Biomaterials 35 (2014) 3356–3364

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Near-infrared fluorescence imaging using organic dye nanoparticles

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ARTICLE INFO

Article history:
Received 11 November 2013
Accepted 1 January 2014
Available online 21 January 2014

Keywords:
NIR-emissive organic nanoparticles
Fluorescence resonance energy transfer
In vitro imaging
In vivo imaging

ABSTRACT

Near-infrared (NIR) fluorescence imaging in the 700–1000 nm wavelength range has been very attractive for early detection of cancers. Conventional NIR dyes often suffer from limitation of low brightness due to self-quenching, insufficient photo- and bioenvironmental stability, and small Stokes shift. Herein, we present a strategy of using small-molecule organic dye nanoparticles (ONPs) to encapsulate NIR dyes to enable efficient fluorescence resonance energy transfer to obtain NIR probes with remarkably enhanced performance for *in vitro* and *in vivo* imaging. In our design, host ONPs are used as not only carriers to trap and stabilize NIR dyes, but also light-harvesting agent to transfer energy to NIR dyes to enhance their brightness. In comparison with pure NIR dyes, our organic dye nanoparticles possess almost 50-fold increased brightness, large Stokes shifts (~250 nm) and dramatically enhanced photostability. With surface modification, these NIR-emissive organic nanoparticles have water-dispersity and size- and fluorescence- stability over pH values from 2 to 10 for almost 60 days. With these superior advantages, these NIR-emissive organic nanoparticles can be used for highly efficient folic-acid aided specific targeting *in vivo* and *ex vivo* cellular imaging. Finally, during *in vivo* imaging, the nanoparticles show negligible toxicity. Overall, the results clearly display a potential application of using the NIR-emissive organic nanoparticles for *in vitro* and *in vivo* imaging.

1. Introduction

In near-infrared (NIR) spectral range, organisms and tissues have low absorption of light and possess low intrinsic autofluorescence. Autofluorescence is the natural emission of light from biological structures. If biomarkers are fluorescent in NIR range, they can be better detected and identified from the surrounding biological environment. Therefore, NIR fluorescence imaging in wavelength range of 700–1000 nm is particularly attractive for early detection of cancers, which is expected to significantly contribute to improved cancer therapy and increased survival rates of patients [1–4].

Currently, the most widely used NIR probes are still organic dyes, which are usually encapsulated in various nanoparticles (NPs) to overcome the intrinsic limitations of conventional NIR dyes including poor hydrophilicity, low photostability, small quantum yield (QY) and instability in bio-environment [5–8]. However, most of these encapsulating nanoparticles including silica NPs, calcium phosphate NPs, and lipoprotein NPs, only act as inert carriers but do not contribute to brightness improvement of the NIR probes [9–12]. Another concern is that Stokes shift of conventional nanoparticle-based dye probes is usually small and optical interferences (light scattering and autofluorescence) caused by biosubstrates often exist, which greatly reduces detection sensitivity [13]. Furthermore, the clearance of inert carriers from patients still remains a great concern. Conjugated polymer dots (Pdots) were then reported to be utilized as the matrix to load organic dyes for imaging, in which Pdots served as light-harvesting agents to transfer their energy to organic dyes to enhance brightness [14,15].

Recently, small-molecule organic dye nanoparticles (SM ONPs) have also been developed as a new class of promising fluorescent probes [16–19], where organic dyes themselves were directly assembled into pure dye nanoparticles without nanocarriers. Despite at early stage, SM ONPs have attracted much attention, because they possess large absorption cross-sections, non-blinking property and favorable biocompatibility. More importantly,

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http://dx.doi.org/10.1016/j.biomaterials.2014.01.004

compared to Pdots, there is great variety and flexibility in materials design and thus tunability in optical properties and functionalities [20,21]. Previously, dye molecules with rigid structures or aggregation-induced enhanced emission (AIEE) properties were used for preparing SM ONPs to avoid concentration quenching of traditional dyes [22]. For example, recently, we reported a type of ultrabright and ultrastable NIR dye nanoparticles which were prepared from a NIR dye of bis(4-(N-(2-naphthyl)phenylamino) phenyl)-fumaronitrile (NPAPF) with AIEE effect [16]. However, if traditional NIR dyes are employed to make nanoparticles, severe quenching will happen.

To address the challenges of developing NIR fluorescence probes with high brightness, large Stokes shift and photo- and bio-environmental stability, herein, we encapsulated an NIR dye into red-emissive ONPs. We hypothesize that the efficient fluorescence resonance energy transfer (FRET) from ONPs to NIR dyes will enable the resultant NPs to possess superior properties for bioimaging. To confirm this, we systematically investigated the optical properties, water-dispersity, photo- and bio-environmental stability and *in vivo* toxicity of the NIR dye doped NPs and tested their application for *in vitro* and *in vivo* imaging.

