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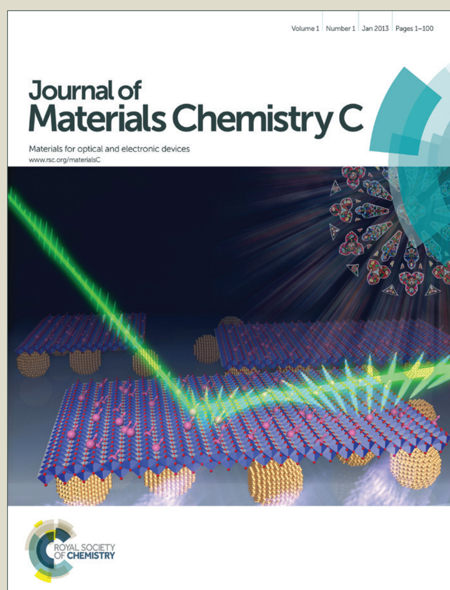

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Full Papers

Upconversion Luminescence Nanoparticles-Based Lateral Flow Immunochromatographic Assay for Cephalexin Detection

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Owing to the near infrared excitation, upconversion luminescence (UCL) rare-earth nanoparticles are very suitable for biological applications for the low background interference. $NaGdF_4$:Yb,Er nanoparticles were firstly synthesized through a replacement reaction at high temperature. In order to improve the upconversion luminescence efficiency, core-shell $NaGdF_4$:Yb,Er@$NaGdF_4$ nanoparticles were then prepared by a seed-mediated method. The core-shell architecture improved the upconversion luminescence greatly and more effectively retained the luminescence during the following phase transfer process by ligand exchange. Upon further coupling with anti-cephalexin monoclonal antibody via "click" reaction, the resultant upconversion luminescence probe was obtained and used in lateral flow immunochromatographic assay (LFIA) to detect antibiotic residue of cephalexin. The results were compared with those achieved by nanoprobe based on gold nanoparticles. More quantitative results were extracted by luminescent intensity analysis. Under optimized conditions, the detection limit of UCL nanoparticles-based LFIA was quite comparable with gold nanoparticles-based LFIA.

Introduction

The lateral flow immunochromatographic assay (LFIA), also known as lateral flow test, has been widely used for environmental monitoring[1], food safety monitoring[2], medical diagnosis[3], especially home testing[4] and point of care testing[5] during past twenty years. The technique of LFIA incorporates immune recognition reaction and capillary action of thin layer chromatography. Colored particle such as colloidal gold has been most widely adopted in LFIA as labels of antibody. By reason of the excellent chemical stability and vivid red color caused by localized surface plasmon resonance of Au nanoparticles, colloidal gold nanoparticles-based LFIA can be used for the qualitative or semi-quantitative detection of various target analytes in solutions through colorimetric read either by naked eyes or with the aid of optical density analysis. Other particles including selenium[6], carbon[7], latex[8], etc, were also applied in LFIA.

Meanwhile, developing new labeling materials provides additional means besides optical density, such as the detection based on magnetic or fluorescent signals, for improving the detection sensitivity[9-11]. For example, luminescent materials including organic dyes and inorganic semiconductor quantum dots (QDs) have emerged as suitable optical labels in LFIA[12, 13]. The high sensitivity of fluorescence detection make them exhibit enormous potential in biological analysis. However, intrinsic limitations remain to be overcome, such as the photobleaching of the probe and the background signals of residues beyond the analyte.

Upconversion luminescence (UCL) rare earth nanoparticles as novel and alternative luminescent materials attract increasing interest in biological labeling and detection[14, 15]. Upconversion luminescence is a nonlinear optical process via a two-photon or multi-photon absorption mechanism, which can convert long-wavelength near-infrared (NIR) radiations into short-wavelength visible light. UCL materials own the attractive luminescence characteristics, such as large effective Stokes shifts, sharp emissions, long fluorescence lifetimes, and high resistance to photobleaching[14]. Since upconversion luminescence is typically absent or low for most materials contrasting fluorescence, the detection based on UCL particles is characterized by high signal-to-noise ratios, which makes the UCL particles potentially useful as labels for LFIA.

