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A simple one-step synthesis of melanin-originated red shift emissive carbonaceous dots for bioimaging

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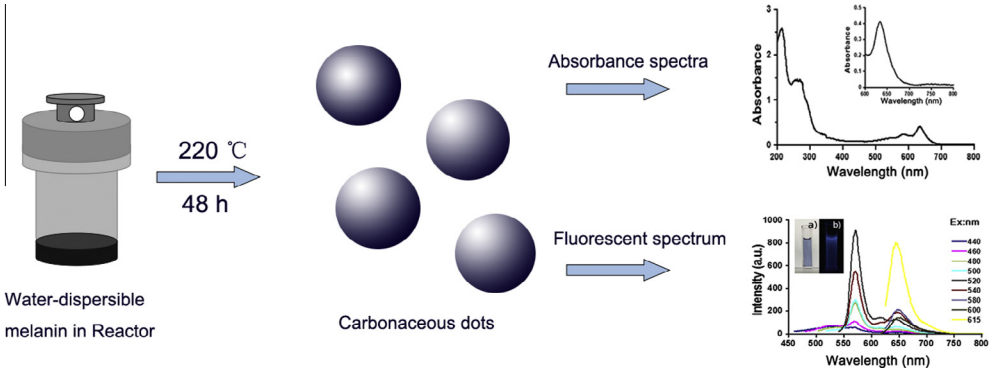
GRAPHICAL ABSTRACT



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ABSTRACT

Carbonaceous dots (CDs) are superior nanomaterials owing to their promising luminescence properties and good biocompatibility. However, most CDs have relatively short excitation/emission, which restrict their application in bioimaging. In this study, a simple one-step procedure was developed for synthesis of melanin-originated CDs (MNPs). The MNPs showed two long red shift emissions at 570 nm and 645 nm with broad absorptions from 200 nm to 400 nm and 500 nm to 700 nm, suggesting the great potential of MNPs in bioimaging. Besides, several experiments indicated that MNPs possessed good serum stability and well blood compatibility. *In vitro*, MNPs could be taken up by C6 cell in a concentration- and time-dependent manner with endosomes involved. In conclusion, MNPs were prepared using a simple one-step method with unique optical and good biological properties and could be used for bioimaging.

1. Introduction

Nanotechnologies have been developed for more than 40 years to deliver therapeutic and diagnostic agents in a safe and more efficient manner [1,2]. Until now, several nanomedicines and nanoprobes have been approved for clinical use. However, the properties of these nanomedicines and nanoprobes are far from fully addressing clinical requirements. Fluorescent carbonaceous dots (CDs) are new kinds of nanomaterials that emerged several years ago [3–7]. CDs has been used in sensoring [8], tumor imaging [9], drug delivery [10] and photothermal therapy [11] because of their intrinsic advantages, including good biocompatibility, high fluorescent intensity, good photo stability, excellent water solubility, simple synthesis and modification [12]. Currently, many

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precursors are used for preparation of CDs, such as silk, graphene, saccharide, amino acid, polymer, and many other carbon contained materials [13–15]. However, the luminescence of most CDs are still with short excitation and emission wavelengths, which is not suitable for *in vivo* imaging because of the poor tissue penetration and high background. Therefore, preparing CDs with red shift luminescence is still a challenge for researchers.

Recently, we prepared CDs from glycine using simple heat treatment method, and the luminescence successfully extended to 500–600 nm [15]. Although it could be used for *in vivo* glioma imaging, the fluorescence intensity at 600 nm was much weaker than that at 500 nm. To further improve the luminescence efficiency with a red-shift wavelength, we developed a one-step method to prepare CDs using melanin as the only precursor. Melanin was a common material in our body, as well as an effective absorber of light [16–18]. Directly hydrothermal method of melanin for 48 h could obtain the melanin originated CDs (MNPs) with a size of 45 nm. Then the luminescence properties of MNPs were carefully evaluated. The particle size, surface groups, serum stability, hemcompatibility and cytotoxicity were performed to characterize the MNPs. Moreover, C6 cell imaging and endosome labeling were also carried out to probe the bioimaging potential of MNPs.

2. Materials and methods

2.1. Materials

Melanin (99%) was purchased from Keddia Reagent (Chengdu, China). DAPI and thiazolyl blue (MTT) were purchased from Beyotime (Haimen, China). LysoTracker Red DND-99 was purchased from Life technologies (Grand Island, NY, 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) and FBS were obtained from Life Technologies (Grand Island, NY, USA). bEnd.3 Cell line was obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All of the other chemicals and reagents were of analytical grade.

