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Novel Upconversion Fluorescent Probe for *Schistosoma japonicum* Cercariae Imaging

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Abstract

Schistosomiasis has influence on people's security and daily life significantly, while cercarial stage of the schistosome life-cycle is the only infectious period. To detect cercarial efficiently, we synthesized rare-earth upconversion nanophosphors UCs-Ni loaded with niclosamide. The fluorescence of UCs-Ni was demonstrated by the fluorescence emission spectra. The cell imaging test illustrated that UCs-Ni was biocompatible, cell-permeable and suitable for cercariae imaging. When it has been utilized for cercariae imaging, we found that the UCs-Ni could penetrate into cercariae.

Keywords: Schistosomiasis; Cercaria; Luminescence; Spectrophotometer; Fluorescence

Introduction

As one of the most serious and neglected tropical diseases [1,2], schistosomiasis have influence people's security and daily life significantly. In recent years, more and more people infect schistosomiasis, which alert us to pay more attention to such kind of diseases.

The cercarial stage of the schistosome life-cycle is the only infectious period, which has the tendency to infect people and mammal. Interestingly, cercarial stage is the most fragile stage of life-cycle, [3] only exterminate cercarial could schistosomiasis be control. However, how to detect cercarial is the main difficulty we encountered.

Fluorescence imaging for small animals has received increasing attention due to offers a unique approach for visualizing morphological details in tissue with subcellular resolution, and becomes a powerful non-invasive tool for visualizing the full range of bio-species from living cells to animals.

The quality of fluorescent probes [4] plays a significant role in labelling the aiming molecules and amplifying the fluorescence signal in fluorescence bioimaging. To our knowledge, there are two kinds of fluorescent probes approximately, conventional downconversion fluorescent materials [5-9] and up-converting rare-earth nanophosphors (UCNPs). Conventional downconversion fluorescent materials usually absorb one higher-energy photon and then emit a lower-energy photon. The higher-energy light is usually associated with ultraviolet (UV) or visible light, which is of possibility to damage the living organisms. What's more, lead to low penetration depth in the biological tissues, which make it difficult to obtain the image of endothelial tissue. Moreover, significant auto-fluorescence from the biological tissues is unavoidable for the conventional downconversion fluorescent materials [10]. All these defects are exposed when utilizing derivatives of niclosamide and praziquantel [11,12] as fluorescent probes to achieve cercaria imaging.

However, rare-earth upconversion nanophosphors (UCNPs) [13-21] with the unique luminescence properties have attracted much attention, which is based on the anti-Stokes luminescence process. Upconversion luminescence (UCL) is a process in which low-energy light (near-infrared), through sequential absorption of multiple photons or energy transfers, is converted to higher-energy light. Under continuous-wave (CW) excitation at 980 nm, some rare-earth nanophosphors exhibit sharp emission lines, large anti-Stokes shift and high photostability [22,23] for the existence of rare-earth. Such properties lead to the perfect features of UCNPs for small animal bioimaging. When excited by continuous-wave near-infrared light, UCNPs display less harmful to small animals, no auto-fluorescence from biosamples and high penetration depth, which result in the success of utilizing UCNPs as probes to make a contribution to the small animal bioimaging.

In this paper, we synthesized UCs-Ni as fluorescent probe to explore no auto-fluorescence cercaria imaging.

Experimental Section

Materials and instrumentations

All of the starting materials (reagents and solvents) were obtained from commercial suppliers and used as received. Praziquantel was purchased from Zhejiang Top Medicine Co., Ltd. (China). The KB cell lines were provided by the Institute of Bio-chemistry and Cell Biology (China). Infected Oncomelania hupensis snails were supplied by the Hunan Institute of Para-sitic Diseases (WHO collaborating center for schistosomiasis control in lakes). ^{1}H NMR spectra were recorded using a Mer-curyplus spectrometer at 400 MHz and 100 MHz, respectively. Fluorescence spectra were measured using an Edinburgh LFS920 fluorescence spectrophotometer. Fourier transform infrared (FT-IR) spectra were measured using a Nicolet Nexus 470 spectrometer with KBr pellets. AFM measurements were performed by using a Nanoscope IIIa (Bruker, Germany). TEM measurements were performed by means of JEM-2010. Fluorescence imaging experiments were performed using an OLYMPUS FV1000 IX81 confocal fluorescence microscope equipped with a 40× oil-immersion objective lens, excitation at 980 nm was carried out with a semiconductor laser and emission was collected at 630 to 670 nm.

