shows Bilirubin treated film as viewed using 254 nm UV light in accordance with the present invention.

Figure 4 shows photographic illustration of luminescence intensity under UV light excitation of (A) The PVDF membrane coated with gold nanoclusters, (B) The same membrane after addition of copper salt and (c) The film in (B) after addition of water.

Figure 5 shows (A) gradual fluorescence intensity quenching of gold nanoclusters on gradual addition of $Cu^{2+}$ and (B) corresponding Stern-Volmer plot, in accordance with the present invention.

Figure 6 shows (A) a steady State luminescence study in solution phase, (B) Result (luminescence) of the same experiment carried out with water and (C) Result (luminescence) of the same experiment carried out with bilirubin in absence of $Cu^{2+}$.

Figure 7 shows graphs for (A) gradual luminescence intensity recovery of gold nanoclusters otherwise quenched in presence of $Cu^{2+}$ and (B) gradual increase of normalized luminescence intensity of the $Cu^{2+}$ quenched gold nanoclusters solution after addition of bilirubin.

Figure 8 shows fluorescence intensity quenching of Au NCs in presence of $Cu^{2+}$ followed by recovery of the same on addition of serum containing bilirubin exceeding normal range.

Figure 9 shows (A) photograph of copper salt treated gold nanocluster film and (B) the same film after thumb impression of a jaundice patient.

Figure 10 shows photograph of copper salt treated gold nanocluster film following thumb impression of a volunteer not affected with hyperbilirubinemia.

## DETAILED DESCRIPTION OF THE INVENTION WITH REFERENCE TO THE ACCOMPANYING FIGURES

**[0020]** The present invention discloses a device for visual detection of bilirubin and method for fabricating the same. The device for visual detection of bilirubin of the present invention is highly sensitive, versatile and robust film-strip based luminescent indicator for detecting and marking excess bilirubin deposition on the human skin as well as in blood serum.

**[0021]** The present device basically comprises chitosan stabilized gold nanoclusters based luminescence source preferably in a form of polymer membrane strip having luminescent chitosan stabilized gold nanoclusters embedded in biopolymer film and $Cu^{2+}$ ions source for treating the strip with $Cu^{2+}$ ions for quenching luminescence intensity of the strip.

**[0022]** The polymer membrane strip of chitosan stabilized fluorescent gold nanocluster (Au NCs) before treating with the $Cu^{2+}$ ions appears as luminescent yellow in the presence of UV light. The luminescence intensity of the strip is quenched after treating the strip with aqueous solution of copper salt having $Cu^{2+}$ ions such as copper sulfate solution and the quenched luminescence intensity is again recovered when the $Cu^{2+}$ ions treated strip comes in contact with solid or liquid medium having bilirubin.

**[0023]** In presence of bilirubin (BR) in solid or liquid phase of the medium that comes in contact with the strip, the luminescence intensity of the strip is restored and the intensity restoration amount depends on the concentration of the bilirubin present in the medium that comes in contact with the strip.

**[0024]** In the present invention, the bilirubin indicating polymer membrane strip is involved to design a diagnostic kit for ready and easy detection of bilirubin content in a person. The kit embodiment basically includes a support platform having thereon said polymer membrane strip based bilirubin indicator. An input means also provided on the kit platform which facilitates the application of blood serum of the person on the polymer membrane strip for allowing liquid phase-bilirubin in the blood serum to form the complex (Cu-BR, A 1:1 complex between copper and bilirubin) with the $Cu^{2+}$ ions of the strip and thereby recovering the luminescence intensity of the strip indicating presence on the bilirubin.

**[0025]** In a preferred embodiment of the present diagnostic kit a touch pad is incorporated for providing the bilirubin indicating polymer membrane strip in contact with thumb skin of the person and allowing solid phase bilirubin deposited in thumb skin to come in contact with the strip and form the complex with the $Cu^{2+}$ ions which recovers the luminescence intensity of the strip indicating the hyperbilirubinemia condition.

**[0026]** The diagnostic kit also comprises intensity detector device for measuring recovered luminescence intensity of the polymer membrane strip. The recovered intensity is detected in presence of UV light after addition of the bilirubin in relation to reduced luminescence intensity of the strip in the presence of copper ions. The bilirubin concentration is determined based on variation of the recovered luminescence intensity with respect to the reduced luminescence intensity.

*Preparation of Chitosan-stabilized Gold Nanoclusters*

**[0027]** Gold nanoclusters were prepared by first adding 1.2 mL of aqueous solution of $HAuCl_4$ (10 mM) to 20 mL of 0.5 % (w/v) chitosan (which was solubilized using 0.1 % glacial acetic acid) under stirring condition. This was followed by addition of 0.8 mL mercaptopropionic acid (0.11 M). Stirring was continued for 30 minutes and the pH of the resulting solution was adjusted below 2.5.

**[0028]** In the present device, the biocompatible and non-immunogenic nature of chitosan makes it an ideal platform for sensing purposes. Moreover, the nontoxic nature of gold (in the form of clusters) may also permit its usage for the said objective.

*Preparation of Gold Nanocluster Containing membrane strip or film:*

**[0029]** The medium or resulting solution (30 mL) containing as-synthesized gold nanocluster was poured onto a petri dish (Tarsons, disposable sterile petridish) and was then allowed to dry overnight in an oven at 55 °C. A film was formed with the approximate dimension of 3 x 3 $cm^2$. The intrinsic luminescent properties of gold nanoclusters remained intact after the formation of the film. The film had yellow-orange luminescence upon excitation with 254 nm UV light. The film could be picked up easily with tweezers.

**[0030]** In a similar way, the as-prepared gold nanoclusters (1 mL) were drop-cast on an approximately 3 x 3 $cm^2$ polyvinylidene difluoride (PVDF) membrane [Figure 1] and the coated membrane was used for further experiments.