2.2. Preparation and characterization of MNPs

MNPs were obtained from directly hydrothermal method of about 1.0 g of water-soluble melanin and 6 mL deionized water at 220 °C for 48 h. After cooled to room temperature, the solution was filtered (pore size 0.45 μm). Morphology of MNPs was captured by transmission electronic microscopy (TEM) (Tecnai G^2 F20 S-TWIN, FEI, USA). Zeta potential was determined by a Malvern Zetasizer (Malvern, NanoZS, UK). Ultraviolet–visible (UV–vis) spectra in water was recorded using a Varian cary 100 conc UV–Vis spectrophotometer (Varian, USA). Fluorecence spectroscopy was recorded using a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Japan). Fourier transform infrared (FTIR) spectra in KBr was collected on a Bruker Vector 22 spectrometer (Bruker, Germany). X-ray photoelectron spectroscopy (XPS) was performed on an AXIS Ultra DLD (Kratos, UK) with Mg Ka radiation ($h\nu$ = 1486.6 eV), with a chamber pressure of 2.2×10^{-9} Torr. Fluorescence quantum yield was measured against Quinine in 0.1 N sulfuric acid (literature QY 0.546 at 365 nm) as a standard using RF-5301PC spectrofluorophotometer (Shimadzu, Japan).

2.3. Stability of MNPs

The stability of MNP was investigated in PBS with different concentrations of FBS, MNPs were suspended in 0%, 10% or 50% FBS and incubated in a shaker (37 °C, 75 rpm). The absorption of MNPs 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. Zeta potential was determined by a Malvern Zetasizer (Malvern, NanoZS, UK) at 0, 1, 2, 4, 8, 12 and 24 h.

2.4. Hemocompatibility

Whole blood was collected from BALB/c mice using heparin as the anticoagulant. After centrifugation at 2000 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 MNPs were added in 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 a positive control while PBS (PH7.4) was used as negative control.

2.5. Cytotoxicity of MNPs

The cytotoxicity of MNPs was evaluated by MTT assay. bEnd. 3 cells (5×10^3 per well) were seeded in 96-well plates and allowed to grow until 60% confluent. MNPs were added into the wells at a series of concentrations ranging from 128 μg/mL to 1 μg/mL for another 24 h incubation, then 10 μL MTT solution (5 mg/mL) was added into each well and incubated for 4 h. After the medium was replaced by 150 μL dimethyl sulfoxide, the absorbance was measured by a microplate reader (Thermo Scientific Varioskan Flash, USA) at 490 nm.

2.6. Cell uptake

For quantitative studies, C6 cells were seeded in 24-well plates at a density of 5×10^5 cells per well and incubated for 24 h. 500 μg/mL, 125 μg/mL and 31 μg/mL of MNPs were added into the wells. After incubation for 0.15 h, 1 h, 2 h and 4 h at 37 °C, the cells were washed three times with PBS, trypsinized, and resuspended in 0.5 mL PBS (pH 7.4). The fluorescent intensity of cells was analyzed by a flow cytometer (Cytomics FC 500, Beckman Coulter, USA).

For qualitative measurement, C6 cells were seeded onto 12 mm coverslips in 6-well plate at a density of 1×10^5 cells per well and incubated at 37 °C for 24 h. Then the cells were treated with different concentrations of MNPs for different time. After incubation, the cells were washed, fixed and stained with 0.5 μg/mL DAPI. then Imaged by a confocal microscope (LSM710, Carl Zeiss Germany).

2.7. Subcellular localization

C6 cells were seeded in plates as described in qualitative measurement of cellular uptake and incubated with 125 μg/mL for 4 h and 0.25 h. At 0.5 h before the incubation ended, LysoTracker Red DND-99 (100 nM) was added. After incubation, the cells were washed, fixed and stained with 0.5 mg/mL of DAPI. Then the cells were imaged by a confocal microscope (LSM710, Carl Zeiss, Germany).

3. Result and discussion

3.1. Characterization of MNPs

The synthetic approaches of CDs have been extensively developed, including top-down and bottom-up methods. The top-down methods are mainly based on the chemical or physical cutting processes of relatively macroscopic carbon structures