Herein, we report a sensitive lateral flow immunochromatographic assay based on UCL nanoparticles. In fact, the antibiotic abuse has become a serious issue worldwide due to the overuse in aquaculture, livestock, etc. In consequence, the antibiotic residues left in food give rise to adverse effects on human health, such as allergic reactions, promoting drug resistance of pathogenic bacteria that may transfer antibiotic resistance genes to human pathogens[16, 17]. Cephalexin (CEX) is a β-lactam antibiotic widely used in veterinary medicine to treat mastitis and other infectious diseases in dairy cows. The residues

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in milk or other edible animal tissues will be harmful to people's health[18]. Therefore, CEX is chosen as a model antibiotic in the current study for antibiotic detection through UCL nanoparticles-based LFIA.

Following on from our previous investigations on the preparation and in vivo application of UCL $NaGdF_4$:Yb,Er nanoparticles[19, 20], the core-shell $NaGdF_4$:Yb,Er@$NaGdF_4$ nanoparticles were synthesized and covalently conjugated with anti-cephalexin monoclonal antibody. The conjugate was employed in LFIA analysis of CEX, and the detection limit achieved was compared with that obtained based on colloidal gold particles.

Materials and Methods

Reagents and materials

The following materials were purchased from Sigma-Aldrich: $GdCl_3·6H_2O$ (450855), $YbCl_3·6H_2O$ (337927), $ErCl_3·6H_2O$ (259256), Oleic Acid (OA, 364525), 1-octadecene (ODE, O806), ammonium fluoride (NH_4F, 216011), tris(2-carboxyethyl) phosphine hydrochloride (TCEP, C4706), polyvinylpyrrolidone (PVP, Mw ~29,000, 9003398), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 161462), and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 56485). A bifunctional ligand, PEG2000 bearing a maleimide group at one end and a diphosphate groups at the other end (mal-PEG-dp) was a customized product provided by Beijing Oneder Hightech Co. Ltd. Bovine serum albumin (BSA, Mw 67,000 kD, Amresco 0332) and polyoxyethylene lauryl ether (Brij35, 9002920) were purchased from Biodee Biotechnology Beijing, Co., Ltd. Polyoxyethylene sorbitan monolaurate (Tween-20), sucrose, disuccinimidyl suberate (DSS), and other analytical grade chemicals such as K_2CO_3, ethanol, cyclohexane, and tetrahydrofuran (THF) were purchased from Sinopharm Chemical Reagent Beijing, Co., Ltd. CEX was purchased from National Research Center for Certified Reference Materials. Goat anti-mouse IgG was bought from Solarbio Science & Technology Co., Ltd. Keyhole limpet hemocyanin (KLH), and ovalbumin (OVA), were all purchased from BoAo Co., Ltd. (Shanghai, China). The nitrocellulose membrane (Sartorius CN 140), glass fiber (Ahlstrom 8964 and GF-06) and absorbent paper were purchased from Jiening Biotech Shanghai, Co., Ltd.

Preparation of $NaGdF_4$:Yb,Er@$NaGdF_4$ nanoparticles

The Yb/Er-doped $NaGdF_4$ nanoparticles were prepared according to our previous reports with some modifications[20]. In a typical synthesis, $GdCl_3·6H_2O$ (0.80 mmol), $YbCl_3·6H_2O$ (0.18 mmol), and $ErCl_3·6H_2O$ (0.02 mmol) were mixed with 14 mL OA and 16 mL ODE in a 100-mL flask. After heating to 150 °C to form a homogeneous solution under nitrogen protection, the solution was cooled down to 50 °C, and 10 mL of methanol solution containing NaOH (2.5 mmol) and NH_4F (3.6 mmol) was added dropwise. The reaction system was then kept under stirring at this temperature for 30 min. Subsequently, methanol in the system was removed under vacuum at 100 °C, and the formed reaction mixture was heated to 300 °C under atmospheric pressure for 1 h. The resultant nanoparticles were precipitated by ethanol, collected by centrifugation, washed with ethanol for three cycles, and finally redispersed in THF.