*Coating of the Gold Nanocluster Containing Film with Copper Sulphate:*

**[0031]** The gold nanocluster coated polyvinylidene difluoride (PVDF) was treated with 0.2 mL $CuS0_4$ (50.2 mM) solution. The film was then dried in the air for 30 min and was observed under UV light. The luminescence intensity of the film got substantially reduced (upon exposure to 254 nm UV light) following addition of the copper salt [Fig 2].

*Interaction of Bilirubin Solution with Copper Sulphate added Gold Nanocluster Containing Film:*

**[0032]** A 0.2 mL aqueous solution of bilirubin (1.1 mg/dL) was added to gold nanocluster containing film which was previously treated with 0.2 mL of CuS04 (50.2 mM) solution. The film was then dried for 30 min and then was viewed using UV light. Recovery of the yellow- orange luminescence of the film was observed [Fig 3]. The intense yellow-orange color indicated recovery of luminescence of the film.

**[0033]** Control experiment with addition of water instead of bilirubin showed little or no effect on the luminescence intensity recovery of the nanoclusters under similar conditions [Fig 4]. The observation further confirmed that bilirubin was instrumental in the luminescence intensity restoration of the gold nanoclusters.

*Solution Phase Assay of Bilirubin Using the luminescence of Gold Nanoclusters:*

**[0034]** The liquid phase assay of bilirubin provided an alternative for high sensitivity detection using the luminescence of the gold nanoclusters. This was achieved by the quenching of photoluminescence of gold nanoclusters by copper ions, followed by recovery of the same upon addition of bilirubin to the system.

**[0035]** For studying the interaction of copper ions with luminescent gold nanoclusters, 0.02 mL of copper sulfate solution (12.5 mg/mL) was gradually added to luminescent gold nanoclusters (0.1 mL in 0.7 mL of glycine buffer maintained at pH < 2.5) till the intensity of luminescence stopped changing [Fig 5 (A)]. Corresponding Stern-Volmer plot was obtained as shown in [Fig 5(B)]. As shown in Fig 5(B), $I_0$ is the luminescence intensity of as-synthesized gold nanoclusters and I is the reduced luminescence intensity of the same on subsequent addition of $Cu^{2+}$ ions. Au NCs means gold nanoclusters.

*Interaction of gold nanoclusters with copper ions and bilirubin:*

**[0036]** For studying the interaction of gold nanoclusters with copper ions and bilirubin, the stabilized gold nanoclusters membrane strip (prepared with 0.1 mL gold nanocluster solution in 0.7 mL of glycine buffer; total volume was 0.8 mL) was treated with 0.1 mL of copper ions (12.5 mg/mL). This caused quenching in luminescence intensity of nanoclusters in the strip. The resulting strip was subsequently treated with bilirubin (1.1 mg/dL). This led to recovery in lost luminescence intensity of the clusters in the strip [Fig 6(A)]. Control experiments were performed with water [Fig 6(B) and bilirubin Fig 6(C)]. Little recovery in intensity of nanoclusters was observed upon addition of water. Whereas in case of bilirubin, the same volume as that of water, caused full recovery in luminescence intensity of the nanoclusters. In case of only bilirubin i.e. without copper added, there was no apparent change in the luminance intensity.

**[0037]** This marks the essentiality of copper as well as bilirubin for the aforementioned process of quenching and recovery to occur.

**[0038]** Similar experiment was performed with different concentration of copper ion and bilirubin [Fig 7(A)] and corresponding Stern-Volmer plot of recovery of luminescence was obtained [Fig 7 (B)]. Another set of experiments was performed in a similar manner to determine the efficiency of bilirubin detection using considerably higher concentration of copper ions. The sensitivity of bilirubin detection using concentration of copper ions as high as that of 477.33 mg/dL was found to be 0.84 mg/dL. The recovery of luminescence intensity of the nanoclusters by bilirubin with respect to copper ions followed an exponential fitting.

**[0039]** Also, similar experiment was performed with blood serum of patents affected with hyperbilirubinemia (6.4 mg/dL as per clinical report). The result indicated substantial recovery of luminescence intensity of gold nanoclusters otherwise quenched in the presence of $Cu^{2+}$ ions. The best sensitivity of bilirubin detection in the said assay was found to be 0.0064 mg/dL [Fig 8].

*Thumb Impression Analysis on copper Salt Treated Gold Nanocluster film*

**[0040]** A volunteer affected with jaundice (bilirubin level 6.2 mg/dL) measured using conventional technique as well as the current solution based technique) gave his thumb impression on the $CuS0_4$ solution treated stabilized gold nanocluster containing film [Fig 9 (A)]. The thumb impression was recorded for a time period of 5 minutes. This had resulted in recovery of luminescence which was clearly observed under UV light [Fig 9 (B)]. Appropriate protocol was followed for recording of the thumb impression.

**[0041]** Control experiment was performed with the thumb impression of a volunteer with no indication of higher bilirubin level, which had no or little effect on the intensity of copper salt and gold cluster containing film [Fig 10].

**[0042]** Thus, the present invention discloses a novel and advanced nanotechnology-based fast and easy device and method for detecting bilirubin level in a person and simultaneously identifying hyperbilirubinemia condition in the person in non-invasive manner. The method invented circumvents the need of blood test for fast analysis and uses primarily thumb impression for identification of hyperbilirubinemia. It is inexpensive and versatile as the analysis can be performed without the need of a typical pathological laboratory setup.

**[0043]** The method advantageously involves the use of luminescent gold nanocluster film, the intensity of which was effectively quenched in the presence of copper salt. The detection method relied on the recovery of luminescent intensity in the presence of bilirubin. The change in intensity was equally effective in liquid medium

as well as in the solid phase. Thus facile detection of bilirubin was possible using blood serum as well as through thumb impression. The best sensitivity of detection of bilirubin in the liquid phase was 0.0064 mg/dL and that in the solid phase was achieved with the concentration of blood bilirubin in serum of the affected patient being 6.2 mg/dL (as per clinical report).

**[0044]** In addition, the liquid phase detection provides a background free high sensitivity method and relying on this it is possible to successfully detect excess bilirubin present in blood serum down to a sensitivity of 0.0064 mg/dL. This is less than the limit of detection using conventional methods, which are generally used in the pathological laboratories.

