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Improving dispersive property, biocompatibility and targeting gene transfection of graphene oxide by covalent attachment of polyamidoamine dendrimer and glycyrrhetinic acid

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ABSTRACT

The surface functional groups of GO have significant effects on the performances of GO-based gene delivery vector. In this work, the polyamidoamine (PAMAM) dendrimer and glycyrrhetinic acid (GA) were tethered onto the GO surface by one-step covalently cross-linking method. The micro-morphology, surface functional groups, and zeta potential of the obtained GO-PAMAM-GA hybrid were characterized and verified. The effects of GA amount in the hybrid on the dispersive property in cell culture medium, in vitro cytotoxicity to human hepatocarcinoma (SMMC-7721) and human embryonic kidney (HEK-293) cells, and gene (plasmid DNA of enhanced green fluorescent protein) transfection capacity were investigated in detail. Under optimal conditions, the obtained hybrid shows small average size (about 160 nm) and has very good dispersive stability (in 30 days) in cellular culture medium. Compared with the GO-PAMAM without GA modification, the GO-PAMAM-GA hybrid exhibits greatly enhanced biocompatibility to the two cell lines. The cellular viability of SMMC-7721 cells still retains about 98% even the concentration of the hybrid up to 200 μg mL^{-1}. The gene transfection capacity of the GO-PAMAM has been improved about 50% through the GA functionalization. Moreover, the GO-PAMAM-GA hybrid possesses targeting gene transfection to SMMC-7721 cells.

1. Introduction

Gene therapy has shown good potential for a number of diseases. One of the critical issues in gene therapy is to choose suitable gene delivery carriers, usually involving the biological and synthetic vectors [1,2]. Biological vectors (such as bacteria, viruses, and etc.) generally show high transfection efficiency but they are at risk of variation or retoxicity, and may lead to excessive immune responses. In contrast to biological vectors, the synthetic vectors, for example, lipids [3,4], polymers [5–7], nanoparticles [8–10], and others, provide several advantages including simplicity of production and high safety. However, the synthetic vectors are limited by the low transfection efficiency. Designing of efficient and low cytotoxicity gene vectors are still one of the urgent issues to address for gene therapy.

In the past decade, graphene oxide (GO) has been widely employed in biological fields involving biosensors [11,12], chemical drug [13–15] or gene [16,17] delivery, photothermal treatment [18,19], and biological imaging [20]. GO-based nanomaterial shows good potential for gene delivery because of its 2D planar structure with a high specific surface area, the versatile surface oxygenated functional groups, and the ability to cross cell membranes easily, which increase the gene loading efficiency and facilitate cellular transfection [16,17,21–25]. Through π–π stacking and hydrogen bonding interactions, ssDNA can be powerfully absorbed onto the surface of GO nanosheets [26]. However, the interactions between GO and dsDNA are relatively weak because nucleobases are arranged inside the double helix structure of dsDNA [27]. It is difficult to directly use non-functionalized GO as gene vectors for plasmid DNA. To address this, one of the most convenient ways to develop GO-based gene vectors is to modify the GO surface with positively charged cationic polymers, being widely used for surface modification of inorganic materials [28]. The polyethylenimine (PEI) [29], branched PEI [30], and disulfide linkage polyethylene glycol (PEG)-PEI [16] have been tethered onto the GO surface. The prepared GO-PEI hybrids have the capacity to efficiently load plasmid DNA, resulting in comparable or even better gene transfection efficiency than PEI while significantly less cytotoxicity. Besides PEI

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functionalization, other cationic polymers such as polyamidoamine (PAMAM) dendrimers have also been utilized for GO modification. PAMAM dendrimers are one of the most promising cationic polymers for biomedical applications. The terminal surface of PAMAM contains a large number of amino groups, being easy to covalently cross-link with the carboxyl groups of GO. More importantly, the terminal amino groups in PAMAM dendrimers are positively charged under physiological conditions, being very favorable for electrostatic immobilization of the negatively charged gene molecules. Gu et al. [31] and our group [32] have reported the use of GO-PAMAM hybrids as protein and plasmid DNA carriers. Wang et al. [33] have developed PAMAM-PEG-functionalized GO for the intracellular delivery of anti-microRNA21. In addition, Sarkar et al. [34] have synthesized different generation PAMAM dendrimers (G1.0, G2.0, and G3.0) to modify GO through "click" chemistry reaction. These works reveal that the PAMAM modification can greatly improve the biocompatibility and gene transfection efficiency of GO-based gene vectors.

Although the functionalized-GO shows great potential for intracellular gene delivery, the factors that influence the gene transfection efficiency and cytotoxicity are still not well understood. Some works [35–37] have demonstrated that the gene transfection efficiency of GO-based nanohybrids may depend on the functional groups, surface potential, particle size, dispersive property, and the cell types, and etc. For example, Wang et al. [35] have synthesized the PEI-PEG-folic acid functionalized GO and investigated the effect of PEI content on the effective size and transfection efficiency. It has demonstrated that the effective size of the GO-PEI-PEG-folic acid decreases with the increase of PEI content, leading to better dispersibility and transfection efficiency. Our previous work [32] has shown that the GO-PAMAM hybrid has relative good dispersive property in ultrapure water; whereas degraded dispersive stability in cellular culture medium. The aggregation of the hybrids in cellular culture medium may influence the entering of GO-PAMAM into cells, leading to poor gene transfection efficiency. On the other hand, the nonspecific interaction of GO-PAMAM hybrid with both normal and cancer cells may limit its application in targeting gene delivery and result in relatively high cytotoxicity [38].

