Biomaterials 35 (2014) 9963–9971

Contents lists available at ScienceDirect

Biomaterials

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

Immunostimulatory oligonucleotides-loaded cationic graphene oxide with photothermally enhanced immunogenicity for photothermal/immune cancer therapy

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ARTICLE INFO

Article history:
Received 30 July 2014
Accepted 23 August 2014
Available online 15 September 2014

Keywords:
Graphene
Immunology
Oligonucleotides
Immunotherapy
Photothermal therapy

ABSTRACT

Graphene oxide (GO) has attracted tremendous research interest due to its excellent electrical, thermal, and mechanical properties. Here, we apply the polyethylene glycol (PEG) and polyethylenimine (PEI) dual-polymer-functionalized GO (GO-PEG-PEI) as the carrier for efficient CpG delivery. GO-PEG-PEI can significantly promote the production of proinflammatory cytokines and enhance the immunostimulatory effect of CpG. In addition, the NIR optical absorbance of GO-PEG-PEI has been further applied to control the immunostimulatory activity of CpG ODNs, showing remarkably enhanced immunostimulation responses under NIR laser irradiation, owing to the photothermally induced local heating that accelerated intracellular trafficking of nanovectors. This is the first demonstration of using the photothermally enhanced intracellular transportation of nanocarriers for light-controllable CpG delivery. In vivo assay demonstrates that the GO-PEG-PEI-CpG complex provides synergistic photothermal and immunological effects under laser irradiation for cancer treatment, which shows the highest efficiency in tumor reduction, implying the excellent therapeutic efficacy of the GO-PEG-PEI-CpG complex in cancer therapy.

1. Introduction

Graphene, a one-atom-thick two-dimensional (2D) layer of sp^2-bonded carbon, has attracted tremendous research interest since its discovery in 2004 [1–4]. Due to their impressive electrical, thermal, and mechanical properties [5–11], graphene and its derivatives, such as graphene oxide (GO) and reduced graphene oxide (rGO), have been extensively studied for applications in a large variety of fields including nanoelectronics, molecule sensing, composite materials, energy research, catalysis, and more recently biomedicine [12–20]. Among these applications, the promise of using graphene and its functionalized derivatives as robust nanocarriers for molecular payloads delivery has become particularly attractive. The payloads can be small drug molecules or large biomolecules, like anticancer drugs [21–25], photosensitizers [26], proteins [14,27], or nucleic acids [28–33]. Recently, graphene-based materials have been reported that they can significantly activate macrophages and trigger the production of proinflammatory cytokines, and thus may be used as the available candidates of immunoadjuvants to promote vaccine efficacy [34–38]. Yet, despite these burgeoning developments, studies focusing on graphene-based platform for vaccines or adjuvants delivery have scarcely been reported [39].

In the past few decades, oligonucleotide therapy has been widely investigated for the treatment of various diseases, such as viral infections, cancer, and neurological disorders [40–43]. In addition to conventional plasmid DNA-based gene therapy, artificial nucleic acids, including aptamers, antisense DNA, and small interfering RNAs (siRNAs) et al., have also been recognized as promising oligonucleotide drugs as a consequence of their easy synthesis with high reproducibility and purity, perfect sequence control, simple operation and conformational polymorphism nature [44–47]. Especially, recent research activities are directed to unmethylated cytosine-phosphate-guanine (CpG) motifs, which act as a type of therapeutic nucleic acids with strong immunostimulatory activities [48]. The mammalian immune system can recognize the CpG oligodeoxynucleotides (ODNs) through Toll-like receptor 9 (TLR9) and secrete a number of proinflammatory cytokines, including tumor necrosis factors TNF-α and interleukin IL-6 [49,50], which are able to stimulate a cascade of innate and adaptive immune responses. Although synthetic CpG ODNs have therefore become a favorable tool for immunotherapeutic

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http://dx.doi.org/10.1016/j.biomaterials.2014.08.036
0142-9612/

applications in both basic research and clinical trials [51,52], the delivery of these synthetic nucleic acids to disease sites still remains a great challenge [41]. For example, since nucleic acids are negatively charged, they cannot easily cross the electronegative cell membrane. Moreover, they can be rapidly degraded by nucleases [41]. Therefore, the development of new materials that can serve as efficacious CpG delivery vehicles capable of efficiently transferring CpG into target cells and preventing CpG degradation is of key importance [53].