**[0045]** In a nutshell, the device of the present invention is superior to the commonly practiced method as it enables prompt, easy and precise detection of bilirubin level in human body and the condition of jaundice and could be used by common mass.

## Claims

1. A diagnostic device or kit for visual detection of bilirubin comprising a polymer membrane strip based bilirubin indicator having a chitosan stabilized gold nanoclusters based luminescence source treated by a $Cu^{2+}$ ions source to quench luminescence intensity of said gold nanoclusters; a UV source; and a variable luminescence intensity detector to visually indicate the quenched luminescence intensity in absence of liquid or solid phased bilirubin samples or the recovered luminescence intensity values in presence of liquid or solid phased bilirubin samples.

2. A diagnostic device or kit as claimed in claim 1, wherein said chitosan stabilized gold nanoclusters comprise film of chitosan stabilized fluorescent gold nanoclusters which is usually luminescent yellow orange in presence of the UV light and said $Cu^{2+}$ ions source comprises copper salt.

3. A diagnostic device or kit as claimed in anyone of claims 1 or 2, wherein the liquid or solid phased bilirubin samples includes blood serum and/or skin.

4. A diagnostic device or kit as claimed in claim 1, wherein the polymer membrane strip based bilirubin indicator comprises said luminescent chitosan stabilized gold nanoclusters embedded in a biopolymer film, said $Cu^{2+}$ ions source for quenching luminescence intensity of the gold nanoclusters on said strip, and indications on recovery of the quenched luminescence intensity in presence of the liquid or solid phased bilirubin samples.

5. A diagnostic device or kit as claimed in anyone of claims 1 to 4, wherein said chitosan stabilized gold nanoclusters are formed on the film involving a chitosan stabilized gold nanoclusters solution obtained of aqueous solution of $HAuCl_4$, chitosan, glacial acetic acid and mercaptopropionic acid having a pH of less than 2.5.

6. A diagnostic device or kit as claimed in anyone of claims 1 to 5, wherein said chitosan stabilized gold nanoclusters comprise a film or casted upon polymeric membrane preferably polyvinylidene difluoride (PVDF) membrane which is treatable with said $Cu^{2+}$ ions source preferably $CuSo_4$ solution.

7. A diagnostic device or kit as claimed in anyone of claims 1 to 6, comprising a support platform for said chitosan stabilized gold nanoclusters based luminescence source on film or polymer membrane strip adapted for said bilirubin sample in liquid phase including the bilirubin source in water or blood serum or in solid phase form including body/thumb (palm) contact of individual with said chitosan stabilized gold nanoclusters;
said $Cu^{2+}$ ions source treated said chitosan stabilized gold nanoclusters for indicating absence or presence of bilirubin based on variable luminescence intensity based on any complex formed with $Cu^{2+}$ ions with bilirubin in said sample.

8. The diagnostic device or kit as claimed in claim 7, comprising a touch pad for providing said polymer membrane strip in contact with thumb of the person and allowing the solid phased bilirubin in thumb skin to form the complex with the $Cu^{2+}$ ions on said gold nanoclusters in case of bilirubin presence and thereby recovering the luminescence intensity of the strip indicating hyper-bilirubinemia.

9. The diagnostic device or kit as claimed in anyone of claims 1 to 8, wherein sensitivity level for bilirubin detection is 0.0064 mg/dL using blood serum and that for bilirubin in water being 0.058 mg/dL.

10. The diagnostic device or kit as claimed in anyone of claims 1 to 9, wherein the thumb imprint on the polymer membrane strip leading to luminescence intensity recovery of the strip corresponds to bilirubin concentration of at least 6.2 mg/dL indicating hyper-bilirubinemia.

11. A process for manufacturing diagnostic device or kit as claimed in anyone of claims 1 to 10, comprising providing a chitosan stabilized gold nanoclusters based luminescence source on a polymer membrane strip based bilirubin indicator; treating the strip with a $Cu^{2+}$ ions source to quench luminescence intensity of said gold nanoclusters; providing a UV source; and involving a variable luminescence intensity detector to visually indicate the quenched lumi-

nescence intensity in absence of liquid or solid phased bilirubin samples or the recovered luminescence intensity values in presence of liquid or solid phased bilirubin samples.

**12.** A process as claimed claim 11, wherein said step of providing said chitosan stabilized gold nanoclusters based luminescence source comprises:

preparing chitosan stabilized gold nanoclusters by adding aqueous solution of $HAuCl_4$ to chitosan followed by addition of mercaptopropionic acid and adjusting pH of resulting solution below 2.5;
preparing chitosan stabilized gold nanoclusters film by drop-casting the resulting solution of the chitosan stabilized gold nanoclusters on the polyvinylidene difluoride (PVDF) membrane strip; and
providing said Cu source comprises providing aqueous solution of copper salt including 0.2 mL $CuS0_4$ (50.2 mM) solution.

**13.** The process as claimed in anyone of claims 11 or 12, wherein said step of preparing chitosan stabilized gold nanoclusters comprises 1.2 mL of aqueous solution of 10 mM $HAuCl_4$ added to 20 mL of 0.5 % (w/v) chitosan under stirring condition with subsequent addition of 0.8 mL mercaptopropionic acid (0.11 M) under 30 minutes continuous stirring for preparing the chitosan stabilized gold nanoclusters.

**14.** The process as claimed in anyone of claims 11 to 13, wherein the preparation of the chitosan stabilized gold nanoclusters film includes pouring synthesized gold nanocluster solution onto a petri dish and allowing to dry overnight in an oven to form the film.

**Patentansprüche**

**1.** Diagnoseeinrichtung oder -kit zur visuellen Detektion von Bilirubin umfassend
einen Bilirubin-Indikator basierend auf Polymermembranstreifen, der eine Lumineszenzquelle basierend auf Chitosan-stabilisierten Gold-Nanoclustern aufweist, die durch eine Quelle von $Cu^{2+}$-Ionen behandelt ist, um die Lumineszenzintensität der Gold-Nanocluster zu löschen;
eine UV-Quelle; und
einen veränderlichen Lumineszenzintensitätsdetektor, um die gelöschte Lumineszenzintensität in Abwesenheit von Bilirubinproben in Flüssig- oder Festphase oder die rückgewonnenen Lumineszenzintensitätswerte in Anwesenheit von Bilirubinproben in Flüssig- oder Festphase visuell anzuzeigen.

