Materials Science and Engineering C 70 (2017) 9–14

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Manipulation of novel nano-prodrug composed of organic pigment-based hybrid network and its optical uses

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ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form 2 August 2016
Accepted 20 August 2016
Available online 22 August 2016

Keywords:
Photosensitizer
Hybrid material
Photodynamic therapy

ABSTRACT

Here we developed the first case of pyropheophorbide-a-loaded PEGylated-hybrid carbon nanohorns (CNH-Pyro) to study tumor targeting therapy. During incubation with living cells, CNH-Pyro exhibited very intense red emissions. The intracellular imaging results were carried out by flow cytometry based on four different kinds of cell lines (including three adherent cell lines and one suspension cell line). Compared with free pyropheophorbide-a, CNH-Pyro demonstrated enhanced photodynamic tumor ablation efficiency during in vitro experiments due to improved biocompatibility of the hybrid nanomaterial and the photothermal therapy effect derived from carbon-network structure. Trypan blue staining experiments supported that the cell fate was dependent on the synergistic effects of both CNH-Pyro and laser irradiations. These results indicated that the chlorin-entrapped carbon nanohorns could provide powerful delivery vehicles for increasing photodynamic efficacy and possess early identification of the disease.

1. Introduction

The incidence of solid tumors has been increasing in recent years and the treatment of the main types of the disease remains a challenging goal. Current biological transport processes have demonstrated that poor penetration and random distribution of drugs would lead to unfavorable therapeutic outcomes in an uncontrollable manner [1]. Therefore, the development of suitable drug-delivery systems especially new nano-sized carriers has aroused considerable interests [2,3]. During the last decade, numerous nanoscale entities such as silica, metal oxides, dendrimers or quantum dots have been designed for the potential uses in biological systems [4,5]. However, it has been found that the role of particle size seems to be a primarily important issue during the interaction with living matter. In particular, the physical shape of the nanoparticles has been closely related to cellular uptake [6]. In this way, a group of carbon nanomaterials with unique structures including carbon nanotubes, graphene, fullerenes and dots has been widely explored as efficient delivery vehicles based on their chemical stability, versatile surface chemistry features and large carrier capacity [7,8].

As an appealing category of non-invasive clinical approach, photodynamic therapy (PDT) has displayed its main advantages such as high effectiveness and reliable treatment with less pain in the suppression of various malignant diseases [9,10]. This technique includes the administration of a photosensitizer drug with the exposure of the tissue under the light irradiation. The existence of highly reactive singlet oxygen will induce cell damage and tumor destruction [11]. Currently, several novel types of photosensitizers like photofrin, porphyrin, phthalocyanine and chlorin have been extensively studied based on their analogous heterocyclic structures [12,13]. Especially those organic pigments (chlorophylls and chlorins) with efficient sensitivities that can be easily available from the rich natural resources have been paid much attention [14]. However, the molecular-based sensitizer has two main drawbacks. Firstly, it has much difficulty in practical uses as tunable drugs due to its low photostability and mechanical strength. New techniques such as the assembly of organic-inorganic hybrid materials have been developed to resolve the problems [15]. In general, encapsulation of an organic chromophore into a nanoscale hybrid host would be beneficial for improving its thermal stabilities and luminescence efficiency. Secondly, to reach tumor region with enough quantity, the photosensitizer should pass through the complex vasculature, enter vessel walls and cross layers of malignant cells. The poor cancer cell uptake and low efficient penetrations would seriously restrict its potential functions in PDT uses [16]. Accordingly, delivery of photodynamic therapy agent through nano-carriers has been paid much attention due to the improved accumulation, permeability and retention effects [2].

In the present work, we explore a new strategy to achieve the organic-inorganic hybrid material based on functionalized carbon nanohorns

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http://dx.doi.org/10.1016/j.msec.2016.08.052
0928-4931/

with PEG surface modifications (CNH-PEG). Pyropheophorbide-a bearing a carboxyl group at the 17^2 position (available from its methyl ester) has been covalently bonded to carbon substrate (abbreviated as CNH-Pyro) through amidation reaction in the presence of EDC and NHS. Although the loading of photosensitizer onto carbon nanostructures has been reported [7,8,17], the assembly of chlorin pigment appended carbonaceous tubules (nanohorns) has never been investigated. In addition, it has been found that the chlorin-functionalized nanohorns exhibited aggregation caused quenching in phosphate buffer saline. Whereas a major red emission band located at 670 nm was clearly identified when the material entered living cells. At cellular levels, photodynamic therapy effects from CNH-Pyro were more powerful than the results of individual carbon nanohorns or chlorin in four different kinds of cells. Furthermore, trypan blue staining experiments also supported the effectiveness of the laser-induced cancer cell apoptosis.

2. Materials and methods

2.1. Chemicals and materials

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-Hydroxysuccinimide (NHS), Poly(ethylene glycol) diamine (H_2N-PEG-NH_2, Mn = 2000) and pyropheophorbide-a were purchased from Sigma-Aldrich. Trypan Blue Solution (0.4%), diamidino-2-phenylindole (DAPI), Fetal bovine serum (FBS), antibiotics penicillin (PS), Dulbecco's modified medium (DMEM), RPMI1640, nutrient mixture F12 Ham Kaighn's modification (F12 K) medium were obtained from Life Technologies. The CellTiter 96®AQueous One Solution Reagent (MTS kit) was obtained from Promega. Single Layer Carbon Nanohorns (CNH) was purchased from Beijing Qingdajiguang technology Development Co., Ltd. All the other chemicals were analytical reagents and used without further purification.

