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Highly photoluminescent N-isopropylacrylamide (NIPAAM) passivated carbon dots for multicolor bioimaging applications

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ABSTRACT

We report a highly luminescent Carbon Dots passivated by nitrogen rich molecules, i.e. N-isopropylacrylamide (NIPAAM), with a QY as high as ca. 94%, which is the highest yield until now. NIPAMM@C-Dots are synthesized directly from the precursors composed of CA, EDA and NIPAAM by hydrothermal reaction. The optical properties of in situ synthesized NIPAAM@C-Dots showed EX-dependent EM at high concentration and EX-independent EM at low concentration. In most cations and anions except Fe^{3+} and Bi^{3+} added to the aqueous solution, NIPAAM@C-Dots exhibit an extremely stable inert behavior. In addition, in order to verify its usefulness as a fluorescent marker, C6 glioblastoma cells and NIPAAM@C-Dots were cultured together and then applied by both confocal bioimaging and flow cytometry analysis. The role of NIPAAM present on or around the C-Dots surface can improve QY values and stability, resulting in reduced cytotoxicity and can be a strong candidate as an alternative to organic dyes.

1. Introduction

Quantum dots (QDs) are much more researched compared to other materials in comparison to their shorter research history. This is due to the fact that it has a narrow emission (EM) band, a high molar extinction coefficient, a high photoluminescence quantum yield, a symmetric EM of adjustable color, broad and strong absorption, reasonable stability, high brightness and light stability, large Stokes shift, and conjugation chemistries that can bind biomolecules [1,2]. Until now, many researchers have published a number of publications that focus on the implementation of QD NPs, particularly in cell marking and imaging applications for applications in the bio-related field [3]. In the case of organic fluorescent dyes, it is difficult to store the sample in the long term due to the photobleaching phenomenon in which the fluorescence efficiency rapidly decreases when exposed to UV or laser. However, QD NPs are relatively strong against photobleaching resulting in long-term sample storage. Moreover, confocal microscopy can be used to measure the specimens for long-time. QD-labeled growth factors [4] and individual membrane resident lipids [5] are able to target and interact with cells with minimal perturbation in the process being sensed. It is generally known that targeting to specific cells can be performed using receptor-ligand interactions by binding ligands that are over-expressed in specific cells to surface nanoparticles with a high surface-to-volume ratio. To efficiently target NP compartments for specific cell compartments, various biomolecules (eg, antibodies, aptamers, peptides, proteins, nucleic acids) can be conjugated and additional QD NPs can be incorporated into and out of the nanostructure [6]. QD NPs are available as a visible tracer of drug delivery system bodies due to its unique optical properties and has been extensively studied as imaging agents at the cellular and animal level [7,8]. Nevertheless, toxic heavy metals included in QD synthesis are causing serious problems with potential risks to human health and environmental pollution. The toxicity of QDs is regarded as a result of the release of toxic elements (e.g. Cd, Pb, In, Se, and Te etc.) from QDs [9]. Cadmium is known as a toxic element that can induce oxidative stress, DNA damage and apoptosis. The potential toxicity is still one of the major issues that limit the advances of QDs into clinical studies. To reduce the toxicity, inert shells are introduced on heavy metal based QDs such as ZnS [10] and polymers [11]. Carbon-based QDs are a new type of fluorescent material that can be applied in many applications due to superiority in water solubility, chemical inertness, low toxicity, ease of functionalization and resistance to photobleaching [12]. Due to