**2.** Diagnoseeinrichtung oder -kit nach Anspruch 1, worin die Chitosan-stabilisierten Gold-Nanoclusters einen Film von Chitosan-stabilisierten fluoreszierenden Gold-Nanoclustern umfassen, der üblicherweise lumineszierend gelborange in Anwesenheit des UV-Lichtes ist, und die Quelle von $Cu^{2+}$-Ionen Kupfersalz umfasst.

**3.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 oder 2, worin die Bilirubinproben in Flüssig- oder Festphase Blutserum und/oder Haut umfassen.

**4.** Diagnoseeinrichtung oder -kit nach Anspruch 1, worin der Bilirubin-Indikator basierend auf Polymermembranstreifen die lumineszierenden Chitosan-stabilisierten Gold-Nanoclusters, die in einem Biopolymerfilm eingebettet sind, die Quelle von $Cu^{2+}$-Ionen zum Löschen der Lumineszenzintensität der Gold-Nanoclusters auf dem Streifen, und Angaben über die Rückgewinnung der gelöschten Lumineszenzintensität in Anwesenheit der Bilirubinproben in Flüssig- oder Festphase umfasst.

**5.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 bis 4, worin die Chitosan-stabilisierten Gold-Nanoclusters auf dem Film unter Verwendung einer Lösung von Chitosan-stabilisierten Gold-Nanoclustern gebildet sind, die von einer wässrigen Lösung aus $HAuCl_4$, Chitosan, Eisessigsäure und Mercaptopropionsäure mit einem pH-Wert kleiner als 2,5 erhalten wird.

**6.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 bis 5, worin die Chitosan-stabilisierten Gold-Nanoclusters einen Film umfassen oder auf eine Polymermembran, vorzugsweise eine Membran aus Polyvinylidendifluorid (PVDF), gegossen sind, die mit der Quelle von $Cu^{2+}$-Ionen, vorzugsweise einer $CuSO_4$-Lösung, behandelt werden kann.

**7.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 bis 6, umfassend eine Stützplattform für die Lumineszenzquelle basierend auf Chitosan-stabilisierten Gold-Nanoclustern auf Film oder auf Polymermembranstreifen, geeignet für die Bilirubinprobe in Flüssigphase einschließlich der Bilirubinquelle in Wasser oder Blutserum oder in Form von Festphase einschließlich Körper-/Daumen-(Handflächen- )kontakts einer Person mit den Chitosan-stabilisierten Gold-Nanoclustern;
die Quelle von $Cu^{2+}$-Ionen behandelte die Chitosan-stabilisierten Gold-Nanoclusters, um die Abwesenheit oder Anwesenheit von Bilirubin aufgrund der veränderlichen Lumineszenzintensität aufgrund jedes Komplexes, der mit $Cu^{2+}$-Ionen gebildet ist, mit Bilirubin in der Probe anzuzeigen.

**8.** Diagnoseeinrichtung oder -kit nach Anspruch 7, um-

fassend ein Berührungspad zum Bereitstellen, dass der Polymermembranstreifen mit dem Daumen einer Person in Kontakt kommt, und zum Ermöglichen, dass das Bilirubin in Festphase in der Daumenhaut den Komplex mit den $Cu^{2+}$-Ionen auf den Gold-Nanoclustern im Falle von Anwesenheit von Bilirubin bildet und somit die Lumineszenzintensität des Streifens rückgewinnt, die Hyperbilirubinämie anzeigt.

**9.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 bis 8, worin der Empfindlichkeitsgrad für die Detektion des Bilirubins 0,0064 mg/dL unter der Verwendung von Blutserum und für Bilirubin im Wasser 0,058 mg/dL beträgt.

**10.** Diagnoseeinrichtung oder -kit nach einem der Ansprüche 1 bis 9, worin der Daumenabdruck auf dem Polymermembranstreifen, der zur Rückgewinnung der Lumineszenzintensität des Streifens führt, einer Bilirubinkonzentration von zumindest 6,2 mg/dL entspricht, die Hyperbilirubinämie anzeigt.

**11.** Verfahren zur Herstellung einer Diagnoseeinrichtung oder eines Diagnosekits nach einem der Ansprüche 1 bis 10, umfassend das Bereitstellen einer Lumineszenzquelle basierend auf Chitosan-stabilisierten Gold-Nanoclustern auf einem Bilirubin-Indikator basierend auf Polymermembranstreifen; das Behandeln des Streifens mit einer Quelle von $Cu^{2+}$-Ionen, um die Lumineszenzintensität der Gold-Nanocluster zu löschen;
das Bereitstellen einer UV-Quelle; und
das Verwenden eines veränderlichen Lumineszenzintensitätsdetektors, um die gelöschte Lumineszenzintensität in Abwesenheit von Bilirubinproben in Flüssig- oder Festphase oder die rückgewonnenen Lumineszenzintensitätswerte in Anwesenheit von Bilirubinproben in Flüssig- oder Festphase visuell anzuzeigen.

**12.** Verfahren nach Anspruch 11, worin der Schritt des Bereitstellens der Lumineszenzquelle basierend auf Chitosan-stabilisierten Gold-Nanoclustern umfasst:

das Vorbereiten von Chitosan-stabilisierten Gold-Nanoclustern durch Hinzufügen einer wässrigen Lösung aus $HAuCl_4$ zu Chitosan, gefolgt von dem Hinzufügen von Mercaptopropionsäure und von dem Einstellen des pH-Werts der resultierenden Lösung unter 2,5;
das Vorbereiten eines Films von Chitosan-stabilisierten Gold-Nanoclustern durch Auftropfen der resultierenden Lösung der Chitosan-stabilisierten Gold-Nanoclusters auf dem Membranstreifen aus Polyvinylidendifluorid (PVDF); und
das Bereitstellen der Cu-Quelle das Bereitstellen einer wässrigen Lösung aus Kupfersalz einschließlich 0,2 mL einer $CuSO_4$ (50,2 mM) -Lösung umfasst.