Subsequent deposition of the $NaGdF_4$ shell followed a similar process for the preparation of $NaGdF_4$:Yb,Er core particles. Briefly, 1 mmol $GdCl_3·6H_2O$ was added to a 100 mL three-neck round-bottom flask containing 4 mL OA and 15 mL ODE, heated to 150 °C under nitrogen protection to form a homogeneous solution, and then cooled down to 80 °C. Thereafter, 0.33 mmol $NaGdF_4$:Yb,Er nanoparticles in 5 mL THF was added. After the removal of THF, 10 mL methanol solution containing NaOH (2.5 mmol) and NH_4F (4 mmol) was added and stirred at 50 °C for 30 min. Then, methanol was removed, and the solution was heated to 300 °C under N_2 protection. The reaction mixture was maintained at this temperature for 60 min under magnetic stirring, and then cooled down to room temperature. The obtained core-shell nanoparticles were precipitated by ethanol, collected by centrifugation, washed with ethanol for three cycles, and finally redispersed in THF for the further experiments.

Preparation of anti-CEX monoclonal antibody

The CEX immunogen, coating antigen, and anti-CEX monoclonal antibody (mAb) were prepared according to previous literature[18]. In brief, CEX immunogen was prepared by conjugating CEX with KLH through DSS. The immunogen of CEX-KLH mixed with Freund's complete adjuvant was used to immunize BALB/c mice. Standard cell fusion techniques were used for generating mAb. CEX-BSA as coating antigen was synthesized through the (EDC/sulfo-NHS)-mediated amidation reaction between CEX and BSA.

Preparation of water-soluble nanoparticles and nanoparticles-mAb conjugate

The water-soluble nanoparticles were prepared through a ligand exchange reaction according to our previous report[19]. Approximately 10 mg of the purified nanoparticles and 100 mg of mal-PEG-dp were dissolved in 5 mL THF, and the reaction took place overnight at 40 °C. After ligand exchange, PEGylated particles were precipitated and washed by cyclohexane, and finally dried under vacuum at room temperature. The obtained nanoparticles were dispersed in water after removing the free ligand by ultra filtration using 100 kD filter (Millipore) at 6000 g. The conjugates of $NaGdF_4$:Yb,Er@$NaGdF_4$ nanoparticles and anti-CEX mAb were prepared by "click" reaction. Typically, anti-CEX mAb (1 mg/mL) was dispersed in 10×PBS buffer, and then subjected to mild reduction by TCEP to convert the disulfide groups in the Fc fragments to thiols. After purified by 30-K MWCO centrifugal devices, the partially reduced antibody was mixed with (mal-PEG-dp)-coated $NaGdF_4$:Yb,Er@$NaGdF_4$ particles in tris-buffered saline (TBS, pH 7.04) by molar ratio of 4:1 (mAb:nanoparticle). The resultant conjugate was transferred into 1×PBS buffer, and stored at 4 °C for further use.

The colloidal gold labeled mAb was prepared according to previous reports[17]. The solution of gold nanoparticles was adjusted to pH 8.2 with 0.1 M K_2CO_3, and then mAb solution was added drop by drop. After shaking for 2 h at room temperature, 10% BSA was added as blocking solution to reduce nonspecific binding. Finally, the solution was centrifuged at 10000 g to remove the unbound antibody and blocking reagent. The obtained conjugate was resuspended in PBS solution containing 10% (w/v) sucrose, 0.01% BSA, and 0.05% Tween-20.