2.2. Preparation of PEG functionalized carbon nanohorns (CNH-PEG)

Firstly, the oxidation of carbon nanohorns was obtained according to the previous report with slight modifications [18]. Briefly, single wall carbon nanohorns (80 mg) was dispersed in 100 mL of hydrogen peroxide aqueous solution (30%) under vigorous stirring. It has been irradiated by a 500-W Xe lamp for 2 h. After cooling to room temperature, the resultant mixture was then centrifuged and washed with water for three times. It has been freeze-dried to obtain carboxyl groups functionalized carbon nanohorns oxide (CNHox). Secondly, CNHox (20 mg) was dispersed in water (10 mL) by sonication for 5 min, and then NHS (57.5 mg, 0.5 mmol) and EDC (77.5 mg, 0.5 mmol) were added into the mixture for activating carboxyl groups for 1 h. In the next step, H_2N-PEG-NH_2 (100 mg, 0.05 mmol) in 2 mL water was added, and the mixture was stirred at room temperature for overnight. The resultants were collected by centrifugation at 13,000 rpm for 15 min, and washed three times with water and ethanol alternatively. The PEG functionalized carbon nanohorns (CNH-PEG) was obtained by freeze-drying.

2.3. Preparation of pyropheophorbide-a (pyro) modified carbon nanohorns (CNH-Pyro)

Pyro (5.32 mg, 0.01 mmol) was dissolved in 5 mL of DMSO, and then NHS (11.5 mg, 0.1 mmol) and EDC (15.5 mg, 0.01 mmol) were added into the mixture for activating carboxyl for 1 h. In the following step, CNH-PEG (10 mg) dissolved in 10 mL DMSO was added and the mixture was stirred in the darkness at room temperature for overnight. CNH-Pyro was precipitated by centrifugation at 13,000 rpm for 15 min, and washed three times with DMSO and water alternatively. The targeted material CNH-Pyro was freeze-dried. For determination of Pyro quantity, the dispersion with CNH-Pyro system was diluted to a certain concentration, and the content of Pyro was calculated by using UV calibration curve at 667 nm. In detail, the absorption signals at different concentrations were collected. The correlation between the UV absorbance at 667 nm and the concentration was normalized by the linear regression equation, which was consistent with the linear relationship. The standard linear curve ($Y = 0.0225 + 0.0071X$ ($R^2 = 0.9978$)) was used for the quantitative analysis. The load efficiency has been obtained from the mass ratio ((weight of loaded Pyro / weight of CNH-Pyro) × 100%).

2.4. Cell lines and cell culture

Four cancer cell lines, Hela (human cervical cancer cell), LL2 (mouse lewis lung carcinoma cell), A549 (Human Caucasian lung carcinoma cell), and THP-1 (human leukemic monocytice cell), were used in this studies. Both Hela and LL2 cells were cultured in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin (PS) and maintained at 37 °C with 5% CO_2. A549 cells was cultured in F12K medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin (PS) and maintained at 37 °C with 5% CO_2. THP-1 cells was incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics penicillin (PS) and maintained at 37 °C with 5% CO_2.

2.5. Flow cytometry

Four kinds of cells were cultured with CNH-PEG, Pyro, or CNH-Pyro at the same Pyro concentration (1 μM) for 2 h, respectively. Then, cells were washed and re-dispersed in PBS, and measured by flow cytometry on a FACS Calibur Flow Cytometer essentially as we described [19,20].

2.6. Dark cytotoxicity and phototoxicity in vitro

The viabilities of HeLa, A549, LL2, and THP-1 cells in the presence of CNH-PEG, Pyro, or CNH-Pyro were measured by MTS kit (with or without laser irradiation). The cells (about 5×10^3 cells/well) within replicate 96-well plates were pre-cultured under standard culture condition for one day. After removal of the medium, cells were incubated with fresh medium which contained CNH-PEG, Pyro, or CNH-Pyro (with Pyro concentration at 10 μM) for another 24 h. For the phototoxicity groups, the cells were treated with a 660 nm laser for 3 min. 20 μL MTS solution was added to each well. After incubated for 2 h, the absorbance at 490 nm was recorded by using a Polarsarmicroplate reader. Moreover, trypan blue staining experiments were carried out by those cells incubated with CNH-Pyro (10 μM for Pyro) for 24 h at 37 °C.

3. Results and discussion

The detailed preparation process is given in Scheme 1. The modified carbon nanohorns employed were made by oxidation in the presence of hydrogen peroxide under irradiation. Poly(ethylene glycol) diamine has been attached to the nano-substrate as the flexible linker and the water solubility or the biocompatibility was improved (abbreviated as CNH-PEG). The coupling between pyropheophorbide-a and amine terminated carbon nanohorns via “amidation” reaction using EDC/NHS as the catalysts has achieved the targeted CNH-Pyro. The absorption properties of CNH-PEG, pyropheophorbide-a and CNH-Pyro were investigated in buffer solution (Fig. S1). Before the encapsulation of chlorin molecule, CNH-PEG displayed no visible absorption signals between 400 and 800 nm. A distinguished broad band at 257 nm could be assigned to typical signal from the pristine nanohorns [21]. The spectrum of free pyropheophorbide-a had absorption maximum at 396 and 667 nm respectively, which were attributed to the intense Soret band and Q_y band. The spectroscopic feature of CNH-Pyro was very similar to pyropheophorbide-a, indicating that the organic pigment has been successfully anchored onto the carbon nanomaterial. However, we could also observe red shifts of Soret (396 → 403 nm) and Q_y bands (667 → 675 nm). Previous studies of light-harvesting organic pigments