Synthesis of UCs-Ni

Synthesis of oleic acid-capped UCNPs (UCs-OA): NaOH (1.2 g, 30 mmol), water (10 mL), ethanol (10 mL), and oleic acid (20 mL)

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were mixed under agitation to form a homogeneous solution. Then 0.6 mmol (total amounts) of rare-earth chloride (1.2 mL, 0.5 mol/L Ln: 85% mol La, 12% mol Yb, 3% mol Er) aqueous solution was added under magnetic stirring. Subsequently, 1.0 mol/L aqueous NH_4F (4 mL) solution was added dropwise to the above solution. The mixture was agitated for about 10 min, then transferred to a 50 mL autoclave, sealed, and hydrothermally treated at 160°C for 8 h. The system was cooled to room-temperature naturally, and the products were deposited at the bottom of the vessel. Cyclohexane was used to dissolve and collect the products. The products were subsequently deposited by adding ethanol to the sample containing cyclohexane solution. The resulting mixture was then centrifuged to obtain powder samples. Pure powders could be obtained by purifying the samples with ethanol several times to remove oleic acid, sodium oleic and other remnants.

Converting to UCs-Ni: A mixture of as-prepared UCs-OA sample (0.1 g), cyclohexane (20 mL), CH_2Cl_2 (10 mL), and 25 mg chlorobenzoic acid were stirred at room temperature for about 3 h. Then PEG-Niclosamide (n=3) 0.7 mmol was added. The resulting mixture was stirred at room temperature for over 48 h. Then the products could be obtained by purifying the samples with ethanol several times.

^{1}H NMR of UCs-Ni (400 MHz, $CDCl_3$, 25°C) ∂: 10.62 (s), 8.86 (d), 8.28-8.33 (d), 8.23-8.24 (d), 8.17-8.20 (d), 7.50 (d), 7.12 (d), 4.46 (t), 3.93 (t), 3.52 (t), 1.58(d), 1.25(d), 0.85(d). IR (KBr) $\nu_{UCs\text{-}Ni}$=3414.3, 2928.7, 1661.49, 1590.08, 1544.9, 1512.73, 1465.1, 1381.82, 1345.5, 1261.5, 1161.65, 1114.7, 1024.79, 944.46, 905.79, 867.11, 813.7, 748.10, 694.55 (Scheme 1).

Nanostructure of UCs-Ni

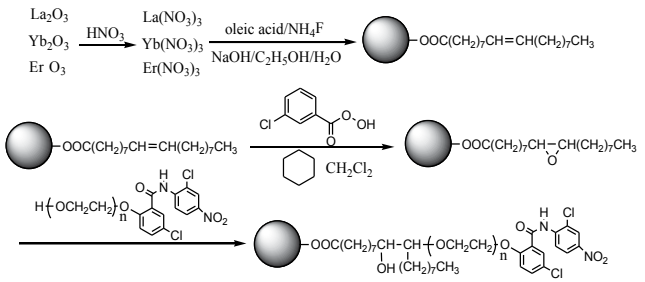
The solutions with the concentration of 2.5×10^{-4} mol/L were prepared by dissolving UCs-Ni into H_2O at room temperature. Then a droplet was dropped onto a freshly cleaved mica surface and allowing the sample to dry. Then AFM measurements were performed by using a Nanoscope IIIa (Bruker, Germany). After dropping the solution onto a copper networks, TEM measurements were performed by means of JEM-2010.

Photophysical properties

The fluorescence emission spectra of UCs-Ni in a diluted solution were studied. The UCs-Ni was dissolved in CH_2Cl_2 with the concentration of 100 μg/mL.

Cell and cercaria imaging experiments

KB cells were plated on 18 mm glass coverslips under an atmosphere of 5% CO_2, 95% air at 37°C to adhere for 24 h. Then the KB cells were stained with a 100 μg/mL UCs-Ni solution in DMSO-PBS (v/v, 1:399) buffer for 10 min. Cercariae were suspended in a 200 μL 5 mg/mL solution of UCs-Ni on a special plate for imaging. Lastly, fluorescence imaging of cercariae was performed by confocal microscopy at 15 min, 30 min and 1 h, respectively.



Scheme 1: Synthesis routes of UCs-Ni

Results and Discussion

Morphology of UCs-Ni

The morphology of UCs-Ni was recorded by AFM and TEM (Figure 1a). The AFM image exhibited the aggregated nanoparticals with the dimension of 15 nm to 25 nm, which was demonstrated by the TEM (Figure 1b).

Fluorescence emission spectra

The photophysical properties of UCs-Ni were investigated before UCs-Ni was used to label KB cells and cercariae. The fluorescence emission spectra of UCs-Ni dissolved in CH_2Cl_2 with the concentration of 100 μg/mL were studied and are shown in (Figure 2). Under excitation at 980 nm, the UCs-Ni exhibited two emission bands at about 510-560 nm and 630-670 nm, which illustrated the strong fluorescence of UCs-Ni.

Cercaria imaging

UCs-Ni with its' biocompatiblity and cell-permeability, had the tendency to label cercariae. We have reported the Anti-cercarial activity of niclosamide and it's derivatives [11]. The interaction between fresh cercariae and UCs-Ni was observed using confocal fluorescence microscopy.

