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Visible-light-excited and europium-emissive nanoparticles for highly-luminescent bioimaging *in vivo*

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ABSTRACT

Europium(III)-based material showing special milliseconds photoluminescence lifetime has been considered as an ideal time-gated luminescence probe for bioimaging, but is still limited in application in luminescent small-animal bioimaging *in vivo*. Here, a water-soluble, stable, highly-luminescent nano-system, Ir–Eu–MSN (MSN = mesoporous silica nanoparticles, Ir–Eu = $[Ir(dfppy)_2(pic–OH)]_3Eu\cdot 2H_2O$, dfppy = 2-(2,4-difluorophenyl)pyridine, pic–OH = 3-hydroxy-2-carboxypyridine), was developed by an *in situ* coordination reaction to form an insoluble dinuclear iridium(III) complex-sensitized-europium(III) emissive complex within mesoporous silica nanoparticles (MSNs) which had high loading efficiency. Compared with the usual approach of physical adsorption, this *in-situ* reaction strategy provided 20-fold the loading efficiency (43.2%) of the insoluble Ir–Eu complex in MSNs. These nanoparticles in solid state showed bright red luminescence with high quantum yield of 55.2%, and the excitation window extended up to 470 nm. These Ir–Eu–MSN nanoparticles were used for luminescence imaging in living cells under excitation at 458 nm with confocal microscopy, which was confirmed by flow cytometry. Furthermore, the Ir–Eu–MSN nanoparticles were successfully applied into high-contrast luminescent lymphatic imaging *in vivo* under low power density excitation of 5 mW cm^{-2}. This synthetic method provides a universal strategy of combining hydrophobic complexes with hydrophilic MSNs for *in vivo* bioimaging.

1. Introduction

Fluorescence imaging is a unique approach for visualizing morphological details in tissue with subcellular resolution that cannot be resolved by other imaging technologies such as radiography, ultrasound, magnetic resonance imaging (MRI) and X-ray computed tomography (CT) [1]. In particular, this time-resolved luminescence imaging technique which employs the long-life phosphorescence of lanthanide ions with the order of milliseconds, is an effective strategy to completely eliminate interference due to scattered light and auto-fluorescence from cells and tissues [2–8]. To date, both inorganic nanocrystals doped with lanthanide ions (Ln^{3+}) and lanthanide complexes have been used in down-conversion luminescence bioimaging [9–13]. However, shielding of the 4f orbitals by the filled $5p^6 6 s^2$ sub-shells results in very low molar absorption coefficients, resulting in the slow development of inorganic lanthanide nanocrystals for down-conversion luminescence bioimaging [2]. Therefore, the introduction of organic ligands plays a notable role in the improvement of absorption and emission efficiency of lanthanide complexes [14–22].

Unfortunately, only a few new water-soluble lanthanide complexes have been used successfully in photoluminescence bioimaging [23–25]. To avoid the reverse transfer from Eu^{3+} to the organic sensitizer (ligand), an efficient sensitization process often requires the energy gap (ΔE) between the triplet state of the sensitizers and the $^5D_0$ level of Eu^{3+} higher than 2500 cm^{-1} [26], which results in the excitation wavelength of the lanthanide complexes within the ultraviolet (UV) region. However, UV excitation light is associated with several significant disadvantages, such as possible photodamage and low light-penetration depth in biotissues [27]. Therefore, it is highly desirable to extend the excitation wavelength of the lanthanide probe toward the visible region.

In the present study, we developed a new europium(III) emissive nano-confined system for achieving visible-light-excited luminescent emission. The emissive nano-confined system is consistent of mesoporous silica nanoparticles (MSNs) as a nanoreactor to *in situ* form insoluble iridium(III) complex–sensitized–europium(III) complex ($[Ir(dfppy)_2(pic–OH)]_3Eu\cdot 2H_2O$, dfppy = 2-(2,4-difluorophenyl)pyridine, pic–OH = 3-hydroxy-2-carboxypyridine, Scheme 1) within the mesoporous channel.

