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Visible-light-driven dynamic cancer therapy and imaging using graphitic carbon nitride nanoparticles

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ABSTRACT

Organic graphitic carbon nitride nanoparticles (NP-g-CN), less than 30 nm in size, were synthesized and evaluated for photodynamic therapy (PDT) and cell imaging applications. NP-g-CN particles were prepared through an intercalation process using a rod-like melamine-cyanuric acid adduct (MCA) as the molecular precursor and a eutectic mixture of LiCl-KCl (45:55 wt%) as the reaction medium for polycondensation. The nano-dimensional NP-g-CN penetrated the malignant tumor cells with minimal hindrance and effectively generated reactive oxygen species (ROS) under visible light irradiation, which could ablate cancer cells. When excited by visible light irradiation ($\lambda > 420$ nm), NP-g-CN introduced to HeLa and cos-7 cells generated a significant amount of ROS and killed the cancerous cells selectively. The cytotoxicity of NP-g-CN was manipulated by altering the light irradiation and the BP-g-CN caused more damage to the cancer cells than normal cells at low concentrations. As a potential non-toxic organic nanomaterial, the synthesized NP-g-CN are biocompatible with less cytotoxicity than toxic inorganic materials. The combined effects of the high efficacy of ROS generation under visible light irradiation, low toxicity, and bio-compatibility highlight the potential of NP-g-CN for PDT and imaging without further modification.

1. Introduction

Photodynamic therapy (PDT) is a compelling anticancer treatment that involves 1) injecting a photosensitizer into a host body and 2) irradiating a photosensitizer with light of an appropriate wavelength to generate highly reactive oxygen species (ROS) [1–5]. The light energy of a particular wavelength is absorbed by the photosensitizer and used to transfer an electron to oxygen to produce toxic free radicals, known as ROS. ROS play vital roles in physiology and pathophysiology. They cause cell damage via oxidative reactions, and excessive levels of ROS are highly cytotoxic to cells, causing cell death through apoptosis (programmed cell death) [6–8].

The major drawback of PDT-based therapy is the poor solubility of the sensitizer in body fluids and photo-bleaching of the sensitizer, which result in a low therapeutic effect [9]. To overcome these limitations, nanomaterials have been incorporated into the sensitizers [10,11]. Certain nanomaterials, such as graphene quantum dots (GQDs), porous silicon nanoparticles, ZnO, and UV-emitting up-conversion-based TiO_2 nanoparticles [12–15], can generate ROS intrinsically and have been used as photosensitizers to overcome the poor water solubility and low selectivity. The metal particles used to perform these studies are highly toxic in nature (over 10 μg/mL) and cannot be used independently. Therefore, metal particles need to be coated with polymers, such as PEG or PVP, before performing the tests, which is the main drawback. Currently, considerable research has been devoted towards the development of nanomaterials with better optical

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properties for more efficient photodynamic therapy (PDT) with low cytotoxicity [5].

In recent years, graphitic carbon nitride (g-C_3N_4), being similar to graphene in structure but with different properties, has attracted considerable attention. Graphitic carbon nitride is an organic two-dimensional, layered conjugated polymer [16] with intrinsic semiconducting properties, biocompatibility [17], and excellent chemical stability [18], which can be used in the visible light range. In the case of graphitic carbon nitride, carbon and nitrogen are bonded covalently to form a heptazine or tri-*s*-triazine structure, which is stacked graphitically, and is highly stable at room temperature and normal pressure. Among the various allotropes, tri-*s*-triazine-based g-C_3N_4 is predicted to be the most stable and energetically favored phase of C_3N_4, which can provide a visible light response and have potential as a photosensitizer for the generation of ROS [19]. Although it is barely formed under normal conditions due to the mass loss at high temperatures (> 500 °C) and incomplete condensation, polymeric derivatives (g-CN) of g-C_3N_4 can be synthesized on a large scale by thermal polycondensation using inexpensive nitrogen-rich molecules, such as melamine, cyanamide, and urea [12]. The inherent high degree condensation of the heptazine ring structure leads to an electronic structure that possesses a band gap in the visible light region with good physiochemical stability [20]. As a polymer, the size and shape of g-CN can be tailored by the polymerization of molecular precursors infiltrated into the solid confinement or bound in molecular crystals [21,22]. Graphitic carbon nitride was only recently found to be useful as a PDT agent [23–25] and as an effective pH-responsive drug carrier. The cumulative effects of the tunable band gap and the optoelectronic properties of g-CN can be utilized effectively as a PDT agent in imaging cancerous cells [26,27]. On the other hand, bulk graphitic carbon nitride has limitations, such as water immiscibility, high recombination rate of electron-hole pairs, and large size, which hinders the application of g-CN in the biomedical fields. To apply bulk g-CN as an effective PDT agent, it is important to develop a method to synthesize nanoparticles with excellent solubility and biocompatibility in body fluids.

This study focused on the preparation of novel graphitic carbon nitride nanoparticles, as an efficient photosensitizer for PDT and its cytotoxic and photosensitization activity was evaluated using circular double strand DNA. Based on its excellent optical properties of reacting in visible light, the PDT cancer treatment was evaluated at the cellular level using nano-size controlled g-CN. Cervical cancer cells were selected as a model sample and NP-g-CN were introduced to cancer cells to confirm the possibility of the PDT agent. The synthesis of homogeneous NP-g-CN as an organic-based PDT agent with excellent biocompatibility and solubility is a promising approach towards the effective cancer treatment.