In this work, the GO-PAMAM-glycyrrhetinic acid (GA) hybrid (Fig. 1) was prepared and used as efficient and targeting delivery vector for plasmid DNA. GA is a pentacyclic triterpenoid metabolite of glycyrrhizin, derived from the root of licorice. It has good bioactivity, for example, anti-inflammatory and antitumor activity, being extensively used in pharmaceutical field for the treatment of a wide range of diseases [39,40]. The GA was selected to functionalize GO due to the follows: i) GA molecules can be easily tethered onto the GO-PAMAM surface by amidation reaction between the carboxyl group of GA and the amino group of PAMAM. The surface of the obtained GO-PAMAM-GA contains amino, hydroxyl, and C═O groups, which may improve the dispersive property of the hybrid in cellular culture medium. ii) GA has remarkable targeting property to hepatocellular carcinoma (HCC) cells [41–43]. The main target binding site of GA is protein kinase C,

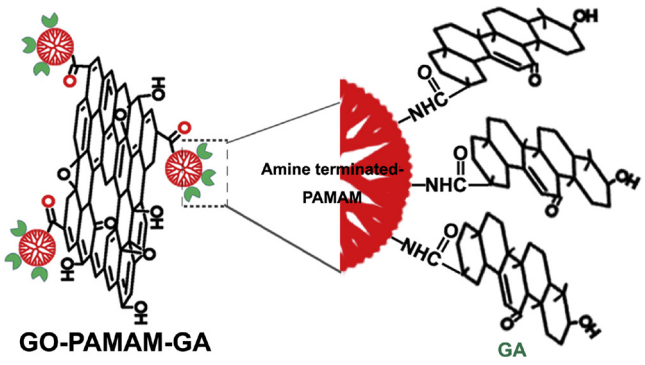

Fig. 1. Schemes demonstrating the GO-PAMAM-GA hybrid.

being expressed more highly in HCC cells than nontumor liver cells. The delivery carriers containing GA exhibit higher HCC therapeutic efficiency with improved safety. He et al. [44] have prepared GA and PEG modified cationic liposomes (CLs) for the delivery of plasmid DNA. The resulting GA-PEG-CLs show high transfection efficiency for HepG-2 cells whereas low transfection efficiency for human embryonic kidney cells (HEK-293) cells, in which the GA receptors are negative-expression. In addition, the GA-functionalized cationic phospholipids [45] and polypropylenimine dendrimer [46] have also shown good siRNA or plasmid DNA condensing ability, low cytotoxicity, and high transfection efficiency. Therefore, the proposed GA and PAMAM-modified GO hybrid may have good dispersive property in cellular culture medium as well as targeting property to hepatocytes. The PAMAM dendrimers and GA were functionalized to the GO surface by one-step covalent linkage (shown in Fig. S1, Supporting Information). The success of the PAMAM and GA modification was verified and characterized by Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), zeta potential measurement, and ninhydrin assay. The GO-PAMAM-GA hybrid exhibits very good dispersive stability in the cellular culture medium. To evaluate the performances of the GO-PAMAM-GA hybrid as gene delivery carrier, human hepatocarcinoma cell (SMMC-7721) and HEK-293 were selected as cell models. The in vitro cytotoxicity of the GO-PAMAM-GA hybrid was evaluated by water-soluble tetrazolium (WST-8) assay. The intracellular transfection efficiency of gene molecules (plasmid DNA of enhanced green fluorescent protein, pEGFP-N1) loaded GO-PAMAM-GA was assessed. Moreover, the effects of GA amount in the GO-PAMAM-GA hybrid on the cytotoxicity and gene transfection efficiency were also investigated.

2. Materials and methods

2.1. Chemicals and instruments

Graphene (diameter, 0.5–2 μm; thickness, 0.8–1.2 nm; single layer ratio, ~80%; purity, 98%) was purchased from Nanjing XF Nano Material Tech Co., Ltd. (China). 18-α-glycyrrhetinic acid (GA), chloroquine disphosphate salt, and amino group-terminated PAMAM dendrimers (generation 4.0) were obtained from Sigma-Aldrich Co. LLC. Ninhydrin was purchased from Sinopharm Chemical Reagent Co., Ltd. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, hydrochloride form) and N-hydroxy-succinimide (NHS) were purchased from Aladdin Industrial Inc. The SMMC-7721 cells were provided friendly by Guangzhou University of Traditional Chinese Medicine. The HEK-293 cells were provided friendly by the College of Life Science in Hunan Normal University. The pEGFP-N1 was transformed to TOP 10 *Escherichia coli* and purified using Hipure Plasmid Filter Midiprep Kit (Invitrogen Corp., Germany). DMEM and fetal bovine serum were purchased from GE Healthcare Life Sciences. 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and OPTI-MEM reduced serum medium were purchased from Life Technologies™ (USA). Trypsinase (0.25% + 0.02% EDTA) was purchased from Life Technologies™ (Canada). The Cell Counting Kit-8 (CCK-8) for WST-8 assays was obtained from Dojindo Laboratories (Japan). Phosphate buffered saline (PBS, pH 7.4, containing 3.49 g L^{-1} $Na_2HPO_4{\cdot}H_2O$ + 0.20 g L^{-1} KH_2PO_4 + 8.00 g L^{-1} NaCl + 0.20 g L^{-1} KCl) solutions were used in the experiments. All other reagents were of analytical grade or better. Milli-Q ultrapure water (> 18 MΩ cm, Milli-pore Co., Ltd.) and fresh prepared solutions were used throughout. In addition, the instruments for characterization of the GO-based hybrid were also listed in the Supporting Information.

2.2. Preparation of the GO-PAMAM-GA hybrid

The GO-PAMAM-GA hybrids containing different amounts of GA were synthesized by one-step covalent cross-linking method using EDC and NHS as coupling reactants. The typical procedures are as follows: the pristine graphene was first treated by mixed acids to prepare GO