**13.** Verfahren nach einem der Ansprüche 11 oder 12, worin der Schritt des Vorbereitens von Chitosan-stabilisierten Gold-Nanoclustern 1,2 mL einer wässrigen Lösung aus 10 mM $HAuCl_4$ umfasst, die zu 20 mL von 0,5% (Gewicht/Volumen) Chitosan unter Rührbedingung hinzugefügt werden, mit darauffolgendem Hinzufügen von 0,8 mL Mercaptopropionsäure (0,11 M) unter einem kontinuierlichen, 30 Minuten langen Rühren, zur Vorbereitung der Chitosan-stabilisierten Gold-Nanoclusters.

**14.** Verfahren nach einem der Ansprüche 11 bis 13, worin das Vorbereiten des Films aus Chitosan-stabilisierten Gold-Nanoclustern das Gießen einer synthetisierten Gold-Nanocluster-Lösung auf eine Petri-Schale und das Trocknen nachtsüber in einem Ofen zum Bilden des Films umfasst.

**Revendications**

**1.** Dispositif ou kit de diagnostic pour la détection visuelle de la bilirubine comprenant un indicateur de bilirubine à base de bande de membrane polymère ayant une source de luminescence à base de nanoagrégants d'or stabilisés avec chitosane traitée par une source d'ions $Cu^{2+}$ pour éteindre l'intensité de luminescence desdits nanoagrégants d'or ;
une source UV ; et
un détecteur d'intensité de luminescence variable pour indiquer visuellement l'intensité de luminescence éteinte en l'absence d'échantillons de bilirubine en phase liquide ou solide.

**2.** Dispositif ou kit de diagnostic comme revendiqué dans la revendication 1, où lesdits nanoagrégants d'or stabilisés avec chitosane comprennent un film de nanoagrégants d'or fluorescents stabilisés avec chitosane qui est généralement jaune orange luminescent en présence de la lumière UV et ladite source d'ions $Cu^{2+}$ comprend du sel de cuivre.

**3.** Dispositif ou kit de diagnostic comme revendiqué dans l'une quelconque des revendications 1 ou 2, où les échantillons de bilirubine en phase liquide ou solide comprennent du sérum sanguin et/ou de la peau.

**4.** Dispositif ou kit de diagnostic comme revendiqué dans la revendication 1, où l'indicateur de bilirubine à base de bande de membrane polymère comprend lesdits nanoagrégants d'or stabilisés avec chitosane luminescents incorporés dans un film de biopolymère, ladite source d'ions $Cu^{2+}$ pour éteindre l'intensité de luminescence des nanoagrégants d'or sur ladite bande, et des indications sur la récupération de l'in-

tensité de luminescence éteinte en présence des échantillons de bilirubine en phase liquide ou solide.

**5.** Dispositif ou kit de diagnostic comme revendique dans l'une quelconque des revendications de 1 à 4, où lesdits nanoagrégants d'or stabilisés avec chitosane sont formés sur le film impliquant une solution de nanoagrégants d'or stabilisés avec chitosane obtenue d'une solution aqueuse de $HAuCl_4$, chitosane, acide acétique glacial et acide mercaptopropionique ayant un pH inférieur à 2,5.

**6.** Dispositif ou kit de diagnostic comme revendiqué dans l'une quelconque des revendications de 1 à 5, où lesdits nanoagrégants d'or stabilisés avec chitosane comprennent un film ou une membrane polymère moulée, de préférence une membrane en difluorure de polyvinylidène (PVDF) qui peut être traitée avec ladite source d'ions $Cu^{2+}$, de préférence une solution $CuSO_4$.

**7.** Dispositif ou kit de diagnostic comme revendiqué dans l'une quelconque des revendications de 1 à 6, comprenant une plate-forme de support pour ladite source de luminescence à base de nanoagrégants d'or stabilisés avec chitosane sur un film ou une bande de membrane polymère adaptée pour ledit échantillon de bilirubine en phase liquide comprenant la source de bilirubine dans l'eau ou le sérum sanguin ou sous forme de phase solide comprenant un contact corps/pouce (paume) de l'individu avec lesdits nanoagrégants d'or stabilisés avec chitosane ;
ladite source d'ions $Cu^{2+}$ a traité lesdits nanoagrégants d'or stabilisés avec chitosane pour indiquer l'absence ou la présence de bilirubine sur la base d'une intensité de luminescence variable basée sur tous les complexes formés avec des ions $Cu^{2+}$ avec de la bilirubine dans ledit échantillon.

**8.** Dispositif ou kit de diagnostic comme revendiqué dans la revendication 7, comprenant une tablette tactile pour fournir ladite bande de membrane polymère en contact avec le pouce de la personne et permettant à la bilirubine en phase solide dans la peau du pouce de former le complexe avec les ions $Cu^{2+}$ sur ledit nanoagrégants d'or en cas de présence de bilirubine et récupérant ainsi l'intensité de luminescence de la bande indiquant une hyperbilirubinémie.

**9.** Dispositif ou kit de diagnostic comme revendiqué dans l'une quelconque des revendications de 1 à 8, où le niveau de sensibilité pour la détection de la bilirubine est de 0,0064 mg/dL en utilisant du sérum sanguin et celui pour la bilirubine dans l'eau étant de 0,058 mg/dL.

**10.** Dispositif ou kit de diagnostic comme revendiqué dans l'une quelconque des revendications de 1 à 9, où l'empreinte du pouce sur la bande de membrane polymère conduisant à une récupération d'intensité de luminescence de la bande correspond à une concentration de bilirubine d'au moins 6,2 mg/dL indiquant une hyperbilirubinémie.