2. Experimental section

2.1. Materials and characterization

NIR712 dye (2,9,16,23-tetra-tert-butyl-29H,31H-phthalocyanine) was purchased from Sigma Aldrich, Inc. Tetrahydrofuran (THF) was ordered from Shanghai LingFeng Chemical Reagent Co., Ltd. High-purity water (resistivity = 18.2 MΩ cm) was produced with a Milli-Q apparatus (Millipore). Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium, folic acid (FA)-free RPMI-1640 and Penicillin-streptomycin solution were obtained from Invitrogen (San Diego, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and poly(maleic anhydride-alt-1-octadecene) (C18PMH) were from Sigma Aldrich (Milwaukee, WI). 4′,6-diamidino-2-phenylindole (DAPI) and LysoTracker Green DND-26 were purchased from Invitrogen. Triethylamine (TEA) and mPEG-NH_2 (5k) were from Sinopharm Chemical Reagent Co. and PegBio (Suzhou, China), respectively. N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were ordered from Fluka. A human nasopharyngeal epidermal carcinoma cell line (KB cell), a human epithelial cervical cancer cell line (HeLa cell), a human fetal lung fibroblast MRC-5 cell line (MRC-5 cell) and a 4T1 murine breast cancer cell line (4T1 cell) were provided by American Type Culture Collection (ATCC).

UV–Vis absorption spectra were obtained on a Perkin–Elmer Lambda 750 UV/Vis/NIR spectrometer. Fluorescence spectra were measured with a Horiba Jobin Yvon luminescence spectrometer (FluoroMax 4). Scanning electron microscopy (SEM) images were taken with a FEI Quanta 200 FEG field emission scanning electron microscope. Transmission electron microscopy (TEM) images were taken by FEI Tecnai G2 F20 S-TWIN. Dynamic light scattering (DLS) measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.). All measurements were performed at room temperature.

2.2. Synthesis of NPAFN

2,3-bis(4′-(diphenylamino)-[1,1′-biphenyl]-4-yl)fumaronitrile (NPAFN) was prepared according to previously reported procedures [23]. A mixture of 4-bromophenylacetonitrile (4.86 g, 24.8 mmol) and iodine (6.35 g, 25 mmol) was purged with N_2 and dry diethyl ether (100 mL) was injected to the mixture with a needle and syringe. Then the temperature of the solution was dropped to −78 °C. Sodium methoxide (2.84 g, 52.6 mmol) and methanol (40 mL) were subsequently slowly added within a period of over 30 min following by stirring for 40 min. Next, the solution was placed in an ice-water bath for 4 h under stirring. Then, 3–6% hydrochloric acid was added dropwisely and the solution was filtered to separate the precipitate. After it, the precipitate was rinsed with cold methanol-water solvent and the filtrate was concentrated to obtain the second crop of product. Through these procedures, a pale yellow solid was obtained with a yield of greater than 90%.

A mixture of bis(4-bromophenyl)fumaronitrile (0.787 g, 2.03 mmol), triphenylamine (4.47 mmol), Cs_2CO_3 (1.95 g, 5.98 mmol) and toluene (13 mL) was degassed and purged with N_2. Pd/P(t-Bu)$_3$ catalyst pre-prepared from $Pd(OAc)_2$ (0.0135 g, 0.06 mmol), P(t-Bu)$_3$ (0.36 g, 0.178 mmol) and toluene (4 mL) was added to the mixture inside a glove box. The reaction mixture was then degassed, purged with nitrogen, heated to 110 °C and maintained for 24 h. The mixture was cooled to room temperature followed by adding water (30 mL) and dichloromethane (50 mL). The organic layer was separated and washed with brine, dried over anhydrous $MgSO_4$ and evaporation under reduced pressure. Finally, the product was purified by column chromatography on silica gel using 1:1 lightpetroleum/dichloromethane as eluant to afford NPAFN (1.036 g) as orange solid. Melting point (°C): 249.8–250.5. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.93 (d, 4H), 7.73 (d, 4H), 7.53 (d, 4H), 7.30 (t, 8H), 7.16 (d, 12H), 7.08 (t, 4H). ^{13}C NMR (400 MHz, $CDCl_3$, δ, ppm): 148.64, 147.43, 143.63, 132.54, 130.33, 129.94, 129.41, 129.28, 127.86, 127.67, 127.04, 126.87, 124.89, 124.01, 123.45, 123.20, 117.03, 77.23, 77.19, 77.17. MS ($C_{52}H_{36}N_4$): calcd, 716.87; found, 716.29.