The emergence of nanobiotechnology has provided unprecedented opportunities to develop effective vectors to transport CpG ODNs into target cells. For instance, various DNA assemblies have emerged as effective carriers for CpG ODN delivery [54–61]. Alternatively, many novel nanomaterials such as nanoliposomes [62], gold nanoparticles [44,63–65], carbon nanotubes [66,67], boron nitride nanospheres [68,69], and lanthanides-based core–shell nanoparticles [70] et al. have also been utilized as efficient carriers for CpG delivery. Although great progress has been achieved in this field, effective methods for facile synthesis of the simple yet efficient CpG delivery vehicles are still highly desirable. In this study, we synthesize the nanosized GO-PEG-PEI nanocomposite (GGI) consisting of GO sheets with covalently conjugated polyethyleneimine (PEI) and polyethylene glycol (PEG), which can be used as a promising carrier of CpG adjuvant. GO-PEG-PEI-CpG nanocomplex (GGIC) can significantly promote the production of proinflammatory cytokines and enhance the immunostimulatory effect. In addition, utilizing the NIR absorbance of GO, we show for the first time that the photothermal heating of GO can efficiently enhance the intracellular delivery of CpG ODNs, which can thus efficiently improve the immune responses (Scheme 1). Furthermore, we also investigate the synergistic effect of GGIC for combining photothermal therapy and immunotherapy in vivo, which shows the highest efficiency in tumor reduction, implying the excellent therapeutic efficacy of GGIC in cancer therapy.

2. Materials and methods

2.1. Materials and measurements

Purified anti-mouse TNF-α, biotin conjugated anti-mouse TNF-α cocktail, TNF-α standard, anti-mouse IL-6, biotin anti-mouse IL-6 and IL-6 standard were purchased from eBioscience. OPD (o-phenylenediamine) substrate was obtained from DingGuo. PEI 25k and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (USA). Graphite was obtained from Sinopharm Chemical Reagent Co., (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Alfa Aesar. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen. The mouse leukemic monocyte macrophage cell line (RAW264.7 cell line) was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai). All other reagents were all of analytical reagent grade and used as received. Nanopure water (18.2 MΩ; Millpore Co., USA) was used throughout the experiment. DNA oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). The sequences were as follows: CpG ODNs: 5′-TCC ATG ACG TTC CTG ACG TT-3′; FAM-labeled CpG ODNs: 5′-TCC ATG ACG TTC CTG ACG TT-3′-FAM.

Fluorescence measurements were carried out by using a JASCO FP-6500 spectrofluorometer (Jasco International Co., Japan). UV–Vis absorption spectra were recorded using a JASCO V-550 UV/Vis spectrophotometer. Atomic-force microscopy (AFM) measurements were performed using Nanoscope V multimode atomic force microscope (Veeco Instruments, USA). Tapping mode was used to acquire the images under ambient conditions. FT-IR analyses were carried out on a Bruker Vertex 70 FT-IR Spectrometer. Fluorescence images were captured using an Olympus BX-51 optical equipped with a CCD camera. Thermogravimetry (TGA) was performed with a Pyres 1 TGA apparatus (Perkin Elmer, MA) at a heating rate of 10 °C/min from 50 °C to 600 °C under a nitrogen atmosphere.

2.2. Synthesis of graphene oxide (GO)

GO was synthesized from graphite powder based on the Hummer's method [71]. Pretreated graphite powder was put into 0 °C concentrated H_2SO_4 (120 mL). Then, $KMnO_4$ (15 g) was added gradually under stirring, and the temperature of the mixture was kept below 20 °C by an ice bath. Successively, the mixture was stirred at 35 °C for 4 h and then diluted with deionized water (250 mL) by keeping the temperature under 50 °C. Water (700 mL) was then injected into the mixture followed by adding H_2O_2 (30 wt%) (20 mL) drop by drop. The mixture was filtered and washed with aqueous HCl solution (v/v 1:10) (1 L) to remove metal ions followed by deionized water to remove the acid. The resulting solid was dried in air and diluted to make a GO dispersion (0.5 wt%). Finally, it was purified by dialysis for 1 week to remove the remaining metal species. Exfoliation was carried out by sonicating the GO dispersion (0.1 mg/mL) under ambient conditions for 1 h.

