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Eco-friendly synthesis of shrimp egg-derived carbon dots for fluorescent bioimaging

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ABSTRACT

Developing synthetic methods to produce carbon dots (CDs) using natural biomass or other readily available carbon sources are currently being explored. We describe a simple and green synthetic method for preparing fluorescent CDs by water extraction from heat-treated shrimp eggs (SE-CDs). The SE-CDs appeared spherical with an average size of 3.25 ± 1.06 nm. Elemental analysis indicate that the SE-CDs have functional groups such as C–OH, C–O–C, C═O, and C–H on the surface which give rise to a series of emissive traps between π–π^* states. SE-CDs also showed a broad emission range with excellent quantum yield of $18.5 \pm 2.6\%$. In addition, when compared with commonly used traditional CdSe and CdTe nanocrystals, SE-CDs were bio-tolerable to cell at high doses (200 $\mu g\,ml^{-1}$) in MTT assay. Thus, SE-CDs are very promising alternatives to semiconductor-based quantum dots for *in vitro* and *in vivo* bioimaging applications.

1. Introduction

Previously, photoluminescent semiconductor-based quantum dots (QDs) have shown several inherent advantages over conventional fluorophore dyes, such as high absorption cross-section, long fluorescent lifetimes, tunable emission peaks and negligible photodegradation, making them useful fluorescent tools for bio-applications (Clapp et al., 2005; Lim et al., 2003). However, the QD surface is typically hydrophobic and thus incompatible with aqueous and biological environments. Consequently, QDs must be subjected to secondary chemical processing to modify the outer surface to make them water soluble (Clapp et al., 2005). This serves the dual purpose of providing a mechanism for adding specific chemical functionalities as well as facilitating the removal of the toxic QD core materials from the environment. As such, functionalized, water-soluble QDs could be well-dispersed in a variety of aqueous buffers, and conjugated with biomolecules (Clapp et al., 2003; Clapp et al., 2005). However, the applied coating on QDs increases their size as compared to standard fluorophores which may be a hindrance in biological systems (Walling et al., 2009). Furthermore, their inherent toxicity makes them non-ideal for biological applications. Thus, substantial effort has gone into developing a smaller, hydrophilic and non-toxic substitute.

Carbon dots (CDs) are carbonaceous nanoparticles that exhibit physical and optical properties analogous to conventional QDs and silicon nanoparticles (Lin et al., 2013; Zhang et al., 2011). Compared to QDs, fluorescent CDs are superior in terms of their aqueous solubility, small size (<10 nm), intense brightness, high photostability, low cytotoxicity, and good biocompatibility (Wang et al., 2011). Additionally, CDs are also relatively easy to functionalize, low-cost, and applicable in large scale (Wang et al., 2013).

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Thus, photoluminescent CDs are promising alternatives to semiconductor QDs for applications such as bioimaging, biosensing, photocatalysis, and drug delivery (Baker and Baker, 2010; Hsu et al., 2012; Hu et al., 2009; Jaiswal et al., 2012; Zhang et al., 2012). Previous studies have reported on the preparation of CDs using natural biomass or other readily available carbon source materials. Liu et al. (2007) fabricated photoluminescent carbon nanoparticles (CNPs) from the combustion soot of candles. Using plant-based biomass, Krysmann et al. (2012) reported an environmentally benign approach to synthesize CNPs based on the pyrolytic decomposition of shredded grass. Hsu et al. (2012) developed a synthetic route for preparing CDs using coffee grounds as the starting material without strong acid or surface passivation. Other related studies of biomass-derived CNPs have been reported, using soy milk (Liu et al., 2007), pomelo peel (Qian et al., 2006), and orange juice (Sahu et al., 2012), as the starting material *via* a variety of processing methods.

Biomass is an abundant and important feedstock for the production of a range of carbonaceous materials including nanoparticles (Krysmann et al., 2012), nanotubes (Shinde and Pillai, 2012), and nanofibers (Qian et al., 2006). However, to the best of our knowledge, no examples have been published where readily-abundant ocean biomass was used as the starting material for fabrication of CNPs. Herein, we report a facile method for fabrication of strongly photoluminescent CDs by water extraction from heat-treated shrimp eggs (SE-CDs). We also demonstrate that the CDs prepared by this method are biocompatible, biodegradable, and are able to permeate a live cell membrane, thereby making them favourable candidates for *in vivo* biomedical imaging applications.

