NOVEL FOLIC ACID BASED CARBON NANODOTS
COMPARATIVE OF THE SYNTHESIS METHOD

Novia Faridatus Sholihah¹, Satya Candra Wibawa Sakti², Jefpri Suprianto Sianturi³, Mochammad Zakki Fahmi⁴

¹,²,³,⁴Department of Chemistry, Faculty of Sains and Technology Airlangga University, Surabaya 60115, Indonesia

Abstract
Folate receptors (FR) are known to be present in various human cancer cells, including ovarian, mammary, lung, kidney, brain, prostate, and throat glands. Carbon nanodots of folic acid can be used as a candidate for a maximum bio-imaging agent because folic acid is expected to directly enter folate receptors on cancer cells. This study were compare the carbon nanodots synthesis process of folic acid precursor via microwave and pyrolysis methods. The favor carbon nanodots was based on the optical properties as well as its graphene-like structure performance. In the pyrolysis method the physical result is a solid blackish brown carbon nanodots, while in the microwave method the result is a solid brownish brown carbon nanodots. The properties of carbon nanodots were identified by UV-VIS spectrophotometer, Spektrofluorometry, Fourier Transform Infrared Spectroscopy (FTIR), Raman Spectroscopy, X-ray Photoelectron Spectroscopy (XPS), and X-Ray Diffraction (XRD). The toxicity assessment were performed by CCK-8 cytotoxicity assay and showed the ability of carbon nanodots as staining agent of HeLa cancer cells. These results were good evidence on promote the carbon nanodots as an eco-friendly and efficient nanomaterial for cancer detection.

Keywords
Carbon Nanodots, Folic Acid, Pyrolysis, Microwave, Staining Agent

1. Introduction
Cancer remains a major public health issue worldwide, which has become one of the leading causes of mortality with its high recurring and death rates [1]. Specific staining of cancer cells is an important goal in cancer research, as it enables direct investigation of cell biology, varied cellular processes, and the effects of therapeutic treatments [2]. The role of cell targeting has generally been focused on the modeling of fluorescent markers with recognition and standards designed to bind on cell-surface receptors [3]. Folate receptors (FR) are known to be present in various human cancer cells, including ovarian, mammary, lung, kidney, brain, prostate, and throat glands, which has been employed as a docking sites for both therapeutic agents and fluorescent dyes. [4].
The application of functional nanoparticles (NPs) in biomedicine has been extensively developed, and nominated as one of the rapid improving and fascinating research directions [5]. The various uses of nanomaterials in the field of therapy and cancer diagnosis include targeting cancer / tumors, contrast agents, drug carriers, photothermal therapy, or as cell killers. The most developed nanomaterials are carbon-based nanomaterials that have sizes less than 10 nm [6]. Carbon nanodots have several advantages, such as low toxicity, strong photoluminescence, and abundant raw materials in nature [7-8]. A particularly attracting property of carbon nanodots has been observed and they can be produced from a wide variety of carbon sources, providing the means to modulate their structural properties.

Folate (or its acid form, folic acid) is essential for all cell functioning, and the high level of this metabolite is found in many cancer cells [9]. For folate receptors, the folic acid (FA) is an ideal ligand because of its high affinity (Kd ~ 10-10 M), and compatibility with organic and aqueous solvents [10]. In addition, folic acid is also ready to be internalized into cells through receptor mediated endocytosis with nonimmunogenicity [11]. This characteristic makes folic acid become a promising agent for the detection of folate specific receptors so that it can detect the location of cancer cells. This study were compared the synthesis process of carbon nanodots folic acid precursor via microwave and pyrolysis methods. The favor carbon nanodots was based on the optical properties as well as its graphene-like structure performance. Comparison of optical properties in the form of UV-VIS spectrophotometer characterization, Photoluminescence (PL), Fourier Transform Infrared Spectroscopy (FTIR), Raman Spectroscopy, X-ray Photoelectron Spectroscopy (XPS), X-Ray Diffraction (XRD). The toxicity assessment result performed by CCK-8 Assay cytotoxicity test and its ability on staining agent of HeLa cancer cell were good evidence on promote the carbon nanodot as eco-friendly nanomaterial for efficient cancer detection.

2. Experimental Section

2.1 Materials: Folic acid was purchased from Sigma–Aldrich, Sodium hydroxide was purchased from Sigma–Aldrich, aquadem, dan aquades.