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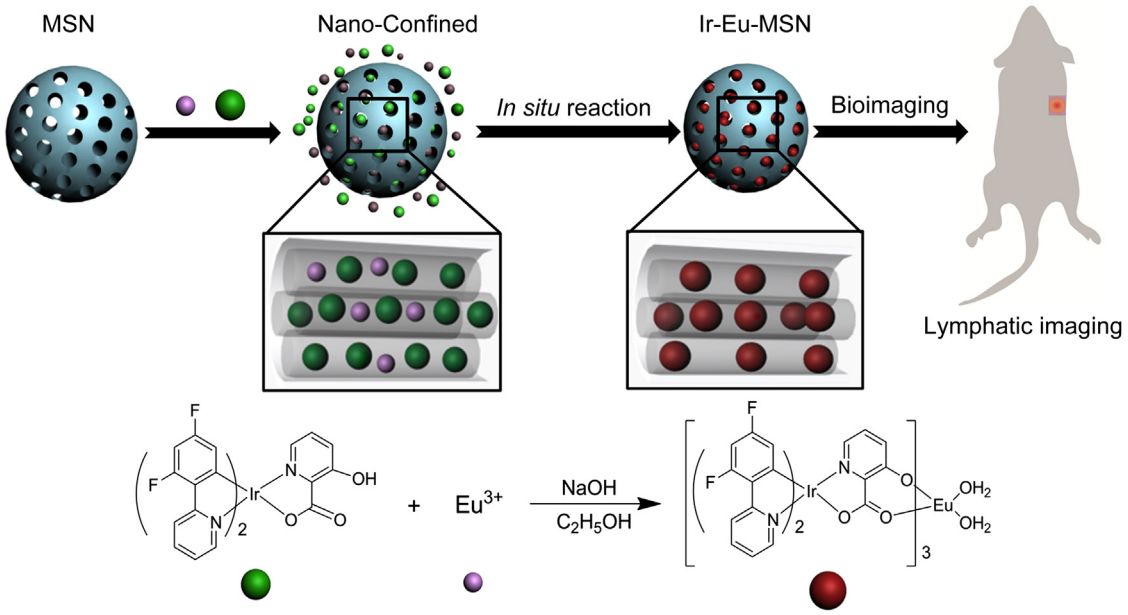

Scheme 1. Schematic illustration of the construction of Ir–Eu–MSN nanosystem *via* nano-confined systems for lymphatic imaging *in vivo*.

Herein, the as-prepared complex $[Ir(dfppy)_2(pic{-}OH)]_3Eu{\cdot}2H_2O$ and nano-confined system (Scheme 1) were abbreviated as the Ir–Eu complex and Ir–Eu–MSN, respectively. Accordingly, the photophysical properties of Ir–Eu–MSN were investigated by UV–vis absorption and fluorescence spectroscopy. Meanwhile, the Ir–Eu–MSN nanoparticles were applied into cell imaging and lymphatic imaging *in vivo* of small animals. In addition, the assessment of cytotoxicity and *in vivo* toxicity of the Ir–Eu–MSN was conducted.

2. Experimental section

2.1. Applied chemicals

Sodium acetate (NaAc), hexadecyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), 2-ethoxyethanol, acetic acid (HAc), triethylamine (Et_3N), ethanol (EtOH), hydrochloric acid (HCl), potassium carbonate (K_2CO_3) and sodium hydroxide (NaOH) were obtained from Sinopharm Chemical Reagent Co. (China). 2,4-Difluorophenylboronic acid, 3-Hydroxy-2-carboxypyridine (pic–OH), tetrakis(triphenylphosphine)palladium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Acros. $IrCl_3{\cdot}3H_2O$ (the Ir content >54.51%) was an industrial product and used without further purification. PBS solution (pH, 7.4) and RPMI 1640 culture solution were obtained from Hangzhou Jinuo Biomedical Co., Ltd. Deionized water was used in all experiments. Other chemicals were used as received without further purification.

2.2. Characterization

^1H NMR spectra were recorded with a Varian spectrometer at 400 MHz. Electrospray ionization mass spectra (ESI-MS) were measured on a Micromass LCTTM system. UV–visible spectra were recorded on a Shimadzu UV-2550 spectrometer. Steady-state emission experiments at room temperature were measured on an Edinburgh instruments spectrometer (LFS-920). The luminescence quantum yield of the Ir–Eu complex in air-equilibrated solution were measured with reference to tris(bipyridine)ruthenium as a standard ($\Phi = 0.028$) [28]. Fourier transform infrared (FT–IR) spectra were measured on a Nicolet Nexus 470 spectrophotometer using KBr technique. Thirty-two scans were collected per sample over the range of 4000–400 cm^{-1}. Transmission electron microscopy (TEM) images were obtained on a JEOL JEM-2010F electron microscope operated at 200 kV. Energy-dispersive X-ray analysis (EDXA) of the samples was performed during high-resolution TEM (HR-TEM) measurement. N_2 adsorption was measured on a Quanta Autosorb-1 system at −196 °C, and samples were degassed at 105 °C under vacuum overnight before measurements.

