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Facile Synthesis of N-Doped Graphene Quantum Dots as Novel Transfection Agents for mRNA and pDNA

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Abstract: In the wake of the coronavirus disease 2019 (COVID-19) pandemic, global pharmaceutical companies have developed vaccines for the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Some have adopted lipid nanoparticles (LNPs) or viral vectors to deliver the genes associated with the spike protein of SARS-CoV-2 for vaccination. This strategy of vaccination by delivering genes to express viral proteins has been successfully applied to the mRNA vaccines for COVID-19, and is also applicable to gene therapy. However, conventional transfection agents such as LNPs and viral vectors are not yet sufficient to satisfy the levels of safety, stability, and efficiency required for the clinical applications of gene therapy. In this study, we synthesized N-doped graphene quantum dots (NGQDs) for the transfection of various genes, including messenger ribonucleic acids (mRNAs) and plasmid deoxyribonucleic acids (pDNAs). The positively charged NGQDs successfully formed electrostatic complexes with negatively charged mRNAs and pDNAs, and resulted in the efficient delivery and transfection of the genes into target cells. The transfection efficiency of NGQDs is found to be comparable to that of commercially available LNPs. Considering their outstanding stability even at room temperature as well as their low toxicity, NGQDs are expected to be novel universal gene delivery platforms that can outperform LNPs and viral vectors.

Keywords: gene delivery; graphene quantum dots; mRNA; pDNA; transfection

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1. Introduction

COVID-19, coronavirus disease 2019, has threatened global society, and various types of vaccines have been developed to overcome the pandemic [1–6]. Gene-based vaccines such as messenger ribonucleic acid (mRNA) with lipid nanoparticles (LNPs) or deoxyribonucleic acid (DNA) with viral vectors have been approved by the United States Food and Drug Administration (FDA) for clinical uses [7–11]. Some pharmaceutic companies have developed the vaccine platform based on mRNAs employing LNPs as a delivery platform, which exhibits a high prevention rate [12,13]; others use viral vectors to transfer spike proteins genes into cells, which are similar to Ebola vaccines [14,15]. The therapeutic strategy that uses genes such as mRNA or plasmid DNA (pDNA) is called gene therapy, which has drawn much attention because it easily expresses desired proteins in the body through their corresponding sequence information to be transcribed and translated into therapeutic proteins to fight various diseases [16–18].

The viral vectors such as adeno-associated virus (AAV) are the main platforms that deliver genes into cells with high transfection efficiency [19,20]. However, they have some drawbacks such as limited size of cavities, high immunogenicity, mutagenesis, and undesired inflammatory responses [21–23]. LNPs, cationic polymers, and inorganic nanoparticles also have been actively recruited as non-viral vectors to overcome such limitations of viral vectors [24]. In particular, LNPs have already been approved by the FDA and have been used to deliver doxorubicin or mRNA [25,26]. However, LNPs also

have drawbacks such as short half-life in the body, harsh storage conditions for stability, and low loading efficiency [27,28]. Therefore, it is of great importance to develop alternative gene delivery platforms to overcome the above-mentioned disadvantages.

Many researchers have tried to transfect genes using carbon-based materials such as graphene oxides (GOs) and graphene quantum dots (GQDs) [29–32]. GOs are the oxidized form of graphene sheets with a size ranging from several hundred nanometers to several micrometers [33]. As GOs are soluble in water and can interact with various drugs and genes, they have been utilized as drug delivery and biosensor platforms. It was previously reported that GOs can be modified with polyethylene glycol (PEG), polyethyleneimine (PEI), or/and targeting moieties to deliver genes into a cell [32,34,35]. Although the GO platform shows a good transfection efficiency, their high toxicity has limited their actual in vivo applications.