**11.** Procédé de fabrication d'un dispositif ou d'un kit de diagnostic comme revendiqué dans l'une quelconque des revendications de 1 à 10, comprenant la fourniture d'une source de luminescence à base de nanoagrégants d'or stabilisés avec chitosane sur un indicateur de bilirubine à base de bande de membrane polymère ;
le traitement de la bande avec une source d'ions $Cu^{2+}$ pour éteindre l'intensité de luminescence desdits nanoagrégants d'or ;
la fourniture d'une source UV ; et
l'implication d'un détecteur d'intensité de luminescence variable pour indiquer visuellement l'intensité de luminescence éteinte en l'absence d'échantillons de bilirubine en phase liquide ou solide ou les valeurs d'intensité de luminescence récupérées en présence d'échantillons de bilirubine en phase liquide ou solide.

**12.** Procédé comme revendiqué dans la revendication 11, où ladite étape de fourniture de ladite source de luminescence à base de nanoagrégants d'or stabilisés avec chitosane comprend :

la préparation des nanoagrégants d'or stabilisés avec chitosane en ajoutant une solution aqueuse de $HAuCl_4$ au chitosane, puis en ajoutant de l'acide mercaptopropionique et en ajustant le pH de la solution résultante en dessous de 2,5 ;
la préparation d'un film de nanoagrégants d'or stabilisés avec chitosane en moulable par coulée la solution résultante des nanoagrégants d'or stabilisés avec chitosane sur la bande de membrane en difluorure de polyvinylidène (PVDF) ; et
la fourniture de ladite source de Cu comprend la fourniture d'une solution aqueuse de sel de cuivre comprenant 0,2 mL de solution de $CuSO_4$ (50,2 mM).

**13.** Procédé comme revendiqué dans l'une quelconque des revendications 11 ou 12, où ladite étape de préparation de nanoagrégants d'or stabilisés avec chitosane comprend 1,2 mL de solution aqueuse de 10 mM de $HAuCl_4$ ajoutée à 20 mL de chitosane à 0,5% (p/v) sous condition d'agitation avec une addition suivante de 0,8 mL d'acide mercaptopropionique (0,11 M) sous 30 minutes d'agitation continue pour la préparation des nanoagrégants d'or stabilisés avec chitosane.

**14.** Procédé comme revendiqué dans l'une quelconque des revendications de 11 à 13, où la préparation du film de nanoagrégants d'or stabilisés avec chitosane comprend le versement d'une solution de nanoagrégants d'or synthétisée sur une boîte de Pétri et le séchage pendant une nuit dans un four pour former le film.

