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Regular Article

iRGD-decorated red shift emissive carbon nanodots for tumor targeting fluorescence imaging

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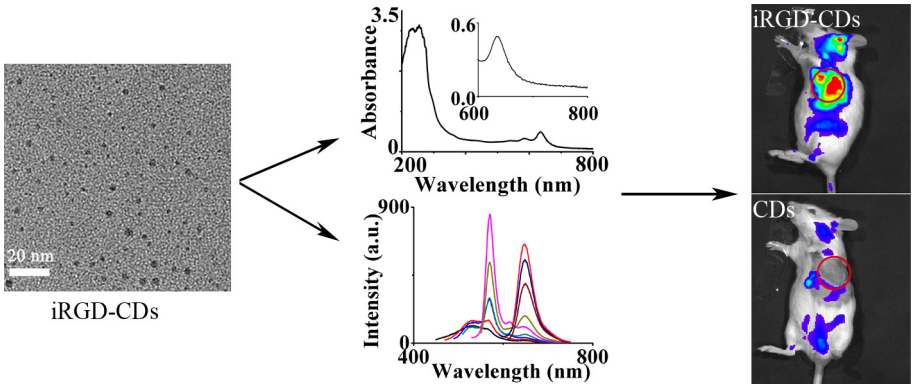
GRAPHICAL ABSTRACT



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ABSTRACT

Carbon nanodots (CDs) have been exhibiting increasing applications owing to their luminescence properties and biocompatibility as imaging probes in diagnosis. However, poor tumor targeting and penetration of CDs is still the biggest challenge limiting their tumor imaging efficacy. To improve the tumor targeting and penetration efficiency of CDs, we developed an active tumor targeting imaging system by simply fabricating a tumor-homing penetration peptide iRGD (CRGDKGPDC) to red shift emissive CDs (iRGD-CDs) with a physical method. Particularly, iRGD-CDs showed a small size and red shift fluorescence signals as CDs, which made iRGD-CDs suitable for *in vivo* fluorescence imaging. iRGD-CDs showed higher cellular uptake *in vitro*, while presented higher penetration and accumulation in tumor tissue *in vivo*, leading to better tumor imaging efficacy. In conclusion, decoration with iRGD could significantly increase the permeability of CDs in tumor vessels and tumor tissue, generating more CDs leaking out from tumor vasculature, consequently improving the sensitivity of tumor imaging.

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1. Introduction

In recent years, carbon-based nanomaterials, including graphene quantum dots, polymer dots, carbon nanotubes, fullerene, nanodiamonds and carbon nanodots (CDs) have been extensively applied in many fields [1–7]. Among them, CDs, the new class of nanomaterials have received considerable attention owing to their intrinsic advantages, including photoluminescence, good water solubility, good biocompatibility, ease of functionalization, low toxicity, low immunogenicity, and resistance to photobleaching [8–14]. Nowadays, CDs hold great potential in sensing, fluorescence imaging, photoacoustic imaging, magnetic resonance imaging, photothermal treatment and drug delivery, which is considered to be a potential eco-friendly alternative to semiconductor quantum dots [15–24]. However, the luminescence of most CDs is still with short excitation and emission wavelengths, which is not suitable for *in vivo* imaging because of high background signal and poor tissue penetration. In the past several years, more and more CDs with red shift luminescence were prepared to reduce the background signal as well as increase tissue penetration, which can be more adapted in *in vivo* fluorescence imaging [25–28]. However, CDs can only passively accumulate in tumor via the enhanced permeability and retention (EPR) effect, which is not sufficient for tumor targeting of CDs [29–32]. Therefore, it is important to develop novel CDs equipped with both red shift fluorescence and active tumor targeting capacity. To improve the tumor targeting and penetration of nanomaterials, various targeting strategies and ligands have been applied to direct them to tumors [33–37]. Among tumor targeting moieties, peptides have been utilized in cancer diagnostics and therapeutics due to their advantages such as low toxicity, low immunogenicity, fast clearance, high specificity, and high versatility [38–40]. As a tumor-homing penetration peptide, iRGD, firstly homes to tumors by initially targeting $\alpha_v\beta_3$ integrin receptors, which are specifically expressed in the tumor vasculature and tumor cells. Then it binds to neuropilin-1 (NRP-1), consequently triggering tissue penetration [30,41–45]. Physically decoration with iRGD or chemically conjugated to iRGD can not only improve tumor penetration of various compositions but also specifically enhanced vascular and tissue permeability in a tumor-specific and NRP-1- dependent manner [46–49]. In this study, we developed an active tumor targeting imaging system only through physically decorating iRGD onto a red shift emissive CDs (iRGD-CDs). Then the luminescence properties, particle size, surface groups, serum stability and hemcompatibility of iRGD-CDs were carefully evaluated. Cellular uptake and cell toxicity were tested on $\alpha_v\beta_3$ and NRP-1 receptors overexpressed 4T1 cells *in vitro* [50,51], and an appropriate control cell experiment was carried out using A2780 cells with low $\alpha_v\beta_3$ [52]. *In vivo* tumor imaging and tissue distribution studies were carried out to elucidate the tumor targeting effect of iRGD-CDs.

