Colloids and Surfaces B: Biointerfaces 151 (2017) 215–223

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Protocols

In vitro cytotoxicity evaluation of graphene oxide from the peroxidase-like activity perspective

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ARTICLE INFO

Article history:
Received 14 November 2015
Received in revised form
29 November 2016
Accepted 18 December 2016
Available online 19 December 2016

Keywords:
Graphene oxide
Hemin
Peroxidase mimics
Cytotoxicity
Reactive oxygen species

ABSTRACT

In this study, PEGylated graphene oxide (PEG-GO)-hemin composite structure was constructed. Hemin in the form of nanoscaled aggregates were immobilized on PEG-GO sheets by the π-π stacking supermolecular interaction. Via catalyzing the oxidation of chromogenic substrates, we elicited the obtained PEG-GO-Hemin composite sheets have much higher peroxidase-like activity compared to hemin or PEG-GO alone, which is due to the introduction of enzyme active center of hemin with high dispersity, the excellent affinity to organic substrate through π-π stacking and/or electrostatic adsorption and the rapid electron transfer capability of PEG-GO. Similarly, PEG-GO-Hemin was found to be able to catalyze the oxidation of low density lipoprotein (LDL) by H_2O_2, resulting in toxicity to porcine iliac endothelial cells (PIECs) in vitro. Furthermore, we also demonstrated that PEG-GO sheets showed enhanced peroxidase activity when met hemin containing proteins including hemoglobin and cytochrome c. High glucose level (HG) in human umbilical vein endothelial cells (HUVECs) can induce cytochrome c to release from the respiratory chain, thus applying PEG-GO under HG condition could cause a much higher peroxidase-like activity, resulting in the production of hydroxyl radical (•OH) and cytochrome c radical (cytochrome c•), which eventually enhance the apoptosis. These results suggest GO has potential hazard for biomedical applications in some pathophysiological conditions.

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1. Introduction

Graphene, a very recent rising star with novel one-atom-thick two-dimensional (2D) graphitic carbon system, has recently emerged as a fascinating material [1–3]. The studies on graphene have attracted considerable attention from both the experimental and theoretical scientific communities [4–6]. In particular, the low cost and mass production of chemically exfoliated graphene oxide (GO) and reduced GO sheets possessing many reactive oxygen-containing groups for further functionalization and tuned properties has been realized [7–9]. The presence of oxygen-containing groups in GO render it strongly hydrophilic and water soluble, and also offer a wide range of possibilities to synthesize graphene-based functional materials for various applications, such as enhanced chemical catalysis, energy conversion and biomedical functions [10–14]. From the catalysis aspect, the peroxidase-like activity of PEGylated graphene oxide (PEG-GO) was weak due to the lack of enzyme active center, but the fact that PEG-GO displays rapid electron transferring and substrate adsorbing capacities in the catalytic reaction endows it great potential in catalytic activity enhancement, making PEG-GO a kind of new functional carrier material to construct organic molecules or nanoparticle-graphene composites. Hemin is a protoporphyrin with Fe^{3+} in a high spin state, and it is the stable oxidized form of heme which is the active catalytic center of natural enzyme such as horseradish peroxidase (HRP) and cytochromes [15,16]. In fact, hemin itself possesses intrinsic peroxidase activity which has been extensively investigated as an enzyme mimics [17]. Inspiring by the characteristics of GO and hemin, PEG-GO and hemin composite structure (PEG-GO-Hemin) with strong peroxidase-like activity was constructed in this paper. The hemin molecules were immobilized on the PEG-GO

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http://dx.doi.org/10.1016/j.colsurfb.2016.12.025
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sheets by a simple wet-chemical strategy through π-π stacking interaction. The as obtained PEG-GO-Hemin exhibited much stronger peroxidase-like activity than hemin alone.

Many researches about GO's biocompatibility and its influence on bodily functions have been proposed [18–26]. The toxicity of GO was generally considered to be related to the interaction of cell membrane and GO [19,20,27], wrapping of cells by GO [21,22] and ROS generation [23,24,28,29]. Prime GO was believe to be toxic while the toxicity can be reduced via functionalizing with hydrophobic molecules including polyethylene glycol (PEG) [14,30], chitosan [31,32], artificial peroxidase [33], fetal bovine serum (FBS) [27] and polyethylenimine (PEI) [34]. In this paper, PEG-GO was chosen because its cytotoxicity is much lower than GO. Moreover, ROS level in cells treated with nanomaterials was closely related to the catalytic activity of the materials [35], hence ROS generation was the most important consideration in our study.

As reported, hemin, the most abundant form of human iron, can intercalate into native LDL and markedly catalyze LDL oxidation by a small amount of H_2O_2 [36]. Although native LDL is not toxic to endothelium, oxidized LDL (ox-LDL) can damage endothelium by denaturing cellular membranes and organelles and thus ox-LDL is one of the most important incentives of atherosclerosis [37–39]. The catalytic activity of hemin was significantly upgraded when combined with GO, implying possible toxicity of GO in vivo by enhanced catalytic oxidation. Here we demonstrated that the PEG-GO-Hemin provoked an enhanced catalytic oxidation of LDL, resulting consequently in an obvious toxicity for endothelial cells. From the peroxidase-like activity evaluation results, we found that in addition to hemin, the enhancement of peroxidase-like activity was also found when PEG-GO met hemin-containing proteins such as hemoglobin and cytochrome c. When exposed to hemin containing proteins such as leaked cytochrome c in human umbilical vein endothelial cells (HUVECs) incubated under high glucose level (HG), PEG-GO accelerated the cell apoptosis due to the elevated oxidation effect induced by hydroxyl radical (•OH) and cytochrome c radical (cytochrome c•) generated from the enhanced peroxidase-like activity. In general, although functionalization with PEG apparently reduced GO's toxicity, the PEG-GO may accelerate the apoptosis in cells under oxidative stress conditions, which is very likely attributed to the enhancement of oxidation effect. Our results indicate a potential hazard for in vivo biomedical application of GO-based composites under oxidative stress-induced pathological conditions such as atherosclerosis and diabetes.