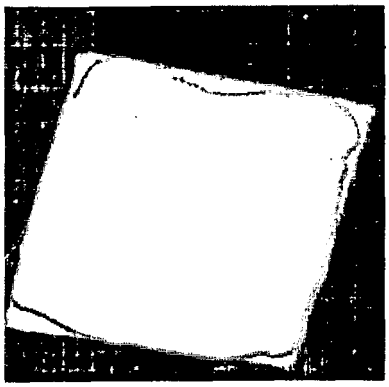

Figure 1

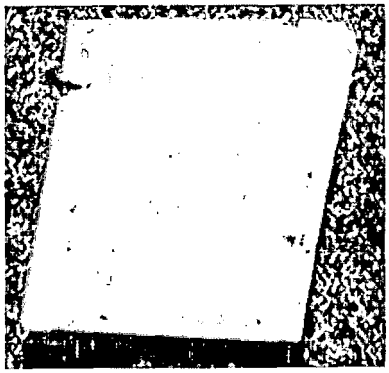

Figure 2

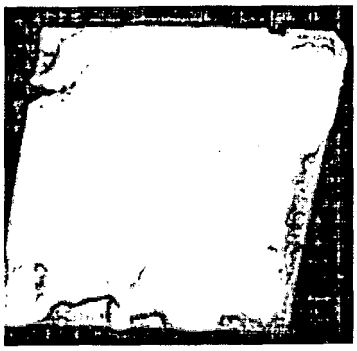

Figure 3

Figure 4A

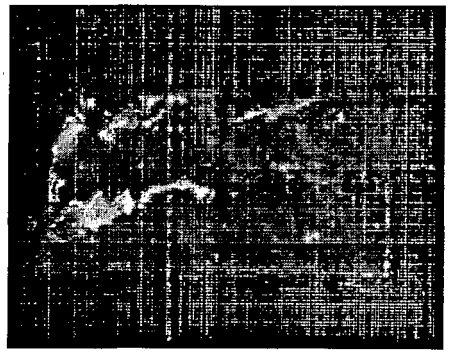

Figure 4B

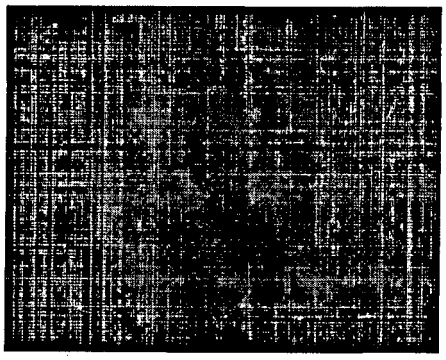

Figure 4C

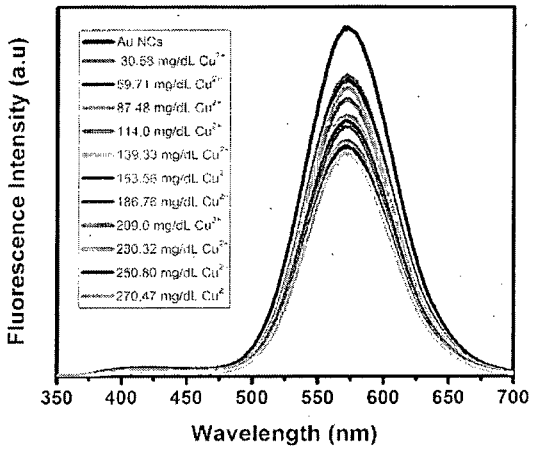


Figure 5A

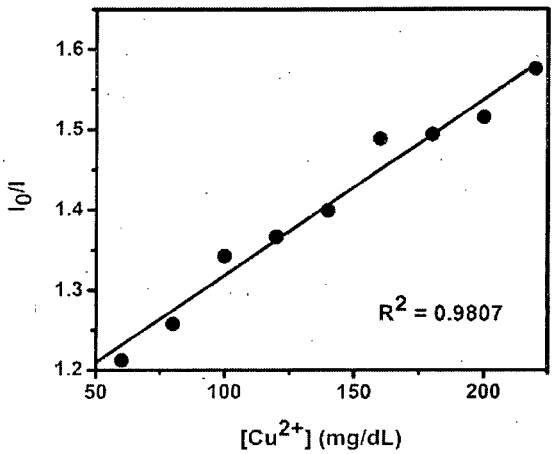


Figure 5B

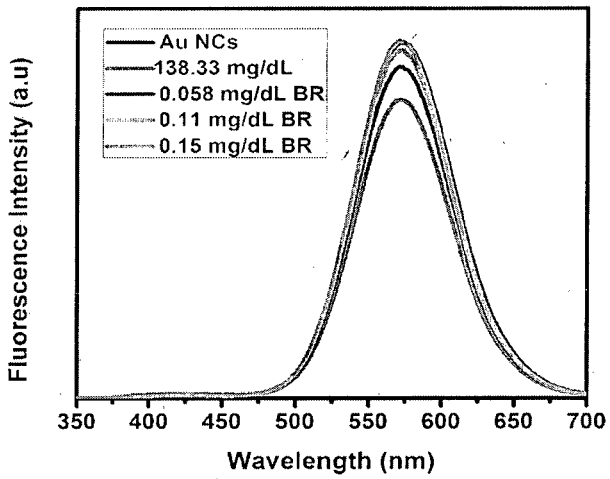


Figure 6A

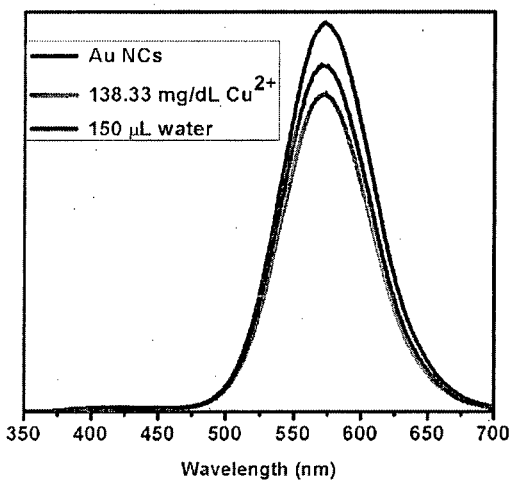


Figure 6B

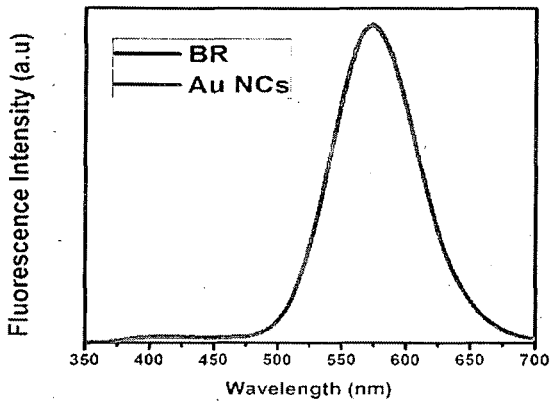


Figure 6C

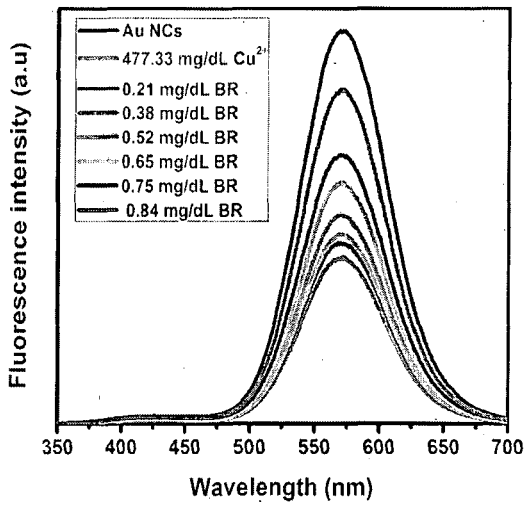


Figure 7A

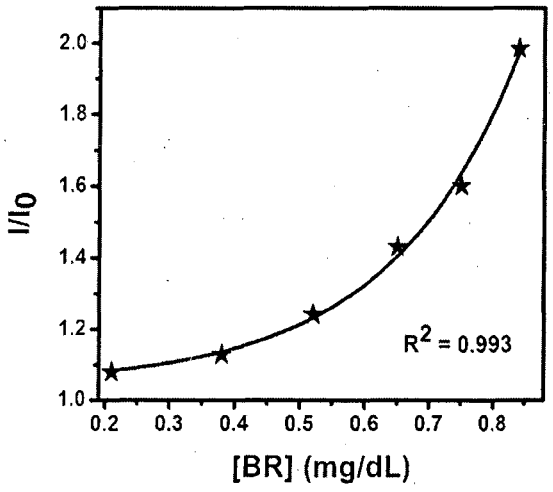


Figure 7B

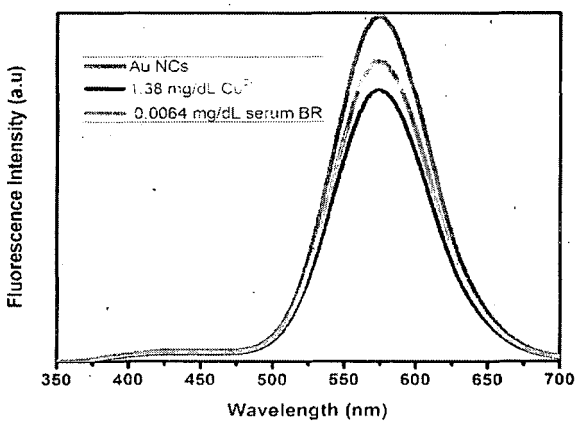


Figure 8

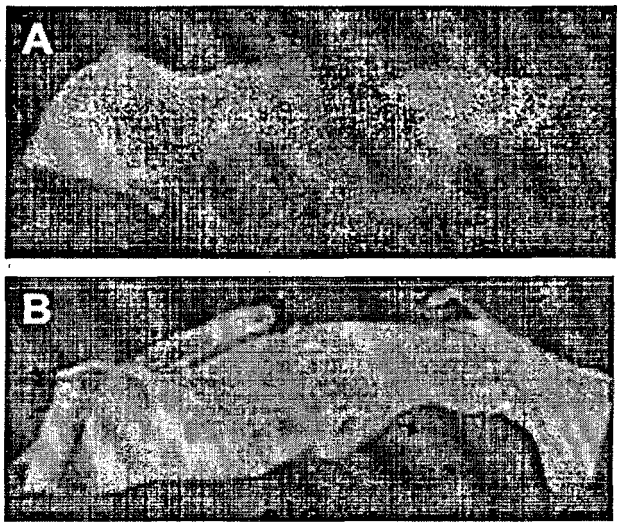


Figure 9

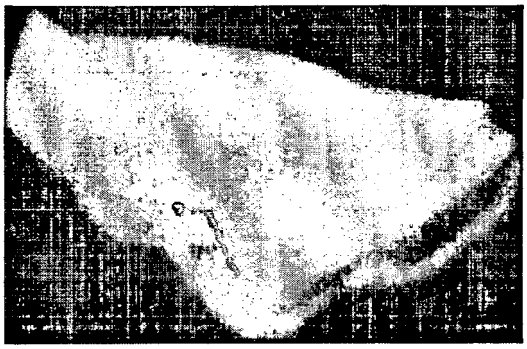

Figure 10

## REFERENCES CITED IN THE DESCRIPTION

*This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.*

### Patent documents cited in the description


- WO 2006138698 A2 **[0011]**


### Non-patent literature cited in the description


- **GNOTH, C. ; S. JOHNSON.** *Geburtshilfe und Frauenheilkunde,* 2014, 661-669 **[0004]**
- Affordable pathogen detection kit launched. The Hindu. 21 February 2013 **[0005]**
- **DEKA, J. ; PAUL, A.** Chattopadhyay. *A. The Journal of Physical Chemistry C,* 2009, vol. 113, 6936 **[0007]**
- **MIRKIN, C. A. ; LETSINGER, R. L. ; MUCIC, R. C. ; STORHOFF, J.** *J. Nature,* 1996, vol. 382, 607 **[0007]**
- **STORHOFF, J. J. ; LUCAS, A. D. ; GARIMELLA, V. ; BAO, Y. P. ; MULLER, U. R.** *Nat Biotech,* 2004, vol. 22, 883 **[0007]**
- **HEMMATEENEJAD, B. ; SHAKERIZADEH-SHIRAZI, F. ; SAMARI, F.** *Sensors and Actuators B: Chemical,* 2014, vol. 199, 42 **[0008]**