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these superior properties, many researchers have been developed synthesis routes including microwave assisted pyrolysis [13], electrochemical exfoliation [14], incomplete combustion oxidation [15,16], laser ablation [17], hydrothermal treatments [18], and plasma treatment [19] and intensive studies on photoluminescence (PL) properties. Especially, carbon dots (CDot) prepared by hydrothermal treatment of grass show a PL quantum yield (QY) of 6.2% [20] and bovine serum albumin and as carbon precursor under the passivation of decanediamine show a PL quantum yield of 11% [21]. The C-Dots prepared by hydrothermal treatment of orange juice showed a PL quantum yield of 26% [22]. C-Dots's PL stabilizes the surface of the particles by surface passivation, which generates surface energy traps and hence higher emission intensities [23]. Therefore, passivation and doping on the surface of C-Dots is a popular approach to enhance the QY of C-Dots [24]. C-Dots passivated with PEG_{1500N} synthesized by laser ablation methods had higher QY than those passivated with PPEI-EI [23]. When coated with ZnO/ZnS and PEG_{1500N}, the QY of C-Dots (λ_{ex}: 440 nm) can be increased to 45 and 50%, respectively [25]. The enhanced QY of Zn-based coating is attributed to a secondary, more effective surface passivation in combination with the PEG passivation agent [12].

In this work, passivation of N-isopropylacrylamide (NIPAAM) to C-Dots yields a QY of approximately 94%, which is the highest QY value so far. The NIPAMM@C-Dots are in-situ synthesized by hydrothermal reaction. By using mixtures of citric acid (CA), ethylenediamine (EDA) and NIPAAM with different ratios, we prepare nitrogen-containing C-Dots samples with various nitrogen concentrations. Briefly, the precursors, i.e. CA, EDA and NIPAAM, were dissolved in water and transferred to Teflon-lined stainless autoclave. The mixture was heated at 160 °C for 5 h. In particular, NIPAAM was introduced as a surface protective agent with nitrogen along with the coating material, and polymers such as C-Dots/carbogenic dots were formed by condensation of CA with EDA.

2. Experiment details

2.1. Materials

Citric acid anhydrous ($HOC(COOH)(CH_2COOH)_2$, 99.9%), ethylenediamine ($NH_2CH_2CH_2NH_2$, 99.5%), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, 98%), silver chloride ($AgCl_2$, 99%) cadmium Chloride hydrate ($CdCl_2{\cdot}xH_2O$ 99.995%), sulphuric acid (H_2SO_4, 95–98%) and calcium chloride (CaCl2, 97%) were supplied by Sigma-Aldrich Chemical Co. (St. Louis, U.S.A.). Quinine sulfate dihydrate ($H_2SO_4{\cdot}_2H_2O$, 99%) were supplied by Acros. Lead(II) Acetate Trihydrate ($C_4H_6O_4Pb{\cdot}3H_2O$, 99%), Manganese(II) chloride tetrahydrate ($MnCl_2{\cdot}4H_2O$, 98%), Copper(II) Nitrate Trihydrate ($Cu(NO_3)_2{\cdot}3H_2O$, 99%) were supplied by Daejung. Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$, 99.5%) and Potassium chloride (KCl, 99.5%) were is supplied by Junsei Chemicals (Chuo-Ku, Tokyo). Sodium Chloride (NaCl, 99.5%), bismuth nitrate pentahydrate ($Bi(NO_3)_3{\cdot}5H_2O$, 99%), cobalt(II) sulfate heptahydrate ($CoSO_4{\cdot}7H_2O$, 99%) and zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$, 98%) were purchased from Samchun chemicals (Kyunggido, South Korea). N-isopropylacrylamide (NIPAAM, 98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All the chemicals was used without further purification. Triple distilled and deionized water is used throughout.