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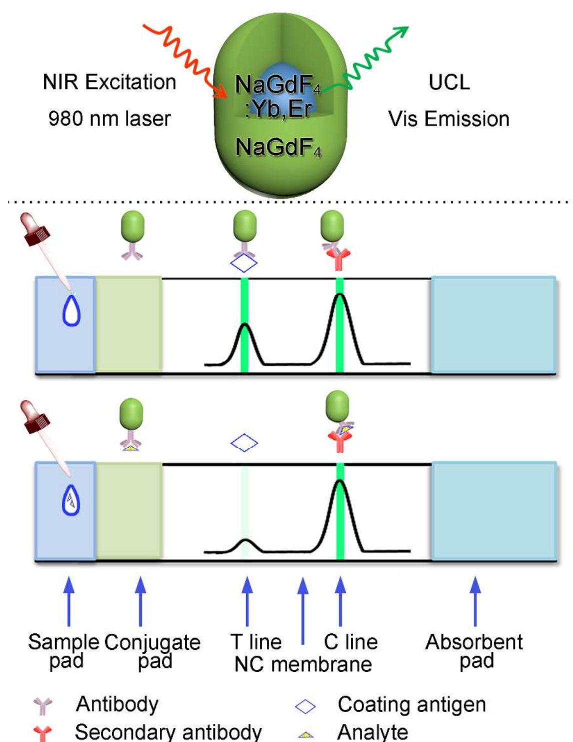

Scheme1. Schematic drawing for the core-shell structured upconversion luminescence nanoparticles (upper panel), and the competitive LFIA principle based on UCL nanoparticles (lower panel).

UCL nanoparticles-based LFIA

The principle and structure of the UCL nanoparticles-based LFIA are shown in Scheme 1. The LFIA strip consists of five components including sample pad, conjugate pad, nitrocellulose (NC) membrane, absorbent pad, and backing card. The sample pad is used to apply sample solution, and the conjugate pad is used to load the particle-labeled antibody. The absorbent pad serves as the liquid sink, and the backing card is used for supporting all of the components. The NC membrane acts as the chromatography matrix. A band of goat anti-mouse IgG and a band of coating antigen (CEX-BSA) were drawn on the NC membrane as control line (C line) and test line (T line), respectively. The sample pad was pre-treated by 1×PBS buffer containing 2% (w/v) Brij-35, and the conjugate pad by PBS buffer containing 0.1% (w/v) BSA, 0.05% (w/v) Tween 20, and 10% (w/v) sucrose, respectively. Then, the as-prepared particle-antibody conjugate solution incubated with 2% PVP for 30 min was spotted onto the conjugate pad. After the aforementioned LFIA components dried, they were assembled with overlaps between the sample pad and conjugate pad, and that between the conjugate pad and the NC membrane being 2 mm to ensure proper solution migration through the strip during the detection procedure.

Competitive assays were run by applying 100 µL of CEX in 1×PBS solution with a series of concentrations to the sample pad in a controlled environment with the relative humidity of 40-50%. After approximate 20 min, the luminescence of the testing strip

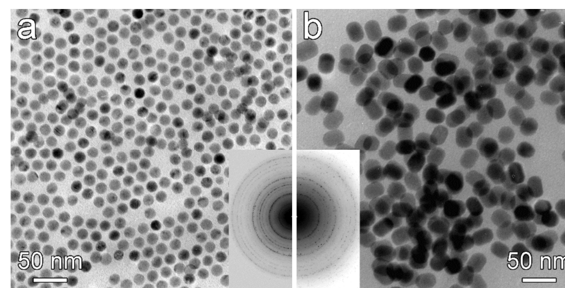

Fig. 1. TEM images of $NaGdF_4$:Yb,Er core particles (a) and $NaGdF_4$:Yb,Er@$NaGdF_4$ core-shell particles (b), insets: electron diffraction patterns of the corresponding nanoparticles.

was recorded under a constant 980 nm excitation by a CW laser. Then, the luminescence intensity was obtained by integrating luminescence signals of both T and C lines.