2. Materials and methods

2.1. Materials

Melamine (Sigma-Aldrich, St. Louis, MO, USA), cyanuric acid (Sigma-Aldrich, St. Louis, MO, USA), lithium chloride (LiCl, Sigma-Aldrich, St. Louis, MO, USA), potassium chloride (KCl, Sigma-Aldrich, St. Louis, MO, USA), and agarose (Promega Corporation) were used as received. Circular double strand DNA (pET22b(+)) was used to evaluate the effects of the prepared models on DNA damage. Red Safe (Invitrogen, Thermo Fisher Scientific Inc.) staining agent was used to stain the DNA for electrophoresis studies.

2.2. Characterization and apparatus

The morphology and structure of MCA and NP-g-CN were characterized by field emission scanning electron microscopy (FE-SEM, S-4300, JITACHI), field emission transmission electron microscopy (FE-TEM, JEM-2100F HR, JEOL) coupled with energy dispersive X-ray spectroscopy (EDS), and wide and small angle X-ray diffraction (XRD, D/MAX-2500, Rigaku). Elemental analysis was conducted using a FLASH 2000 from Thermo Scientific manufacture. The ^{13}C cross polarization/Total Suppression of Spinning Sidebands nuclear magnetic resonance (CP/TOSS NMR) spectra were obtained using a solid-state FT-NMR spectrometer (Bruker, DSX 400 MHz). The Fourier transform infrared (FT-IR, JASCO 4700 plus) spectra were obtained from an average of 12 scans with a 4 cm^{-1} resolution from 4000 to 600 cm^{-1}. The diffuse reflectance UV–vis spectra were obtained from Shimadzu UV2100 UV–vis spectroscopy. The photoluminescence measurements were performed using UniRAM of UniNanoTech. The light irradiation experiments for PDT were carried out using a 1 kW high-pressure Hg lamp from a force lamp connected to a manufactured voltage supply. The wavelength of light was controlled by a cut-on-glass filter to allow the penetration of long wavelength light (≥ 420 nm).

The DNA vectors were analyzed by an electrophoresis method using Mupid-2plus from Koreaciotech. To indicate and quantify the ROS of NP-g-CN, microplate fluorometry made from Safire (Tecan) was used. The cells were cultured in a CO_2 incubator (BB15, Thermo Scientific) with a 5% CO_2 flow. Cell imaging was conducted by BIO-TEM (Tecnai G2 Spirit, FEI Company) at the Korea Basic Science Institute (KBSI). In addition, confocal imaging (LSM 510 Meta, Carl Zeiss) was used to verify the existence of NP-g-CN inside the cells. The diode lasers of confocal microscopy were configured with the point Source i-flex 2000 (405 nm, 25 mW, Laser Class 3 B) and an objective lens (EC Plan-Neofluar 20 × /0.50 M27).

Cytotoxicity studies were carried out on fibroblast cells (cos-7, ATCCCRL-1651, KCTC) derived from monkey kidney tissues (control) and HeLa cells (CH18805, KCTC). The cells were cultivated in 1 × phosphate buffered saline (PBS, Thermo Fisher Scientific Inc.), Dulbecco's Modified Eagles' Medium (DMEM, Gibco, Lifetechnology Inc.), 10% Fetal bovine serum (FBS, Gibco, Lifetechnology Inc.), 1% Penicillin-Streptomycin (Gibco, Lifetechnology Inc.) as the cultivation medium. The cultivated cells were counted using a standard 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide assay (Cell Counting Kit-8, Sigma-Aldrich) and the ROS were measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA, Merckmillipore), sodium hydroxide (NaOH, Junsei Chemical), and peroxidase from horseradish (HRP, Sigma-Aldrich).

2.3. Material synthesis

2.3.1. Bulk graphitic carbon nitride (Bulk g-CN)

Melamine (analytical grade, 10 g) was heated in an alumina crucible for 4 h at 550 °C under a nitrogen atmosphere. After cooling to room temperature, bright yellow powders of bulk graphitic carbon nitrite (Bulk g-CN) were recovered and used for PDT and the bio-imaging test was performed without further purification [21].

2.3.2. Melamine-cyanurate adduct (MCA)

Rod-like g-CN was synthesized using a 1:1 hydrogen-bonded network between melamine and cyanuric acid. Saturated clear solutions of cyanuric acid (0.523 mmol) and melamine (0.523 mmol) in water (25 mL) were prepared by sonication. Equimolar solutions were mixed to produce a white precipitate and the mixture was then treated hydrothermally in a Teflon line reactor for 12 h at 150 °C. After the reaction, the precipitate was filtered, washed, and dried at 100 °C [28].

2.3.3. Graphitic carbon nitride nanoparticle (NP-g-CN)

MCA (1 g), KCl (1.375 g), and LiCl (1.125 g) were mixed together using a mortar and pestle and heated to 550 °C (2.3 °C min^{-1}) for 12 h under a nitrogen atmosphere. The resulting solid was washed with hot water to remove the remaining salt, filtered, and washed sequentially with water and ethanol. After drying, the yellow-green power of NP-g-CN was obtained.