2.2 Analytical methods: Daihan Scientific furnace and commercial microwave were used to prepared carbon nanodots, UV/visible absorption spectra of carbon nanodots in aqueous solutions were measured on a JASCO V-550 UV/visible spectrophotometer. Fluorescence emission spectra were recorded on a LS 55 Fluorescence Spectrometer. The photoluminescence quantum yields (PL QYs) of the carbon nanodots were calculated using the following equations: QY=QYR6G IQD/IR6G(ηchloroform/ηethanol) where I and η denote the integral PL intensity and the optical density and reflective index of the solvent, respectively. X-ray photoelectron spectroscopy (XPS) of dried carbon nanodots was performed on an X-ray photoelectron spectrometer XPert MPD with an AlKa X-ray source and monochromator. The X-ray beam size was 500 mm, survey spectra were recorded with pass energy (PE) 150 eV, and high-energy resolution spectra were recorded with PE 20 eV. The XPS results were processed with the XPSpeak program. XRD spectra were investigated by a Rigaku 18 kW rotating anode source X-ray diffractometer with the Cu Kα1 line (λ = 1.54 Å). Fourier transform infrared (FTIR) spectra were measured on Shimadzu IRTracer-100 spectrometer using KBr pellets of the dried carbon nanodots samples. The data was collected and the final data were obtained using Origin software.
2.3 Synthesis of carbon nanodots:

For pyrolysis method, Folic acid (10 mg) heated at 270°C for 1.5 h. The resulting solid color was changed from light yellow to brown, indicating the formation of carbon dots.

For Microwave method, Folic acid (10 mg) was mixed with sodium hydroxide solution (2 tetes) and then heated at high temperature using the microwave. The resulting solid color was changed from light yellow to brown, indicating formation of carbon dots.

2.4 Cytotoxicity Test with CCK-8 Assay

This cytotoxicity test uses breast cancer cells (HeLa cells) which are inserted into DMEM culture media with cell density of 3x10^4 cells/μL. Cells were placed in 96 well plates and incubated for 24 hours at 37 °C with 5% CO₂ content. After 24 hours, the media is removed and washed with Phosphate Buffer Saline (PBS). The carbon nanodots were added to each well with a concentration of 12.5; 25; 50; 100; 200; and 400 μg/mL. As a negative control, some wells are only added with solvents. Next, 10 μL of CCK-8 reagent was added and re-incubated for 4 hours until orange formazan was formed. The reaction was stopped with the addition of a reagent stopper, then an ELISA multiplate reader was analyzed at a wavelength of 450.0 nm.

2.5 Cell Culture and Observation of Intracellular Location of QDs with Confocal Microscopy

The HepG-2, HeLa and MCF-7 cells were cultured in Eagle’s Minimum Essential Medium (containing 1.5 g/L sodium bicarbonate) supplemented with 1% L-glutamine, 1% antibiotic anti mycotic formulation, and 10% fetal bovine serum. To induce cell expansion and senescence, the cells were cultured in a humidified 5% CO₂ atmosphere at 37 °C. HepG2 cells were seeded in a six-well plate in 2 mL of culturing medium 24 h before carbon nanodots feeding. After 1 h of incubation with 300 μL of carbon nanodots, the cells were washed three times with PBS and then fixed with 75% alcohol for 10 min. Then, the fixed cells were incubated for 20 min at room temperature with 2 mL (0.05 μg/mL) DAPI in PBS for nucleus staining. Fluorescence images were acquired by confocal laser scanning microscopy.

3. Results and Discussion

3.1 Synthesis and characterization

3.1.1 Synthesis of Carbon Nanodots Folic Acid with Pyrolysis Method

The carbon nanodots were prepared by simple one-step synthetic for route in which 0.01 gr of folic acid as a precursor of carbon nanodots. Furthermore, folic acid was heated using Daihan Scientific furnace with a ratio of time of 2 hours and 2 minutes at 270 °C. This process produces blackish brown solids. The process of changing color from pale yellow to brown black shows the formation of carbon nanodots. The mechanism of carbon synthesis reaction is shown in Figure 1.A. The carbon nanodots was produced as graphene reaction with the structure of benzene which is formed from the reaction of dehydration during pyrolysis, causing carbon nanodots difficult to dissolve in water.
3.1.2 Synthesis of Carbon Nanodots Folic Acid with Microwave Method

The carbon nanodots was synthesized from folic acid by the microwave method. The 0.01 gr folic acid mixed with NaOH and heated to 90 °C using a commercial microwave with a ratio of 2 hours and 2 minutes at high temperatures. This process produces the crust in the brown vial bottle. During the carbonization process, the reaction mixture changed color from pale yellow to brown shows the formation of carbon nanodots. The color of the crust produced by the microwave method is brighter when compared to the pyrolysis method. It is possible for the carbonization process to be less than perfect. The mechanism of the carbon nanodots synthesis reaction is shown in Figure 1.B