Characterization

Transmission Electron Microscopy (TEM) measurements were carried out with a JEM-100CXII microscope operating at an accelerating voltage of 100 kV for characterizing the UCL nanoparticles. The concentration of the rare earth elements of the nanoparticles was determined by inductively coupled plasma atomic emission spectrometer (ICP-AES) produced by Jiangsu Skyray instrument Co., Ltd after the particles were dissolved with concentrated nitric acid. The UCL spectra were recorded on a Cary Eclipse fluorescence spectrophotometer equipped with a 980 nm CW laser diode (2 W) serving as the excitation source. Dynamic light scattering (DLS) was carried out at 298.0 K with a Zetasizer Nano ZS (Malvern) equipped with a solid-state He-Ne laser (λ= 633 nm) for monitoring the variation of the hydrodynamic size of the nanoparticles before and after antibody conjugation. The test strips were excited by the 980 nm fiber laser with a beam size of 2 cm at a distance of 10 cm to fully cover the detection area. Optical images were acquired by using a digital camera (EOS450D, Canon), and then processed by using ImageJ 1.42q software for analyzing the luminescence signal from the T and C lines.

Results and Discussion

1. Structural analysis of the UCL nanoparticles

TEM was used to characterize the as-prepared $NaGdF_4$:Yb,Er and $NaGdF_4$:Yb,Er@$NaGdF_4$ core-shell nanoparticles. As shown in Fig. 1, the core is rather monodispersed and spherical particle showing an average size of 15.9 ± 0.6 nm, while the core-shell particles become slightly ellipsoidal shape exhibiting an average size of 30.5 ± 2.0 × 22.4 ± 1.3 nm. Meanwhile, the monodispersity remains unchanged, which indicates that no secondary nucleation occurred during shell coating process. Further electron diffractometry studies reveal that the shell coating doesn't alter the crystalline structure of the core, i.e., hexagonal structure that is favorable for upconversion luminescence. The following ICP-AES measurements reveal the ratios of Gd:Yb:Er in the core and core-shell particles are of 81.1:16.6:2.3 and 86.7:11.7:1.6, respectively. These composition

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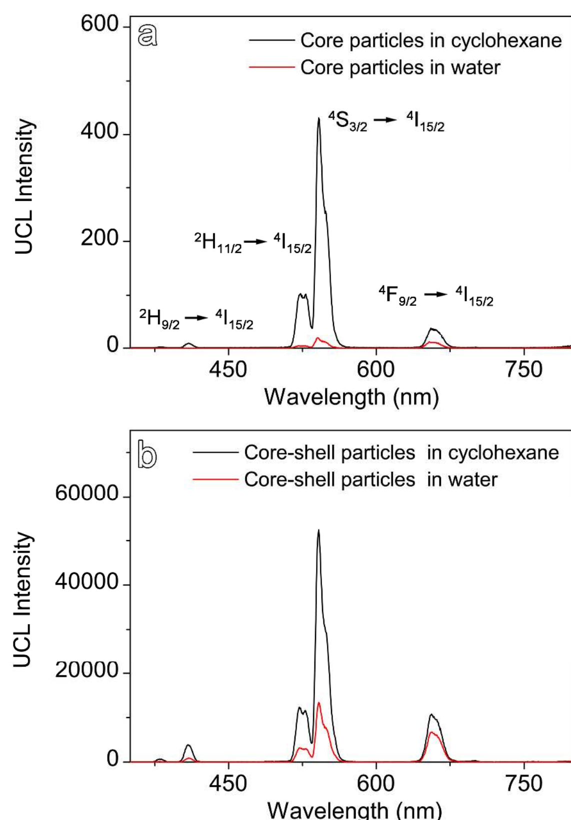

Fig. 2. Normalized luminescence spectra of $NaGdF_4$:Yb,Er core (a) and $NaGdF_4$:Yb,Er@$NaGdF_4$ core-shell nanoparticles (b) dispersed in cyclohexane and water, respectively, recorded upon constant excitation by a CW 980 nm laser.

data are used for charactering the luminescence enhancement effect in the following section.