2. Materials and methods

2.1. Materials

All chemicals used in the experiment were analytical grade reagent and were used as received. Polyethylene glycol (PEG) modified graphene oxide (PEG-GO) was provided by Nanjing Nanoeast Biotech Co. Ltd., which were synthesized from natural graphite by Hummers' Method and conjugated with amine-terminated six-armed PEG molecules via EDC chemistry [14,40,41]. Hemin (ferriprotoporphyrin chloride), 2,2′-azinobis (3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), 3,3′,5,5′-tetramethylbenzidine (TMB), glucose oxidase (GOD), 30% H_2O_2, glucose, fructose, maltose, lactose, low density lipoprotein (LDL L-7914), lactoferrin (L-4040), ferritin (F-7879), transferrin (T3309), hemoglobin (H-2625) and cytochrome c (C-3438) were obtained from Sigma-Aldrich. PIEC and HUVEC were purchased from the Shanghai Cell Bank. Glacial acetic acid, anhydrous sodium acetate were purchased from Sinopharm Chemical Reagent Co. Ltd. Dipotassium hydrogen phosphate, sodium phosphate dibasic, sodium chloride, and tetramethyl ammonium hydroxide were reagents from Shanghai LingFeng Chemical Reagent Co. Ltd. Deionized water used throughout all experiments was purified with the Millipore system.

2.2. Synthesis of PEG-GO-Hemin composite sheets

PEG-GO-Hemin composite sheets were prepared as followed: PEG-GO was suspended in deionized water under ultrasonication (output power 180 W) for 10 min to obtain homogeneous single layered PEG-GO sheets. Meanwhile, hemin was suspended in deionized water (pH 7.8) under ultrasonication (output power 180 W) for 10 min to obtain homogeneous hemin solution. Then 20.0 mL of the homogeneous PEG-GO dispersion (7.5 μg/mL) was mixed with 20.0 mL of hemin aqueous solution (7.5 μg/mL), with a weight ratio of PEG-GO to hemin of 1:1, and the mixture was ultrasonicated for 30 min (output power 180 W). The dispersion was purified by an Ultrafiltration system for removing the excessive free hemin which was not immobilized on GO sheets. After that the PEG-GO-Hemin composite sheets were obtained and dispersed in water.

2.3. Characterization of PEG-GO-Hemin composite sheets

A JEOL JEM-2100 transmission electron microscope (TEM) was employed to observe the morphology of the PEG-GO-Hemin composite sheets and hemin. Agilent PicoPlus atomic force microscopy (AFM) was used to measure the thickness of the PEG-GO-Hemin composite sheets. Ultraviolet visible (UV-vis) absorption spectra were recorded on an UV–vis spectrophotometer (Shimadzu UV-3600, Japan). The peroxidase-like activity and the Michaelis constants were measured by a Bio-rad 680 microplate reader. An Optima 5300DV Inductive Coupled Plasma Emission Spectrometer (ICP) was used to detect the load rate of hemin on PEG-GO-Hemin composite sheets. A Brookhaven Zetaplus dynamic light scattering was used to measure the hydrodynamic size and zeta potential of PEG-GO-Hemin composite sheets. Infrared (IR) spectroscopy (NEXUS870) was used to characterize the structure of PEG-GO. Raman spectroscopy (JY HR800) using an Nd-YAG laser source operating at a wavelength of 532 nm was applied at room temperature. Pyris 1 thermal gravimetric analyzer was employed for investigating the rate process of reduction of PEG, GO and PEG-GO.

2.4. Peroxidase-like activity of PEG-GO-Hemin and kinetic analysis

The catalytic reaction was carried out in the NaAc-HAc buffer (0.2 M, pH 3.6) with 0.622 mM TMB (or 0.396 mM ABTS) and 852 mM hydrogen peroxidase as substrates, using PEG-GO-Hemin composite sheets as peroxidase-like mimic enzyme. The absorbance values at 650 nm for TMB oxidized product (or 405 nm for ABTS oxidized product) in the reaction system catalyzed by PEG-GO-Hemin composite sheets, PEG-GO, and hemin. Each at the same Fe concentration was recorded for 3 min with the microplate reader for comparing their activities.

Steady state kinetic assays were carried out using a microplate reader at room temperature. The reaction system was NaAc-HAc buffer (0.2 M, pH 3.6) in a 96-well plate containing H_2O_2 and TMB (or ABTS) as substrates and PEG-GO-Hemin composite sheets, PEG-GO or hemin as peroxidase-like enzyme. The kinetic assay of TMB (or ABTS) as the substrate was performed by adding 32 μL 30% H_2O_2 and different amounts (0.5, 1, 2, 4, 6, 8, 10 μL) of TMB solution (10 mg/mL, dissolved in DMSO) or ABTS solution (10 mg/mL, dissolved in deionized water). The kinetic assay of H_2O_2 as the substrate was performed by adding 10 μL TMB or ABTS and different amounts (0, 2, 4, 6, 8, 16, 32 μL) of 30% H_2O_2 solution. All the reactions were monitored in timescan mode at 650 nm for TMB or 405 nm for ABTS using the microplate reader.

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