3.2 Characterization

In figure 2a, The ultraviolet–visible absorption spectrum of the sample show that pure folic acid has λmax of 255 nm and carbon nanodots of folic acid with a pyrolysis method for 2 hours (CD-FAP1) had a maximum wavelength of 0 nm and carbon nanodots of folic acid with a pyrolysis method for 2 minutes (CD-FAP2) had a maximum wavelength of 259 nm. While the carbon nanodots of folic acid with the microwave method for 2 hours (CD-FAM1) had a maximum wavelength of 256.4 nm and carbon nanodots of folic acid with a microwave method for 2 minutes (CD-FAM2) had a maximum wavelength of 257 nm. The largest absorbance of carbon nanodots is FAP1 carbon nanodots.

In samples FAM1 and FAM2 the absorption patterns formed in the microwave method have two absorption peaks namely peak 1 at a wavelength of 259 nm and peak 2 at a wavelength of 280 nm. The absorption peak 1 at this wavelength shows the electron π → π * transition corresponding to the aromatic sp² carbons within the carbon nanodots core and absorption peak 2 shows the electron transition n → π * (surface state) [12]. FAM1 and FAM2 have the same wavelength with the range of 256-280 nm, but FAM2 carbon nanodots have greater absorbance compared to FAM1, this is related to the carbonization process with the microwave method on FAM2 carbon nanodots is better than FAM1. the samples of pyrolysis method, FAP1 and FAP2 carbon nanodots, showed absorption pattern with one absorption peak at a wavelength of 259 nm. The absorption peak at this wavelength indicated the electron transition π → π * (core). On the graph of the pyrolysis method on FAP1, the structure of the carbon nanodots that form the surface does not appear to absorb the peak. However, the peak surface conditions are still present only when the peak UV-Vis test is unreadable due to the carbonization process on carbon nanodots were too high.

The spectrofluorometric analysis was used for samples that could produce fluorescence. The analyzed samples were FA pure, CD-FAP1, CD-FAP2, CD-FAM1 and CD-FAM2 in NaOH. All samples were analyzed at 320 nm excitation wavelength and emission wavelength ranges from 340 to 400 nm. As shown in fig.2b, the pure folic acid exhibit maximum emission at 398.5 nm and the carbon nanodots FAP1 and FAP2 has maximum emission peak exhibit centered at 435 and 475 nm when excitation at 320 nm. And the carbon nanodots FAM1 and FAM2 maximum emission peak exhibits centered at 442 and 481 nm when excitation at 320 nm. The FAM1 and FAM carbon nanodots demonstrates that the pale-yellow carbon solution emitted light blue luminescence under UV light for microwave method. and The FAP1 and FAP2 samples in figure 2b show that a pale-brownish carbon nanodots solution emits a green light under UV light for the pyrolysis method.

The surface functional states and components of the CDs were investigated by Fourier transform infrared spectroscopy (FTIR) and X-ray
photoelectron spectroscopy (XPS). The FTIR spectrum (Fig. 2c) showed the absorption bands at 3440 and 2995 cm\(^{-1}\) represent the typical stretching vibrations of O-H/N-H and C-H, respectively. Simultaneously, the absorption band at 1626 cm\(^{-1}\) is attributed to the stretching vibration of C=O. Several sharp peaks at 1511, 1411 and 1031 cm\(^{-1}\) correspond to the vibrations of N-H, C=C and C-N, respectively.

Corroborating the reaction Scheme in Figure 1A and 1B, the raw XPS data depicted peaks corresponding to carbon, nitrogen, and oxygen. The survey of XPS spectrum reveals three prominent peaks of C1s at 284 eV, N1s at 400 eV and O1s at 531 eV [13-15]. The typical C1s spectrum of CD-FAP (Fig. 2d) exhibits four distinct peaks located at 284, 285.8, 287.4 and 288.6 eV, which correspond to C=C, C-N, C-O and C=O groups and the typical C1s spectrum of CD-FAM (Fig. 2e) exhibits three distinct peaks located at 283.9, 284.8, 285.8 eV, which correspond to C=C, C-C/C=H, C-N groups. The O1s spectrum of CD-FAP (Fig. 2f) exhibits two peaks at 530.2 and 532.4 eV, representing C=O and C-OH/O=O=C-OH and the O1s spectrum of CD-FAM (Fig. 2g) exhibits two peaks at 530.9 and 532.2 eV, representing C=O and C-OH/O=O=C-OH. The N1s spectrum of CD-FAP (Fig. 2h) can be deconvoluted into two components at 399.9 and 401.2 eV, attributed to C-N/C=N and N-O, respectively. The N1s spectrum of CD-FAM (Fig. 2i) can be deconvoluted into two components at 402 and 403.3 eV attributed to C-N/C=N and N-O respectively. The analytical results of FTIR and XPS demonstrate that there existed effective -NH\(_2\) and -COOH groups on the surface of as-synthesized carbon nanodots, which can be directly conjugated with FA without further modification.