2. Materials and methods

2.1. Materials

Melanin (99%) was purchased from Alfa Aesar (Ward Hill, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) was purchased from Beyotime Institute Biotechnology (Haimen, China). Rabbit anti-CD34 polyclonal antibody was obtained from eBioscience, Inc., (San Diego, USA). Rabbit neuropilin-1 polyclonal antibody (NRP-1) was purchased from 4A Biotech Co., Ltd. (Beijing, China). Rabbit anti-integrin beta-3 was obtained from Abcam Ltd. (Hong Kong, China). Cy3-conjugated donkey anti-rabbit secondary antibody and Cy3-conjugated donkey anti-rat secondary antibody were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, USA). Plastic cell culture dishes and plates were obtained from Wuxi NEST Biotechnology Co. Ltd (Wuxi, China). Dulbecco's Modified Eagle Medium cell culture medium (DMEM) containing 10% of fetal bovine serum (FBS), 100 U mL^{-1} of penicillin G and 100 U mL^{-1} of streptomycin sulfate were obtained from Life Technologies (Grand Island, USA). 4T1 cell line and A2780 cell line were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Female BALB/c mice (20 ± 2 g) were purchased from Laboratory Animal Centre of Southern Medical University (Guangzhou, China) and were maintained under standard housing conditions. All animal experiments were performed under the guidelines evaluated and approved by the ethics committee of Southern Medical University, China.

2.2. Preparation and characterization

iRGD-CDs were prepared by adsorbing iRGD onto CDs obtained from previous study [53]. Briefly, 1.0 g of water-soluble melanin was dissolved in 6 mL of deionized water and the reaction mixture was heated at 220 °C for 48 h. After cooled to room temperature, the solution was filtered (pore size 0.45 μm) and then iRGD at the concentration of 3.8 mg/ml was added into the above-mentioned CDs solution. Morphology of iRGD-CDs was captured by transmission electronic microscopy (TEM) (JEM-2100F, Japan). Ultraviolet–visible (UV–vis) spectra of iRGD-CDs and CDs in water were recorded using a Varian cary 100 conc UV–vis spectrophotometer (Varian, USA). Fluorescence spectra of iRGD-CDs and CDs were recorded using a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Fourier transform infrared (FT-IR) spectra of iRGD-CDs and CDs in KBr was collected by using a Nexus 670 (Thermo Nicolet, USA).

2.3. Stability and hemocompatibility

The serum stabilities of iRGD-CDs and CDs were investigated in PBS with different concentrations of FBS. iRGD-CDs and CDs were suspended in 0%, 10% or 50% FBS and incubated in a shaker (37 °C, 100 rpm/min). The absorption of iRGD-CDs and CDs at 490 nm was detected by a microplate reader (Multiskan MK3, Thermo, USA) at 0, 1, 2, 4, 6, 8, 10, 12 and 24 h. In addition, fluorescence spectra of iRGD-CDs incubated with 10% FBS was recorded at 0, 2, 4, 12 and 24 h.

Whole blood was collected from BALB/c mice using heparin as the anticoagulant. After centrifugation at 2500 rpm for 5 min, the red blood cells were resuspended in PBS (pH = 7.4) to get 2% erythrocyte stock dispersion (ESD). Different concentrations of iRGD-CDs and CDs were added into 2% ESD and incubated at 37 °C for different time. The absorption at 490 nm was detected by a microplate reader (Thermo Scientific Varioskan Flash, USA). 1% of Triton X-100 was used as positive control while PBS (pH = 7.4) was used as negative control.

2.4. Cytotoxicity

MTT assay was used to investigate the cytotoxicity of iRGD-CDs and CDs. 4T1 cells were seeded into the 96-well plates at a density of 1×10^5 per well. After 24 h incubation, iRGD-CDs and CDs were added into each well with final concentrations from 128 μg/mL to 1 μg/mL. After another 24 h incubation, 10 μL MTT solution (5 mg/mL) was added into each well and incubated for 4 h, then the medium was replaced by 150 μL dimethyl sulfoxide and the absorbance was measured by a microplate reader (Thermo Scientific Varioskan Flash, USA) at 490 nm.