2.3. Preparation of GO-PEG-PEI (GGI)

For preparation of GO-PEG-PEI conjugate, a GO solution (~0.5 mg mL^{-1}) was mixed with 4-arm PEG–NH_2–10k (0.5 mg mL^{-1}) under sonication for 5 min. The mixture was added with EDC (0.5 mg mL^{-1}) following another 5 min sonication, and then was stirred gently at room temperature for 10 min. After being sonicated with PEI (Mw: 25 kDa, 2.0 mg mL^{-1}) for 5 min, the mixture was stirred at room temperature for 6 h following the second time addition of EDC (1 mg mL^{-1}). After that, the mixture was washed 3~5 times with nanopure water, obtaining GO-PEG-PEI resuspended in water. For the preparation of GO-PEI and GO-PEG, the whole procedure was same to that for GO-PEG-PEI except no addition of PEG or PEI, respectively.

2.4. Preparation of GO-CpG (GC), GO-PEG-CpG (GGC), GO-PEI-CpG (GIC) and GO-PEG-PEI-CpG (GGIC) complexes

Assigned amounts of GO, GO-PEG, GO-PEI or GO-PEG-PEI conjugate were mixed with CpG ODNs. After being incubated at room temperature for 20 min, the complexes could be formed.

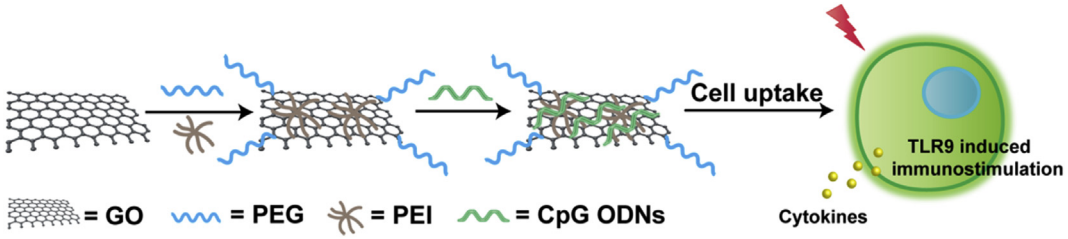
2.5. Cell culture

The murine macrophage-like RAW264.7 cells were grown at 37 °C in an atmosphere of 5% (v/v) CO_2 in air, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, 1.5 g/L $NaHCO_3$, 100 units/ml penicillin, 100 mg/mL streptomycin, 4.5 g/L glucose and 4 mM glutamine. The media was changed every three days, and the cells were digested by trypsin and resuspended in fresh complete medium before plating.

2.6. Cytotoxicity assays

MTT assays were used to probe cellular viability, MTT assays were used to probe cellular viability. RAW264.7 cells were seeded at a density of 5000 cells/well (100 μL total volume/well) in 96-well assay plates. After 24 h incubation, the as-prepared GO-PEG-PEI and GO-PEI, at the indicated concentrations, were added for further incubation of 24 h. To determine toxicity, 10 μL of MTT solution (BBI) was added to each well of the microtiter plate and the plate was incubated in the CO_2 incubator for an additional 4 h. Then the cells were lysed by the addition of 100 μL of DMSO. Absorbance values of formazan were determined with Bio-Rad model-680 microplate reader at 490 nm (corrected for background absorbance at 630 nm). Three replicates were done for each treatment group.

To evaluate the safety of laser irradiation, the cell viability of RAW264.7 cells was also measured by the MTT assay. Briefly, RAW264.7 cells were seeded at a density of 5000 cells/well (100 μL total volume/well) in 96-well assay plates. After 24 h incubation, the cell medium was replaced with fresh medium containing GO-PEG-PEI.



Scheme 1. Schematic showing the synthesis of GGIC and its immunostimulatory effect.