2. Optical properties of UCL core-shell nanoparticles

The UCL spectra of $NaGdF_4$:Yb,Er core and $NaGdF_4$:Yb,Er@$NaGdF_4$ core-shell nanoparticles dispersed in cyclohexane and water are shown in Fig. 2. The major emissions locating at 409, 521, 541, and 655 nm can be attributed to radiative relaxations from $^2H_{9/2}$, $^2H_{11/2}$, $^4S_{3/2}$, and $^4F_{9/2}$ states to the $^4I_{15/2}$ state of Er^{3+}, respectively. Compared to core particles dispersed in cyclohexane, the UCL intensity of the major emissions of core-shell ones are unanimously improved by the shell coating and the integrated UCL is remarkably enhanced by a factor of 136. The luminescence improvement effect offered by the $NaGdF_4$ shell can be explained, according to literature[21], by reducing the non-radiative pathways associated with surface defects and depressing the non-radiative emission associated the organic surface ligands bearing alkyl chains. Furthermore, the overall UCL intensity of the $NaGdF_4$:Yb,Er core particles drops by a factor of 14.2 after being transferred into water through PEGylation. In contrast, the overall luminescence intensity of the core-shell $NaGdF_4$:Yb,Er@$NaGdF_4$ particles only drops by a factor of 3.3, which suggests that the $NaGdF_4$ shell can greatly help to preserve the luminescence through the phase transfer process. In addition, the core-shell structure is more favorable for

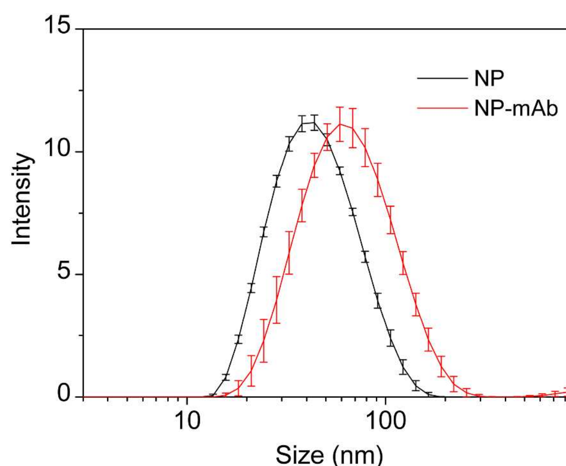

Fig. 3. The hydrodynamic size distribution profiles of the PEGylated core-shell nanoparticles recorded before and after the conjugation reaction with antibody.

maintaining the radiative relaxation from $^2H_{11/2}$ and $^4S_{3/2}$ states to the $^4I_{15/2}$ state of Er^{3+} according to the spectroscopy results. This is very favorable for the detection sensitivity of LFIA based on UCL particles, because the green emissions are very sensitive to naked eyes and Si-based detectors as well.

3. Conjugate of antibody and UCL nanoparticle

The conjugation reaction between anti-CEX mAb and UCL nanoparticle was investigated by DLS. As shown in Fig. 3, the intensity-weighted mean hydrodynamic size of different samples, denoted as NP and NP-mAb, were 41.1 nm and 61.6 nm, respectively. The reasonable increment in hydrodynamic size and no additional light scattering peaks appeared upon conjugate indicated the coupling reaction took place in a controllable way. After storing for more than 4 months at 4 °C, no precipitation is observed and the hydrodynamic size of conjugate is increased slightly (Fig. S1†). Further spectra results reveals that the upconversion luminescence nearly no change after storing so long time (Fig. S2†).