2. Materials and methods

2.1. SE-CDs (carbon dots) synthesis

Carbon dots were fabricated by placing 5 g of fresh shrimp eggs in a clean glass dish, heating in an oven at 180 °C for 25 min, and then cooling the sample to room temperature. This process results in the conversion of the shrimp egg constituents to form carbon dots. Subsequently, cold-water extraction and ultrasonic extraction methods were used to prepare a stock solution of carbon dots from the heat-treated shrimp eggs. The procedures were performed as follows: 0.1 g treated shrimp eggs were dispersed into a micro tube with 1 ml pure water, and immediately put into an ultrasonic bath (Delta DC200H) for 5 min (40 kHz ultrasound frequency, 200 W power output) at room temperature to separate the egg shell and accelerate the extraction rate of carbon dots. Finally, the solution was centrifuged at 10,000 rpm for 10 min and the supernatant liquid filtered through a 0.45 μm PVDF syringe filter (Millipore Millex-HV) to yield the final shrimp-egg CDs (SE-CDs) product sterile filtration.

2.2. Characterization methods

Topographic images of SE-CDs were obtained using an atomic force microscope (AFM) (MFP-3D™, Asylum Research, Santa Barbara, CA, USA) operating in AC (tapping) mode under ambient conditions. A silicon cantilever (Olympus, AC240-TS) with a nominal spring constant of 2 N m^{-1} was used for all images, at a scan rate of 1.0 Hz and an image resolution of 512 × 512 pixels.

Transmission Electron Microscopy (TEM) images of CD samples were acquired using a JEOL JEM-2100 operated at 200 KV. The TEM sample was prepared by depositing a droplet of SE-CD/H_2O solution onto a Carbon composite TEM grid with copper 200-mesh (Ted Pella Inc, CA, USA) and allowed to dry.

The elemental composition and chemical state of SE-CDs was investigated by X-ray photoelectron spectroscopy (XPS) using a JEOL JPS 9010 MX equipped with a monochromatic Mg Kα X-ray (1253.6 eV) radiation source. Samples were prepared by depositing an aliquot of SE-CD/H_2O solution onto clean gold substrates at room temperature.

Transmission Fourier transform infrared (FTIR) spectra were acquired using a Bruker 66 v/s FTIR spectrometer (8 cm^{-1} resolution, 256 scans, sample compartment vacuum pressure was 0.12 hPa). Double-sided polished silicon (100) wafer substrates were cut into 20 mm × 20 mm pieces using a diamond-tipped stylus. Spectra from a plasma-cleaned silicon wafer sample were collected before each measurement to obtain the background spectrum. Samples were prepared by depositing an aliquot of SE-CD/H_2O solution onto clean double-sided polished silicon wafer substrates at room temperature.

The UV–vis absorption spectrum was recorded with by a UV–Vis spectrophotometer (JASCO V-630, Japan) using a 1-cm path length quartz cell. Photoluminescence (PL) spectra measurements were acquired using a HITACHI F-4500 Fluorescence Spectrometer. The excitation wavelengths were 280–500 nm at 20 nm intervals in the excitation domain. Emission spectra were recorded from 200 to 800 nm.

The quantum yield was calculated according to the experimental method described by Liu et al. (2009) using the equation:

$$\varphi_x = \varphi_{std} \left(\frac{m_x}{m_{std}}\right) \left(\frac{\eta_x}{\eta_{std}}\right)^2$$

where "φ" is the quantum yield, "m" is slope, "η" is the refractive index of the solvent, the "x" indicates the unknown sample, and "*std*" refers to the quinine sulfate standard solution in 0.1 M H_2SO_4. The "m" is the slope from the plot of integrated fluorescence intensity *vs* absorbance. The refractive index of the solvent was 1.333 for 0.1 M H_2SO_4, and the sample solution was 1.396 for water.

2.3. In vitro cytotoxic assay

SK-Hep-1 cells were harvested and seeded in a 96-well culture plate (1×10^4 cells/100 μl/well) overnight at 37 °C and 5% CO_2 in a humidified incubator. Cells were then treated with various concentrations (0, 10, 20, 40, 100, and 200 μg/ml) of SE-CDs and CdTe or CdSe QDs (in 100 μl) for 24 h. Subsequently, the cell survival was analyzed by MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay.

2.4. Intracellular uptake

Human hepatoma cell line SK-Hep-1 was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum. Cells were seeded in tissue culture plates at 4×10^4 cells/well and incubated overnight. Cells were then treated with SE-CDs (200 μg/ml: 1/5 dilution) for 24 h. For the fluorescent gene delivery, 0.5 μg plasmid DNA: pEGFP and pDsRed (CLONTECH) were pre-incubated with 3 μl lipofectamin (Invitrogen) in 100 μl OPTI-MEM® I medium (GIBCO) for 30 min. Subsequently, this DNA-lipofectamin complex was added to the cells and incubated for 24 h.

Prior to the imaging experiment, the cells were washed three times with PBS and fixed with 4% paraformaldehyde. Subsequently, cellular internalization of SE-CDs and expression of fluorescent proteins were detected using an inverted optical fluorescence microscope (Leica, DMIL Germany) through the filters: DAPI, FITC and R-DIL. Image processing and projections were performed using Photoshop 5.5 (Adobe Systems, Mountain View, CA).