



Water-soluble, lignin-derived carbon dots with high fluorescent emissions and their applications in bioimaging



Aye Aye Myint^{a,b}, Won-Kyu Rhim^c, Jwa-Min Nam^c, Jaehoon Kim^{b,*}, Youn-Woo Lee^{a,*}

^a School of Chemical and Biological Engineering, College of Engineering and Institute of Chemical Processes (ICP), Seoul National University, Seoul 151-744, Republic of Korea

^b School of Mechanical Engineering, School of Chemical Engineering, and SKKU Advanced Institute of Nano Technology, Sungkyunkwan University, 2066, Seobu-Ro, Jangan-Gu, Suwon, Gyeong Gi-Do 16419, Republic of Korea

^c Department of Chemistry, Seoul National University, Seoul 151-747, Republic of Korea

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ABSTRACT

We report the synthesis of water-soluble and environmentally friendly fluorescent carbon dots (CDs) using the renewable resource lignin as a precursor, which is a waste emitted from pulp and paper industries and lignocellulosic biofuel production, using a compressed liquid CO₂ antisolvent method followed by carbonization and chemical oxidation with an acid mixture (H₂SO₄:HNO₃ = 1:3, v/v) at 90 °C for 8, 16, and 24 h. The as-synthesized CDs emitted bright yellow fluorescence (FL) under argon laser excitation at 458 nm with excitation-wavelength-independent and excitation-wavelength-dependent FL emission characteristics. Moreover, the CDs exhibited excellent stability in a highly concentrated NaCl solution, photostability with pH-dependent FL emission activities, and cellular imaging capability with very low cytotoxicity. Notably, the FL emission intensities of the as-synthesized CDs increased with longer reaction times, indicating high degree of oxidation on the CD surface and consequently, high quantum yield of 13% was achieved. Therefore, the as-synthesized CDs can be used as promising fluorescent probes for bioimaging applications *in vitro* and *in vivo*.

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Introduction

As a novel class of fluorescent carbon nanomaterials, carbon dots (CDs) have attracted increasing attention in diverse applications, including bioimaging [1–9], drug delivery [5–9], sensing [9–11], photocatalysis [12–14], and electronic nanodevices [15] owing to their fascinating and favorable properties such as water solubility, chemical stability, environmental friendliness, biocompatibility, low toxicity, excellent photostability, non-blinking photoluminescence, tunable photoluminescence, versatile surface functionality, and easy accessibility as compared to traditional fluorescent organic dyes, semiconductor quantum dots, and other carbon nanomaterials (e.g. graphene and carbon nanotubes) [12]. Typically, CDs are quasi-spherical carbon nanoparticles (CNPs) with sizes below 10 nm and are collectively defined as a carbogenic core with surface functional groups [16–18]. CDs are comprised of an amorphous to nanocrystalline core with predominantly graphitic or turbostratic sp²-hybridized carbon

whose surface is modified with oxygenic moieties [19–22]. In fact, the physico-chemical and optical properties of CDs are governed by their high aspect ratio, surface defects, and surface functionalities, which also strongly depend on the carbon precursors and synthetic routes [23,24].

Over the past decade, various synthesis routes have been developed to synthesize CDs and control their morphology and/or size; these synthesis routes can generally be classified into two kinds of approaches: “top-down” and “bottom-up” [13,23]. Although the previous approaches could produce high-quality CDs, they have some limitations. For example, the precursors for CDs were based on petroleum-based products and the synthetic routes typically involved complicated, multi-step operations and used a large amount of organic solvents, which produced chemical wastes as byproducts. Therefore, it is highly desirable to develop an environmentally friendly and economically sustainable route for practical-scale production of CDs. Furthermore, with the recent advances in materials science and nanobioengineering, carbon nanomaterials from natural resources have become highly attractive for biomedical applications because of their biodegradability, biocompatibility, and large-scale availability at a low cost [18]. Importantly, high-quantum-yield CDs is a crucial in its

* Corresponding authors.

E-mail addresses: jaehoonkim@skku.edu (J. Kim), ywlee@snu.ac.kr (Y.-W. Lee).

efficient utilization of fluorescent bioimaging, sensing, drug delivery and therapy [25] and the development of high fluorescence (FL) intensity of CDs is still challenging [1]. Hence, it is highly desirable to develop a green, sustainable, cost-effective, and scalable method to synthesize CDs from renewable carbon precursors with suitable functional groups.

