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Croconaine nanoparticles with enhanced tumor accumulation for multimodality cancer theranostics

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ABSTRACT

A novel nanoparticle self-assembled by polyethylene glycol (PEG) modified croconaine dye (CR780) is presented for photoacoustic (PA)/near-infrared (NIR) fluorescence imaging-guided photothermal therapy (PTT). The simple PEGylation made CR780 amphiphilic, and led to their self-assembly into well-defined and uniform nanostructures with size tunable by controlling the assembly conditions. The CR780-PEG5K not only displayed the strength of small molecules (including rapid distribution to different organs, fast renal clearance and minimal accumulation to normal tissues), but also demonstrated the advantages of nanomaterials (including high physiological stability, multimodal theranostic ability, high tumor accumulation and retention). These facilely synthesized molecular nanoprobes showed great clinical translation potential as a versatile theranostic agent.

1. Introduction

Nanomedicine has been extensively investigated in recent years for cancer treatment due to their unique physicochemical properties and ease of functionalization [1–3]. The enhanced permeability and retention (EPR) effect has led to the preferential accumulation of nanoscopic agents in tumor tissues, resulting in better theranostic efficacy and reduced side effects [4–6]. A series of nanoagents, including iron oxide nanoparticles (NPs) [7], quantum dots [8] and gold NPs [9] have been developed for theranostic application; however, most of them are struggling to enter into clinical trials, partially due to their structurally complexity and potential toxicity to the normal tissues [10]. There is a pressing need to develop novel biocompatible and effective theranostic NPs which is favorable for clinical translation.

Photothermal therapy (PTT) is a promising cancer therapeutic approach which uses the photothermal conversion agents (PTCAs) to generate heat for cancer cell ablation upon near-infrared (NIR) laser irradiation [11–14]. Most effective PTCAs are inorganic NPs such as gold nanorods [15,16], graphene based NPs [17,18] and copper sulfide NPs [19,20] which are difficult to enter clinical practice. Alternative PTCAs are organic NIR dyes with small molecular weight, clearly defined structure and reliable synthetic procedures [21,22]. Although NIR dyes have demonstrated their capability as *in vivo* photo-diagnostics (such as fluorescence and photoacoustic (PA) imaging) and phototherapies (such as photothermal and photodynamic therapy) [23,24], many of them, including FDA approved indocyanine green (ICG), suffer from photobleaching and poor tumor accumulation [25]. Recently, croconaine dyes have demonstrated intense NIR absorption [26,27], high photothermal conversion efficiency as well as excellent chemical and thermal stability, proving to be promising candidates for PTT [28]. However, their insolubility in aqueous solution make them difficult for *in vivo* biomedical use [29–31].

Chemical conjugation of polyethylene glycol (PEG), a FDA approved hydrophilic polymer, has been widely used to improve the physicochemical properties of both small molecules (e.g. drugs [32] or NIR dyes [33]), and macromolecule (e.g. proteins [34] or

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DNA [35]). Here, we found that through PEGylation, CR780 (a croconium dye, 2,5-bis[(4-carboxylicpiperidylamino) thiophenyl]-croconine) became amphiphilic and could self-assemble into monodispersed NPs in aqueous environment with size tunable by controlling the assembly conditions. The 5.1 nm croconaine NPs formed by CR780-PEG5K in the presence of serum have at least four advantages: 1) They demonstrated excellent water solubility and impressive chemical stability in physiological environment with no morphology change within 24 h. 2) Due to the EPR effect, these NPs showed improved tumor accumulation. Meanwhile, they are renal clearable with nearly no accumulation in the normal tissues. 3) They are naturally NIR fluorescent/PA imaging and PTT agents. 4) The fabrication process is straightforward and reliable, favorable in clinical use. We believe that these resulting NPs represent a new generation of theranostic agents, capable of diagnosis, therapy and monitoring therapeutic response, promising for clinical translation.

2. Materials and methods

2.1. Materials and instrumentation

Methyl isonipecotate, 1-ethyl-(3-dimethyllaminopropyl)carbodiie hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), *N*,*N*-diisopropylethylamine (DIEA), 1-hydroxy-benzotriazole monohydrate (HOBT) and croconic acid were purchased from J&K Scientific Ltd. (Beijing, China). Thiophene-2-thiol, 4,6-diamidino-2-phenylindole (DAPI), 4-nitrophenyl chloroformate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and Calcein-AM were obtained from Sigma-Aldrich Co. Ltd. (MO, USA). 2-(2-(2-Aminoethoxy)ethoxy)ethanol and methoxy poly(ethylene glycol) (mPEG, M_w = 2 kDa or 5 kDa) were purchased from Shanghai Seebio Biotech (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Penicilline-Streptomycin solution were purchased from HyClone Inc. All other chemicals were purchased from Sigma Aldrich in analytical grade and used without further purification unless noted. The ^{1}H NMR spectra were performed on Bruker-Avance II NMR spectrometer at 600 MHz using tetramethylsilane as the internal standard. Electron-spray ionization mass spectra in positive mode (ESI-MS) data were recorded on a Bruker Esquire 3000t spectrometer. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was obtained on Bruker microFlex MALDI—TOF-MS, located in College of Chemistry and Chemical Engineering, Xiamen University. The fluorescent spectra were performed with a Horiba FluoroMax4 equipped with automatic polarizers. The spectra were further analyzed in FluorEssence (Horiba, Kyoto, Japan) or in Origin 9.0 Pro (Origin Lab, Northampton, MA). Dynamic light scattering (DLS) measurements were obtained on a DelsaNano C (Beckman Coulter, Fullerton, CA) instrument equipped with a laser diode operating at 658 nm. The particle ζ-potential measurement was carried on by a DelsaNano C particle analyzer equipped with a 30 mW dual laser diode (658 nm). Transmission electron microscopy (TEM) images were obtained on a JEOL 1200EX operating at 100 kV. The samples were prepared by depositing aqueous solution (4 μl) onto carbon-coated copper grids and removing the excess solution by filter paper.