2.2. Characterization

Excitation and emission spectra are examined by Fluorescence Spectrophotometer with SHIMADZU, RF-5301PC equipped with a 150 W xenon lamp. Luminescence is measured at an angle of 90° with respect to excitation light beam. The resulting particles are diluted with distilled water and measured at 25 °C. The relative fluorescence quantum yield (Φ) of the C-Dots was measured according to the procedure of a reported method and calculated using the equation of $\Phi_x = \Phi s_{td} I_x A_{std} \eta^2_x / I_{std} A_x \eta^2_{std}$. The optical densities were measured on Mecasys Optizen Pop UV–Vis spectrophotometer. In the equation, I_x and I_{std} are the fluorescence intensities of the C-Dots and the standard, and A_x and A_{std} are the optical densities of the C-Dots and the standard, respectively. Quinine sulfate in 0.1 M H_2SO_4 was selected as a standard with a quantum yield $\Phi_{std} = 0.54$ at 360 nm. η_x and η_{std} are the refractive index of the C-Dots and the standard, respectively. The absorbencies of all the samples in 1.0 cm cuvette were kept under 0.05 at the excitation wavelength to minimize re-absorption effects.

The chemical composition of synthesized polymers was analyzed by an ALPHA FTIR with a Platinum attenuated total reflection (ATR) (Bruker Optics, USA). The samples were dried in oven at 50 °C overnight and grinded into fine powder with a motor and pestle prior to measurement. Spectra were measured with a resolution of 1 cm^{-1} and the wavenumber range was 500–4000 cm^{-1}.

An FEI/Philips, Tecnai F30, 300 kV field emission source TEM instrument, equipped with STEM, an HAADF detector, and XEDS was used to perform TEM bright field high resolution imaging analysis. The software used was Tecnai G2 Digital Micrograph (DM) and Tecnai Imaging & Analysis (TIA). Samples were mounted on Quantifoil holey carbon grids by placing a few droplets of a methanolic suspension of the pigment, followed by air drying.

For XPS analysis, a drop of particle solution is placed on freshly cleaned (plasma cleaner) silicon oxide plates and left for 2 h to dry at ambient conditions. This step is repeated until a complete layer of C-Dots nanoparticles was formed on the substrate and the substrate Si signal was minimized during XPS analysis. XPS measurements are performed approximately 2 h after drying the sample. XPS measurements are performed using a PHI 5000 VersaProbe II Scanning XPS Microscope with a focused monochromatic Al Kα X-ray source (117.40 eV) operating at 26.6 W. Beam diameter is set to 100 μm. The focused source is scanned over a rectangular 400 × 300 μm^2 area of a sample.

2.3. Synthesis of C-Dots by hydrothermal reaction

In a typical experimental procedure was as following: C-Dots were prepared by directly reacting the CA, EDA and NIPAAM. 2 mmol CA, 6 mmol EDA and 2 mmol NIPAAM were dissolved in 20 mL double distilled water (DDW) and located in 20 mL tubular vial and rubber stopper was inserted to vial followed by sealing with aluminum seal by vial clamper The sample was transferred to Teflon-lined stainless steel autoclave (50 mL volume). Ensure that the rubber stopper was tightly contact with Teflon cap and further with outer stainless cap. The precursor loaded autoclave was heated hydrothermally at 160 and 200 °C for 5 h with a heating rate of 10 °C min^{-1}. The tubular vial resists the pressure without rupturing up to 200 °C. The dependence of NIPAAM was measured by changing the molar ratio of 1–3 against CA. The reaction time was varied for 5 and 12 h. After heating the samples for predefined duration, the autoclave was allowed to cool at room temperature. The detailed experimental conditions and procedures are available in Table 1.

2.4. Immunofluorescence and confocal microscopy

The potential for bioimaging of C-Dots was tested using C6 glioblastoma cells. Approximately 1×10^5 C6 glioblastoma cells were deposited on each coverslip (diameter = 18 mm) to form a sparsely distributed layer of cells to ensure good exposure to C-Dots. C6 glioblastoma cells were cultured using the DMEM growth medium with 10% fetal bovine serum at 37 °C with 5% CO_2. All the C6 glioblastoma cells were incubated until approximately 70% confluence was achieved. The mixture of carbon dots in the DMEM medium was added to each well. After 4 h of incubation in 5% CO_2 at 37 °C, the C-Dots loaded C6 cells were washed twice with PBS to remove extracellular C-Dots and