4. UCL nanoparticles-based LFIA for CEX detection

In the following LFIA experiments, the competitive reaction approach is adopted for detecting the model antibiotic CEX. As shown in Scheme 1, when an aqueous solution without CEX is applied onto the sample pad, the NP-mAb conjugates are rehydrated, consequently released into the migrating liquid and then migrate across NC membrane driven by capillary force. As the immobilized CEX-BSA on the T line can specifically bind with the NP-mAb conjugates, test signal under illumination will be observed at the T line. When CEX is present in the sample solution, it competes with CEX-BSA immobilized on the T line to bind with NP-mAb conjugates. Consequently, the signal of T line becomes inversely proportional to the amount of CEX in the sample. While the C line is functionalized with a secondary antibody and captures the NP-mAb conjugates irrespective of the presence of CEX. But when C line signal disappears, the test becomes invalid.

Fig. 4 shows the LFIA results based on UCL nanoparticles comparing with the colloidal gold nanoparticles-based LFIA

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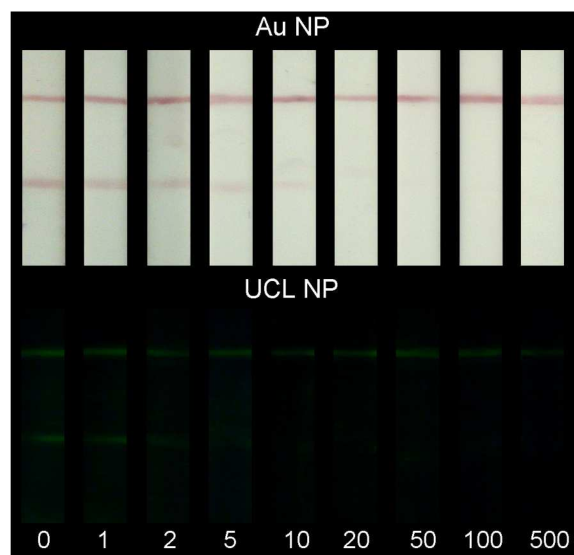

Fig. 4. Photographs for a group of test strips of LFIA based on Au nanoparticles and UCL nanoparticles, respectively, for detecting CEX in different concentrations (ng/mL) shown below.

results in CEX detection. The visual detection limit is defined herein as the minimum target analyte concentration required for the T line to show no obvious staining effect. Following this definition, the visual detection limit achieved by both UCL NP-mAb and Au NP-mAb was around 10 ng/mL, below the detection limit legislated by the European Union (EU) and Food and Drug Administration (FDA)[17].

To quantitatively extract the detection limit, the luminescence of both the T and C lines was carefully imaged after CEX solutions with gradient concentrations of 0, 0.5, 1, 2, 5, 10, 50, 100, and 500 ng/mL went across them. Then the luminescence images were analysed by integrating the cross-section of T line and C line by using Image J. In a similar way, the Au nanoparticles-based LFIA images were analyzed by integrating the optical density of both T line and C line. In both cases, the T line signal was normalized with respect to C line signal recorded from the same strip. As shown in Fig. 5, the signal intensity of the T line of UCL nanoparticle-labeled and Au nanoparticle-stained LFIA strips significantly decreases against the concentration of CEX. Linear fitting of the dose-response curves reveals that the linear response range is from 0.5 to 100 ng/mL for both UCL NP-mAb and Au NP-mAb probes, with a correlation coefficient being of 0.9983 and 0.9836, respectively. By defining the detection limit as the minimum concentration of analyte required for inducing 10% relative optical signal decrease[22], as guided by the dashed line, it was determined as 0.3 ng/mL for Au NP-mAb and 0.6 ng/mL for UCL NP-mAb, respectively. Although the detection limit remains comparable with colloidal gold nanoparticles-based LFIA achieved under optimized condition according to previous work[18], the UCL particles-based LFIA may still be favorable for detecting actual sample due to the low background signal for upconversion luminescence.