2.2. The preparation of CR780, sCR780 and CR780-PEG5K

Synthesis of CR780. 2,5-bis[(4-carboxylic-piperidylamino)thiophenyl]-croconine (CR780) was synthesized according to a previously described method [27]. The synthetic route of compound CR780 is shown in Sheme S1. Briefly, a mixture of 2.15 g (15 mmol) of methyl isonipecotate, 1.16 g (10 mmol) of thiophene-2-thiol were mixed in toluene (10 mL) and refluxed with vigorous stirring under nitrogen atmosphere for 2 h. After the reaction, the mixture was cooled down to room temperature. The resulting compound methyl 1-(thiophen-2-yl) piperidine-4-carboxylate (1) was purified by column chromatography on silica gel using hexane and ethyl acetate (5/1, v/v) as the eluent. A pale yellow solid was obtained with a yield around 75%.

Then, 0.45 g (2 mmol) of product 1 and 10 mL of 0.5 M sodium hydroxide solution were refluxed for 1 h. After cooling to room temperature, the reaction mixture was acidified with 10% acetic acid forming a white precipitate, which was then filtered and dried under vacuum to afford 1-(thiophen-2-yl)piperidine-4-carboxylic acid (2) in the form of a white solid (0.37 g, yield 85%).

Next, 211 mg (1 mmol) of compound 2 and 71 mg (0.5 mmol) of croconic acid were dissolved in the mixture of n-butanol and toluene (10 mL, 1:1) and stirred at 120 °C for 1 h. The mixture was filtered after cooling down, and washed with methanol and dried under vacuum to obtain 230 mg of pure CR780 (yield 90%) as a black solid. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) 8.523 (s, 2H), 7.038 (d, J = 6.0 Hz, 2H), 4.015 (d, J = 13.4 Hz, 4H), 3.524 (t, J = 11.2 Hz, 4H), 2.676 (s, 2H), 2.054 (d, J = 10.2 Hz, 4H), 1.746 (q, J = 11.2 Hz, 4H). MS (ESI): m/z = 529.85 $[M+H]^+$; calculated m/z = 528.6 for $C_{25}H_{24}N_2S_2O_7$. All spectral data match well with those previously reported.

Synthesis of sCR780. Sodium salts of CR780 were prepared *via* a widely used method [36]. Briefly, sodium bicarbonate (1.00 mmol, 0.0840 g) was added to an aqueous solution (10 mL) of the CR780 (1.00 mmol), and the mixture was stirred for 30 min rigidly. The solvent was removed by rotary evaporator until a black precipitate was formed. The solid products were purified by recrystallization from H_2O/EtOH mixture.

Synthesis of CR780-PEG5K. CR780 (0.16 g, 0.32 mmol), EDC·HCl (0.25 g, 1.28 mmol), and HOBt (0.18 g, 1.28 mmol) were dissolved in 50 mL of DMF and then DIPEA (0.5 mL, 3.0 mmol) was added into the solution. The mixture was stirred in an ice bath for 30 min, followed by the addition of mPEG5K—NH_2 (1.6 g, 0.32 mmol). After stirring at room temperature for 72 h, the solvent was evaporated to yield black crude product. The crude was then purified by precipitation from methanol into cold diethyl ether to give CR780-PEG5K (yield 72%). CR780-PEG132 and CR780-PEG2K were synthesized in the similar way, using mPEG132-NH_2 and mPEG2-K—NH_2 instead of mPEG5K—NH_2.

CR780-PEG132 ^{1}H NMR (600 MHz, CD_3OD): δ (ppm) 7.717 (s, 2H), 7.702 (s, 2H), 7.515 (dd, J = 7.8 Hz, 2H), 7.446 (dd, J = 7.8 Hz, 2H), 3.684 (t, J = 4.8 Hz, 4H), 3.635—3.654 (m, 16H), 3.600 (s, 2H), 3.359 (t, J = 4.8 Hz, 4H), 3.128 (t, J = 4.8 Hz, 4H).

CR780-PEG2K ^{1}H NMR (600 MHz, CD_3OD) and CR780-PEG5K ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 8.611 (s, 2H), 6.934 (s, 2H), 4.161 (d, J = 11.4 Hz, 4H), 3.744 (d, J = 4.8 Hz, 4H), 3.621—3.644 (m, repeat ethylene units), 3.380 (t, J = 4.8 Hz, 4H), 3.354 (s, 3H), 3.344 (s, 2H), 2.026 (d, J = 13.2 Hz, 4H), 1.897 (m, 4H).

2.3. NIR laser-induced PTT effect

The aqueous solutions of sCR780, ICG, IR806, CR780-PEG5K (30 μM) and PBS in 1.5 mL Eppendorf tubes were irradiated upon a NIR laser (808 nm, Stone laser, Shenzhen) at power density of 1 W/cm^2 for 5 min. Real-time thermal imaging was acquired using a FLIR A × 5 camera (FLIR Systems Inc., Wilsonville, OR) and quantified by FLIR Examiner software. The photothermal conversion efficiency (η_T) was calculated based on the following equation [37].

$$\eta_T = \frac{hA(T_{max} - T_{amb}) - Q_0}{I(1 - 10^{-A_\lambda})}$$

where h is the heat transfer coefficient, A is the surface area of the