2.3. Synthesis of 2-(2,4-difluorophenyl)pyridine (dfppy)

Generally, 2,4-difluorophenylboronic acid (4.34 g, 27 mmol), 2-bromopyridine (3.95 g, 25 mmol), tetrakis(triphenylphosphine)palladium(0) (0.8 g) and potassium carbonate (8.63 g, 62.5 mmol) were added into 150 mL three-neck flask, and then a mixture of THF (40 mL) and H_2O (40 mL) also was injected into reaction system. The mixture was heated for 24 h under N_2 atmosphere at 70 °C. After cooling to room temperature, the mixture was extracted by CH_2Cl_2 in a separating funnel and the water phase was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4. After removing the solvent under reduced pressure, the crude product was purified by column chromatography using pure CH_2Cl_2, and a slightly yellow liquid dfppy was obtained. Yield: 85%. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.70 (d, $J = 4.4$ Hz, 1H), 7.98 (dd, $J = 16.0$, 7.8 Hz, 1H), 7.73 (s, 2H), 7.25 (s, 1H), 6.95 (dt, $J = 19.9$, 9.5 Hz, 2H).

2.4. Synthesis of $[Ir(dfppy)_2(pic{-}OH)]$

Complex $[Ir(dfppy)_2(pic{-}OH)]$ (Scheme 1) was synthesized according to the previous literature [29]. A mixture of 2-ethoxyethanol and water (3:1, v/v) was added to a flask containing $IrCl_3{\cdot}3H_2O$ (1 mmol) and 2-(2,4-difluorophenyl) pyridine (dfppy, 2.2 mmol). The mixture was heated under reflux for 24 h. After cooling to room temperature, the solid precipitate was filtered to give crude iridium(III) chloro-bridged dimer $[Ir_2(dfppy)_4Cl_2]$. To the mixture of $[Ir_2(dfppy)_4Cl_2]$ (0.2 mmol), 3-hydroxy-2-carboxypyridine (pic–OH, 0.44 mmol) and K_2CO_3 (0.6 mmol) were added to flask containing solvent of 2-ethoxyethanol, and then the slurry was refluxed for 24 h. After cooling to room temperature, a precipitate was collected by filtration and was chromatographed using CH_2Cl_2/CH_3OCH_3 (4:1, v/v) to give yellow solid $[Ir(dfppy)_2(pic{-}OH)]$. Yield: 53%. ^1H NMR (DMSO-d^6, 400 MHz): δ (ppm) 13.59 (s, 1H), 8.52 (d, $J = 5.7$ Hz, 1H), 8.28 (dd, $J = 17.1$, 8.5 Hz, 2H), 8.08 (dt, $J = 16.7$, 8.2 Hz, 2H), 7.71 (d, $J = 5.7$ Hz, 1H), 7.68–7.61 (m, 1H), 7.57–7.43 (m, 2H), 7.37 (t, $J = 6.6$ Hz, 1H), 7.31–7.21 (m, 1H), 6.98–6.67 (m, 2H), 5.68 (dd, $J = 8.7$, 2.3 Hz, 1H), 5.51 (dd, $J = 8.6$, 2.3 Hz, 1H). Anal. Calcd for $C_{28}H_{16}F_4IrN_3O_3$: C, 47.32; H, 2.27; N, 5.91. Found: C 47.21, H 2.36, N 5.83. Exact Mass: 711.0757. ESI-MS: m/z, 750.09 ($M + K^+$).

2.5. Synthesis of $[Ir(dfppy)_2(pic{-}OH)]_3Eu{\cdot}2H_2O$ (Ir–Eu complex)

The synthetic routine of the Ir–Eu complex was shown in Scheme S1. 50 mL ethanol was add into a solvent of in a 100 mL flask containing $[Ir(dfppy)_2(pic{-}OH)]$ (54 mg, 0.75 mmol) and $Eu(NO_3)_3{\cdot}6H_2O$ (12 mg, 0.25 mmol), and the mixture was heated at 80 °C. Then, a solution of NaOH (aq, 0.1 M) was added into reaction solution, and the reaction solution was neutralized to pH = 6–7 and reacted for 12 h at 80 °C. After cooling to room temperature, yellow precipitate was filtered and washed by mixed solvent (ethanol/water = 95:5) three times to give $[Ir(dfppy)_2(pic{-}OH)]_3Eu{\cdot}2H_2O$. Exact Mass: 2319.1460, MADIA-TOF-MS: m/z 2319.5604.

2.6. Synthesis of Ir–Eu complex-functionized MSN (Ir–Eu–MSN)

The synthesis of MSNs was based on the method reported by Yu's group with modifications [30], and synthetic routine of Ir–Eu–MSN was shown in Scheme 1. Briefly, 15 mL ethanol was add into a solvent of in a 50 mL flask containing complex $[Ir(dfppy)_2(pic{-}OH)]$ (5.4 mg, 0.75 mmol), and the mixture was refluxed for 30 min. Next, a stock of 2 mL ethanol containing 80 mg MSN was added into reaction system and stirring for 30 min. Then, an ethanol solution of $Eu(NO_3)_3.6H_2O$ (1.1 mg, 0.25 mmol) was poured into reaction solution and also stirring for 30 min. Last, the