As shown in Figure 3, The UCs-Ni was taken up by cercariae via the tegument at 15 min with the low fluorescence intensity (Figure 3a). Then, the fluorescence intensity in the cercarial continually increased during the period from 30 min to 1 h (Figures 3b,3c). What's more, the cercarial body even the viscera were coated with UCs-Ni evenly, which emitted green fluorescence. Even if the test time was extended, UCs-Ni was still mainly located at the cercarial from the inside out and the fluorescence intensity increasing slightly. In a word, no obvious fluorescence signal was detected in the initial 15 min, but a more significant fluorescence signal appeared 30 minutes later. With extending the time to 1 h, the fluorescence of the cercariae continued to increase. As the changes mentioned above, we speculated that the accumulation of drugs in the tegument was less in 15 min as the drug action time was short, which lead to low fluorescence signal. However, with the extending of reaction time, the drug penetrated into cercarial body and viscera, and then gathered more, which resulted in stronger fluorescence signal. All the results indicated that UCs-Ni could penetrate into cercariae easily, which was proved to be a perfect labelling material.

To further understand the drug's action in cercariae, we also carried out a control experiment using UCs-OA (morphology of UCs-OA). We selected images stained by UCs-OA after 1 h. The fluorescence signal of

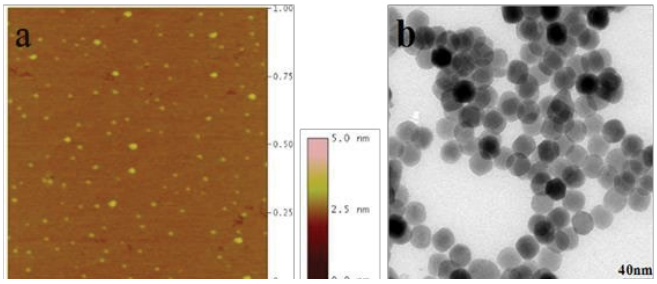


Figure 1: (a) AFM image of UCs-Ni nanoparticles; **(b)** TEM image of UCs-Ni nanoparticles.

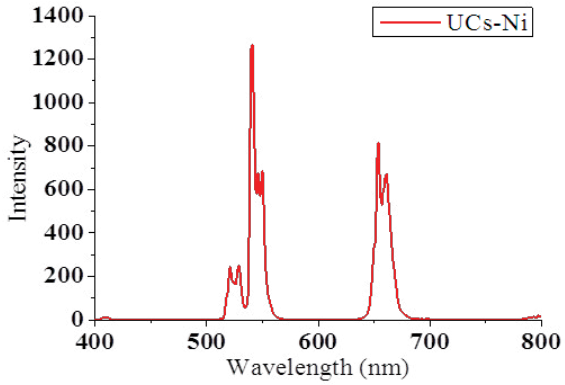


Figure 2: The fluorescence emission spectra of UCs-Ni, excitation at 980 nm.

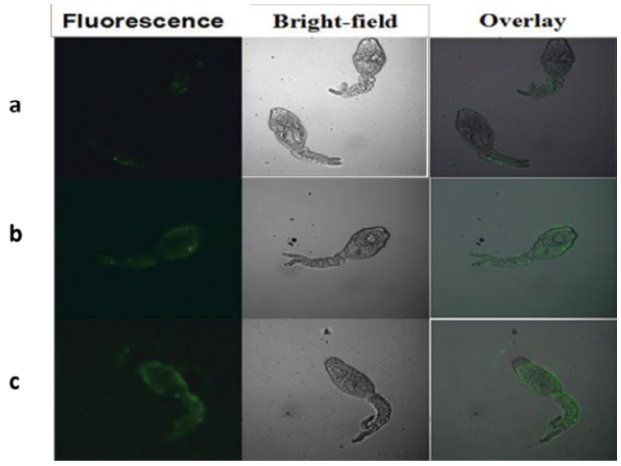


Figure 3: Confocal bright-field, fluorescence and overlay images of cercariae incubated with UCs-Ni in PBS buffer at room temperature for **(a)** 15 min; **(b)** 30 min and **(c)** 1 h. All excitation wavelengths were 980 nm and emission was collected at 630 to 670 nm.

UCs-OA was localized around the head and the tail mainly, while the fluorescence signal was remarkably weak in other parts. It is concluded that the ability for imaging of UCs-Ni is much different and stronger than UCs-OA.

Conclusion

In summary, we synthesized and characterized rare-earth upconversion nanophosphors UCs-Ni. The fluorescence of UCs-Ni was demonstrated by the fluorescence emission spectra. The cell imaging test illustrated that UCs-Ni was biocompatible, cell-permeable and suitable for cercariae imaging. Interestingly, the UCs-Ni could penetrate into cercariae and kill cercariae specifically, which may become a perfect labelling material in the field of biomedicine.

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