In recent years, many efforts have been devoted to develop facile and green chemistry-based methods for the preparation of fluorescent CDs using various ecofriendly materials of natural products and waste derived from biomass as the carbon source; this includes apple juice [2], *Saccharum officinarum* (sugar cane) juice [3], *Solanum tuberosum* (potato) [4], dried shrimp [5], *Phyllanthus acidus* [9], *Chionanthus retusus* fruit extract [11], unripe peach [26], unripe *Prunus mume* fruit extract [27], waste frying oil [28], food wastes [29], and dairy waste [30]. As the largest natural aromatic biopolymer on earth and a major component of lignocellulosic biomass, lignin is an ideal candidate for preparing bulk aromatic chemicals and value-added carbon materials because of its high carbon content with unique aromatic structure and chemical composition [31–33]. However, there are some difficulties associated with lignin utilization as carbon-based nanomaterials due to its intrinsic nature of complex aromatic structure and different properties based on its source of origin or type of separation/production methods [34,35]. To date, only three studies have been documented for producing fluorescent CDs from lignin. Chen et al. [36] reported as the first study for the synthesis of CDs using lignin through a hydrothermal treatment with H_2O_2 as an oxidizing agent. However, they did not describe the source or type of lignin and quantum yield of the resulted CDs, which could not fully discuss the advantages of lignin as the carbon source of fluorescent CDs. Very recently, Niu et al. [37] reported a simple and green molecular aggregation method for preparing CDs (size: ~ 2.4 nm) with a quantum yield of 1.68% in water and 2.47% in the solid state from cellulytic enzyme lignin and used as fluorescent probes for cellular imaging. Similarly, Rai et al. [38] reported the synthesis of water-soluble reduced fluorescent CDs (size: ~ 4.9 nm) with a quantum yield of 47.3% using sulfur containing lignosulfonate lignin as a carbon source. Sulfur acted as a doping agent through microwave irradiation after reduction with $NaBH_4$. The reduced fluorescent CDs used as a fluorescent probe for nano-drug-carrier and cellular imaging. Despite the inspiration of the achievements, they did not report yields of CDs products which is an important in a large-scale production. Here, we report the first study of a facile, cost-efficient and scalable method for the production of water-soluble fluorescent CDs (~ 2.4 nm) with bright yellow FL emission under argon excitation at 458 nm and tunable FL characteristics and excellent chemical- and photo-stability using commercial kraft lignin as a precursor. The chemical structure and functional groups present in kraft lignin are different with the types of lignins used in the previous reports. For instance, kraft lignin includes hydrophilic aliphatic thiol groups with a low sulfur content (1–2 wt%) as compared to lignosulfonate lignin (sulfur content: 3.5–8.0 wt%). Even though cellulytic enzyme lignin contains basic subunits and linkages that are similar to those of kraft or lignosulfonate lignin, but the chemical structure of cellulytic enzyme lignin is very similar to sulphur-free native lignin [39,40]. Previously, we demonstrated that kraft lignin can be utilized to produce lignin nanoparticles (LNPs) [39] using compressed liquid carbon dioxide ($clCO_2$) as an antisolvent and hierarchically porous nanocarbon [41] via thermostabilization and carbonization of the LNPs. Herein, to explore the potential biomedical applications of lignin-derived carbons, we synthesized highly water-soluble and fluorescent CDs with high quantum yield (QY) directly from lignin. The synthetic approach for producing CDs from lignin consisted of two steps: first, CNPs were

synthesized using the $clCO_2$ antisolvent method followed by thermostabilization and carbonization. Subsequently, the as-synthesized CNPs were oxidized to produce water-soluble and fluorescent CDs. The oxidation conditions were adjusted to control the physicochemical, surface state, optical, and photoluminescent (PL) properties of the synthesized CDs. Thus, we attempted to gain an understanding of the origin of PL emission behavior in the CDs. Owing to potential applications in bio-imaging and related therapies, cytotoxicity tests and cell imaging of the synthesized CDs were conducted using HeLa cells (breast cancer cell lines), which are commonly used as model cells for bioimaging.

Materials and methods

Materials

Ethanol (99.9%), NaOH (98.0%) in bead form, HCl (35–37%), and HNO_3 (60%) were purchased from Samchun Pure Chemical Co. (South Korea), and H_2SO_4 (98%) was purchased from PFP (Japan). All the chemicals were analytical grade and used as received. Kraft (Alkali) lignin was purchased from Sigma-Aldrich (USA) and used as received. Rhodamine B (for fluorescence), coumarin 153 (dye content 99%), phosphate buffer solution (PBS, pH: 7.4, BioReagents), NaCl ($\geq 99.5\%$), and dialysis tubing (high-retention seamless cellulose tubing, 99.99% retention) with a molecular-weight cut-off (MWCO) of 12.4 kDa were purchased from Sigma-Aldrich. Cell culture media (Gibco™), such as Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS), and a blue fluorescence dye Hoechst 33342 (Invitrogen™) were supplied by Thermo Fisher Scientific (USA). Deionized water (DI) was prepared using a Milli-Q water purification system (Millipore, USA).

Synthesis of water-soluble fluorescent CDs from lignin

Fluorescent CDs were synthesized from lignin via a two-step approach. The synthetic scheme of fluorescent CDs is shown in Fig. 1A. First, CNPs were synthesized using commercial kraft lignin by first preparing LNPs via a $clCO_2$ antisolvent method followed by subjecting the as-synthesized LNPs to thermostabilization and carbonization processes [32,33]. The schematic diagram of $clCO_2$ antisolvent apparatus and detailed description of the experimental procedures for synthesizing LNPs and CNPs from kraft lignin are provided in the supplementary data (Fig. S1). The acid oxidation of the as-synthesized CNPs was carried out using a slight modification of the method documented in previous reports on the synthesis of carbon nanostructures, CDs, and graphene quantum dots (GQDs) from various carbon sources such as graphite, carbon nanotubes, and carbon fibers [42–45]. In brief, 100 mg of CNPs was added to a 20 mL acid mixture ($H_2SO_4:HNO_3 = 3:1$, v/v) in a 250 mL borosilicate bottle, and the solution was sonicated for 1 h. The mixture of CNPs and acids was thereafter heated to 90 °C in an oil bath under vigorous stirring for 8, 16, and 24 h. The oxidation time was controlled to elucidate the effects of reaction time on the physicochemical properties of the CDs. After cooling down to 25 ± 2 °C, the reaction mixture was dispersed in 100 mL of DI water and filtered through a G4 Pyrex glass filter (IWAKI, Japan) to remove unreacted carbon residues. The filtrate was further dialyzed in a dialysis bag (MWCO: 12.4 kDa) against DI water for 2 d until the pH value of DI water was ~ 7.0 to remove the excess acid. Subsequently, the water in the dialyzed CD solution was evaporated under vacuum, and the obtained wet powder was dried in a vacuum oven to achieve a dry powder of CDs. The percentages of the overall recovery yields of CDs were calculated using Eqs. (1) and (2).

For raw kraft lignin as a starting material,

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