- **SANTHOSH, M. ; CHINNADAYYALA, S. R. ; KAKOTI, A. ; GOSWAMI, P.** *Biosensors and Bioelectronics,* 2014, vol. 59, 370 **[0009]**
- **S. K. SAILAPU ; A. K. SAHOO ; S. S. GHOSH ; A. CHATTOPADHYAY.** Hierarchical Logic Structures Based on Responsive Fluorescent Gold Nanoclusters. *Small,* 2014, vol. 10, 4067-4071 **[0010]**
- **C. BANERJEE ; J. KUCHLYAN ; D. BANIK ; N. KUNDU ; A. ROY ; S. GHOSH ; N. SARKAR.** Interaction of gold nanoclusters with IR light emitting cyanine dyes: a systematic fluorescence quenching study. *Physical Chemistry Chemical Physics,* 2014, vol. 16, 17272-17283 **[0010]**

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|---|---|---|---|
| 专利名称(译) | 胆红素的视觉检测装置 | | |
| 公开(公告)号 | EP3302449B1 | 公开(公告)日 | 2020-04-01 |
| 申请号 | EP2016802704 | 申请日 | 2016-06-02 |
| [标]申请(专利权)人(译) | 印度INST TECH古瓦哈蒂 | | |
| 申请(专利权)人(译) | 印度理工大学，古瓦哈提 | | |
| 当前申请(专利权)人(译) | 印度理工大学，古瓦哈提 | | |
| [标]发明人 | CHATTOPADHYAY ARUN<br>PAUL ANUMITA<br>BASU SRESTHA<br>SAHOO AMARESH KUMAR | | |
| 发明人 | CHATTOPADHYAY, ARUN<br>PAUL, ANUMITA<br>BASU, SRESTHA<br>SAHOO, AMARESH KUMAR | | |
| IPC分类号 | A61K31/00 A61B5/00 G01N33/72 G01N21/64 A61B5/145 A61B5/1455 A61K49/00 | | |
| CPC分类号 | A61B5/0071 A61B5/14546 A61B5/1455 A61K49/0004 A61K49/0065 G01N33/728 G01N21/643 G01N21/6447 G01N2021/6432 G01N2021/6439 | | |
| 优先权 | 631KOL2015 2015-06-04 IN | | |
| 其他公开文献 | EP3302449A1<br>EP3302449A4 | | |
| 外部链接 | Espacenet | | |


摘要(译)

本发明公开了用于视觉检测胆红素的诊断装置或试剂盒。 所述诊断装置或试剂盒包括基于壳聚糖稳定的金纳米团簇的发光源，用于猝灭所述金纳米团簇的发光强度和在胆红素存在下恢复淬灭的发光强度的Cu 2+离子源。 所述装置能够通过视觉或从血清中通过拇指印象无创地检测高胆红素血症。


Figure 1

Figure 2

Figure 3