The XRD pattern is used to determine the structure of crystals and purity materials. Solids of carbon nanodots synthesized by the pyrolysis and microwave methods were analyzed in the angle of 2\(\theta\) range between 5–40°. The XRD pattern for pure folic acid was compared to that of the sample FAP1, FAP2, FAM1 and FAM2 carbon nanodots (Fig. 2j). From this picture, the sample has a peak at an angle of 2\(\theta\) over 20°. The pure folic acid show peaks at an angle of 2\(\theta\) 20.50 °. CD-FAP1 and CD-FAP2 show peaks at an angle of 2\(\theta\) 18.06 °, 22, 21 °. Samples CD-FAM1 and CD-FAM2 produced peaks at an angle of 2\(\theta\) of 22.62 °, 22.54 °. The data indicates that an amorphous product formed suggesting that the loss of a water molecule from the surface of the solid has occurred leaving an amorphous anhydrous material or a lower carbonate. This amorphous anhydrous material then decomposes. Further, from the standard library of XRD patterns, it was shown to have a similar pattern to that of carbon, establishing the fact that this residue is indeed carbon [5].

Raman scattering data corroborated the presence of graphitic carbon as the most prominent component of the carbon nanodots (Figure 2k). The CD-FAP1 spectrum shows a peak D band and G band at 1329.79 cm\(^{-1}\), 1519.39 cm\(^{-1}\), the variation of CD-FAP2 shows the peak D band and G bands at 1335.19 cm\(^{-1}\), 1566.72 cm\(^{-1}\), CD-FAM1 shows the peak band D band and G band at 2051.02 cm\(^{-1}\), 2369.7 cm\(^{-1}\), and the CD-FAM2 shows the peak band D band and G band at 1335.19 cm\(^{-1}\), 1556.23 cm \(-1\). D band discusses the imperfections of carbon structures that reflect the structure and carbon paired from sp3 carbon atoms obtained by amorphous graphite during the oxidation process. Through the G band obtained from vibrations in the sp2 carbon atom field. The intensity ratio of D band and G band (ID / IG) is very vulnerable to carbon political changes that can be used by several factors such as doping, defects, substrate, and so on.
Figure 1. Carbon nanodots synthesis from folic acid A) Scheme depicting one-step synthesis of the C-dots using the microwave method B) Illustration of the synthetic of the FA-CDs using the pyrolysis method based fluorescence bioimaging platform for the targeted imaging of FR+ cancer cells.
Figure 2. A) Ultraviolet-visible (UV/Vis) absorption spectrum of the Cdots in aqueous solution. B) Fluorescence spectra of carbon nanodots C) FTIR spectrum of CDs. D) C1s XPS of CD-FAP. E) C1s XPS of CD-FAM. F) O1s XPS of CD-FAP. G) O1s XPS of CD-FAM. H) N1s XPS of CD-FAP. I) N1s XPS and of CD-FAM. J) XRD pattern of FA and CD-FA. K) Raman scattering data corroborated the presence of graphitic carbon as the most prominent component of the carbon nanodots.
4. Conclusion

Selective imaging of cancer cells expressing the folate receptor on their surface was achieved by carbon nanodots prepared from folic acid as the carbon source. The carbon nanodots were synthesized in a simple single-step process without conjugation of free folic acid; spectroscopic data confirmed the display of folic acid residues on their surface. Fluorescence microscopy and quantitative analyses demonstrated that the folic acid-derived carbon nanodots effectively targeted cells expressing the folate receptor on their surface, thereby providing a means for selective microscopic imaging of cancer cells. Competition experiments further indicated potential use of the carbon nanodots assay for assessing the activity of folate receptor agonists and antagonists. Of particular importance is that the folic acid-derived C-dotswere not cytotoxic; this opens avenues for cancer cell imaging and analysis.

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