2.3. Synthesis of folic acid conjugated C18PMH-PEG (C18PMH-PEG-FA)

C18PMH-PEG was synthesized as stated in literature [24]. To prepare C18PMH-PEG-FA, C18PMH-PEG was firstly functionalized with amine group. In detail, C18PMH (1eq), mPEG-NH_2 (5k) (1eq) and NH_2-mPEG(5k)-BOC (Polymere, Germany) (0.5eq) were mixed in dichloromethane under agitation. EDC (2eq) and TEA (8eq) were then added to the solution under magnetic stirring. After stirring for 24 h at room temperature, the dichloromethane solvent was blown dry with a N_2 flow. Subsequently, 2 mL of trifluoroacetic acid was added and actively stirred for 3 h at room temperature to de-protect the Boc group. After the evaporation of the TFA solvent, the residual solid was dissolved in water, which was dialyzed for 2 days in a dialysis bag (MWCO = 14 kDa) to remove unreacted PEG polymers and other reagents. After lyophilization, the final product (C18PMH-PEG-NH_2) in white solid was stored at −20 °C for future use.

The folic acid conjugated C18PMH-PEG was prepared by conjugating the amine-functionalized C18PMH-PEG-NH_2 with activated FA. Briefly, 35 mg of FA was mixed with 15 mg EDC and 23 mg NHS in 5 mL of anhydrous DMSO for 15 min at room temperature. 20 mg of C18PMH-PEG-NH_2 in 5 mL of DMSO was added afterwards (molar ratio of NH_2/FA/EDC/NHS = 1:2:2:5). After stirring at room temperature for 8 h, water was added and the product was purified by dialysis. The final product was lyophilized and stored at −20 °C until use.

2.4. Preparation and functionalization of NIR712-doped NPs

NIR712-doped NPs were synthesized through a facile solvent exchange approach. NPAFN was first dissolved in THF to prepare a 1×10^{-3} M stock solution. Different amounts of NIR712 dye were added to 0.15 mL of the stock solutions. The mixed solutions were each injected to 5 mL of aqueous solution at 30 °C under vigorous stirring at 1000 rpm. After stirring for 3 min, the samples were stabilized for 24 h.

For modification of NIR712-doped NPs, 2 mg of C18PMH-PEG was dispersed in 10 mL of water by sonication. Then 300 μL of solution was added into 5 mL of NPs suspensions followed by ultrasonication for 5 min. After that, solution was kept for use at room temperature. C18PMH-PEG-FA functionalization was executed referring to the former. To evaluate the stability of the NPs, evolutions of their diameter and fluorescence in phosphate buffered saline (PBS), serum and Na_2CO_3–$NaHCO_3$, Na_2HPO_4-citric acid buffer solution of different pH values were recorded at different times. All measurements were performed at room temperature.

2.5. Determination of quantum yield

Fluorescence quantum yield was measured using a Perkin-Elmer Lambda 750 UV/Vis/NIR Spectrometer and luminescence spectrometer. We made use of 2,7-di(4-(diphenylamino)phenyl-2,1,3-benzothiadiazol-7-yl)-9,9′-spirobifluorene (Spiro-BTA) excited at 468 nm (QY = 0.45) as a standard to measure the fluorescence quantum yields of NIR712-doped NPs in suspensions. Quantum yield was calculated according to the following equation:

$$\eta_s = \eta_r \left(\frac{A_r}{A_s}\right)\left(\frac{I_s}{I_r}\right)\left(\frac{n_s^2}{n_r^2}\right)$$

η, A, I and n refer to quantum yield, absorbance, fluorescent intensity and refractive indices, respectively. The subscripts s and r refer to the sample and the reference solutions respectively. In our experiments, A_r = 0.05, A_s = 0.05, n_s = 1.333 (H_2O, 20 °C), n_r = 1.4 (THF, 20 °C). It was obtained by integrating the emission spectra over 680–850 nm for NIR712-doped NPs.

2.6. Cell culture

KB cells were cultured in folic acid free RPMI-1640 and normal RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2. The former was considered as a positive group and the latter as a negative group. 4T1 cells, MRC-5 cells and HeLa cells were cultured in normal RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7. Cytotoxicity assays

KB, MRC-5 and HeLa cell lines were grown into a 96-well cell-culture plate at 10^4–10^5/well and then incubated for 24 h at 37 °C under 5% CO_2. After incubating the cells with different doses of NPs, the standard MTT assay was performed to measure the cell viabilities by using untreated cells as a control. In this paper, all of the error bars indicate the standard deviation (SD) of the results.