Conclusion

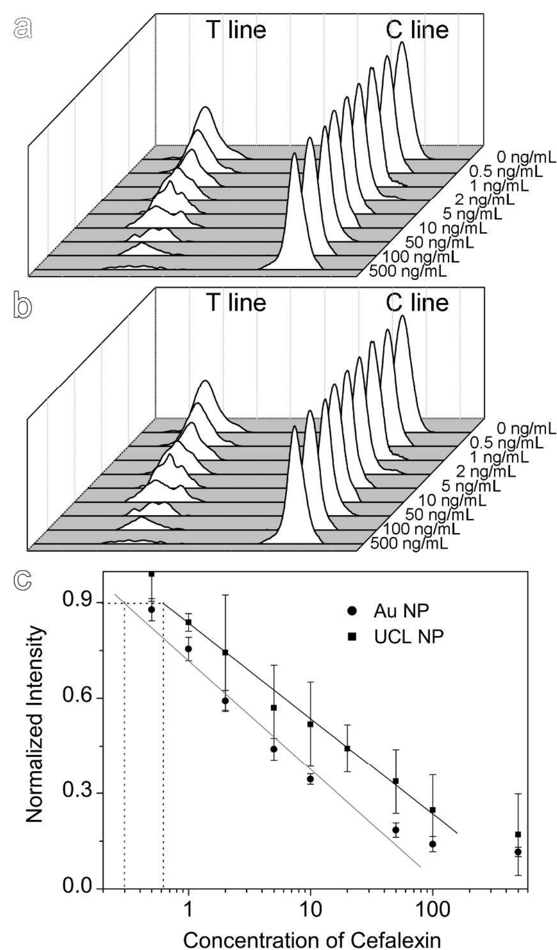

Fig. 5. Optical density profiles of T line and C line recorded by using Au NP-mAb (a), luminescence intensity profiles of T line and C line recorded by using UCL NP-mAb (b) after applying a series of standard solutions with different CEX concentrations (N=4), and dose-response curves for CEX based on quantitative analysis using Au nanoparticles and UCL nanoparticles respectively, as antibody labels (c).

A sensitive LFIA method is successfully established for detecting antibiotic CEX through the use of core-shell structured UCL nanoparticles. Owing to the $NaGdF_4$ shell coating, the luminescence of the underlying $NaGdF_4$:Yb,Er core is remarkably increased and better preserved through the phase transfer process upon PEGylation, which is beneficial for the high sensitivity of CEX detection. As a result, a visual detection limit of approximately 10 ng/mL is achieved through upconversion luminescence. Further quantitative analysis reveals that the linear detection range is about 0.5 to 100 ng/mL, with a sensitivity approaching 0.6 ng/mL. Based on these investigations, it can be concluded that the UCL nanoparticles-based LFIA becomes an alternative approach for Au nanoparticles-based LFIA, and may become useful for the actual sample detection due to the low UCL background.

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Notes and references

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†Electronic supplementary information (ESI) available: Shows the chemical stability and the optical stability of the conjugate of UCL particles with mAb. See DOI: 10.1039/b000000x

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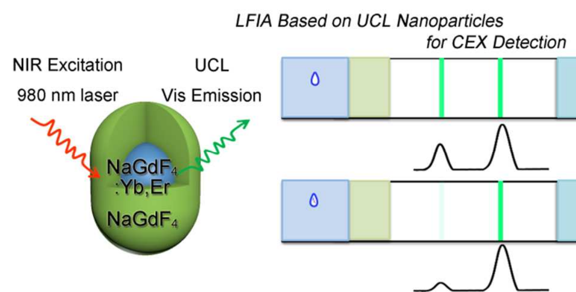

Upconversion luminescence core-shell nanoparticles were used as labels of antibody in lateral flow immunochromatographic assay for sensitive detection of cephalexin.

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