On the other hand, GQDs with a size of 1~10 nm that are functionalized with hydroxyl, carbonyl, alcohol, carboxylic groups, etc. exhibit relatively low toxicity [36]. This enabled the various applications of GOs in nanomedicine and bio-imaging fields, taking advantages of unique biological, chemical, and optical properties, amphiphilicity, and high biocompatibility [37]. In our previous reports, we found that GQDs with hydrophobic cores and hydrophilic edges interact with α-synuclein fibrils and dissociated them into monomers [38]. Additionally, we showed that GQDs exhibit low cytotoxicity and do not affect microbiome environment when fed to mice via oral administration [39]. GQDs also can be loaded with various drug molecules and genes through physicochemical conjugation between their functional groups [40–44]. Like GOs, GQDs need additional functionalization to interact electrostatically or covalently with therapeutic molecules or genes [45,46]. However, their relatively complicated synthetic processes has been a hurdle for practical applications [47,48], and the covalent modifications of genes possibly lower the gene activities [49]. In addition, most of previous studies have been focused on the delivery of pDNAs [50–53] as the intracellular distribution of GQDs near the nucleus is supposed to be advantageous for pDNA transfections [54,55]. Recently, Ya et al. reported the first study on the delivery of mRNAs using GQDs [56]. However, in this case, the GQDs required post-modifications with PEI via covalent bonding catalyzed by ethylenediamine (EDA) and dicyclohexycarbodiimide (DCC), which resulted in lower transfection efficiency than that of LNPs. In addition, the PEI-modified GQDs were incapable of delivering other types of genes such as pDNA.

GQD is a promising drug delivery platform that can deliver a wide range of small molecules, peptides, and genes into cells. It was found that GQDs localize in the late endosome or the lysosomes around the Golgi apparatus and nucleus after incubating cells with GQDs, which indicated that the cellular uptake of GQDs happens via endocytosis [55,57–59]. Furthermore, GQDs entered cells through diverse pathways related to endocytosis like caveolae-mediated endocytosis after drug loading. It is well known that GQDs interact with various types of drug molecules like doxorubicin and curcumin through electrostatic interaction or π—π interaction, where the loaded drugs don't affect cellular uptake efficiency [42,60,61].

Herein, we synthesized positively charged NGQDs as gene transfection agents, utilizing the microwave-assisted hydrothermal reactions between polyethyleneimine (PEI) and citric acid precursors. It was expected that the negatively charged mRNA and pDNA interact electrostatically with the positively charged NGQDs. Indeed, NGQDs showed an excellent transfection efficiency even at ~200 times lower concentrations than their CC50 (50% of cytotoxic concentration ~125 μg/mL). To the best of our knowledge, these are the first applications of the as-synthesized NGQDs capable of transfecting both mRNA and pDNA without additional chemical modifications, which is expected to enable the cost-efficient large-scale synthesis that is essential for future clinical applications.

2. Materials and Methods

2.1. Synthesis of NGQDs

200 mg of citric acid (Sigma Aldrich, St. Louis, MO, USA) and 50 mg of PEI (branched, Mw 1800, Polyscience, Warrington, UK) were added to 15 mL of distilled water. After 30 min of sonication, a transparent solution was placed into the center of a microwave (MWO-2027, 800 W, Midea, Foshan, China). 30 s of a microwave-assisted hydrothermal reaction was iterated about 10 times until the reaction solution turned yellow. The product solution was filtered with 200 nm and 20 nm disc filters (Anodisc^{TM}, GE Healthcare Life Sciences, Chicago, IL, USA) and dialyzed for 5 days with a 3.5 kDa dialysis tube (Snakeskin^{TM}, Thermo Fisher Scientific, Waltham, MA, USA) for removal of the remaining reactants. The finished solution was lyophilized for 2 days.

2.2. Characterization of NGQDs

The morphology and size distributions of NGQDs were analyzed with a Cs-corrected transmission electron microscope (Cs-TEM; JEM-ARM200F, JEOL Ltd., Tokyo, Japan). The zeta potential was measured by a zeta potential analyzer (Zetasizer NanoS, Malvern Instruments, Malvern, UK). The functional groups of NGQDs were characterized by Fourier transform infrared (FT-IR; Vertex-80V, BRUKER, Billerica, MA, USA) and X-ray photoelectron spectroscopy (XPS; AXIS-His, Kratos Analytical Ltd., Manchester, UK). A Raman spectrometer (in Via Raman microscope, Renishaw, Wotton-under-Edge, UK) was used to identify the D and G bands of the graphene in the NGQDs. The absorbance of the NGQDs was analyzed with an ultraviolet-visible (UV-Vis) spectrophotometer (S-3100, Scinco, Seoul, Korea). The emission spectra at various excitation wavelengths were acquired with a spectrofluorometer (FP-8300, Jasco Inc., Tokyo, Japan).

2.3. Loading Capacity

To analyze the ratio of NGQDs to genes, 100 ng of mRNA (CleanCap® EGFP mRNA, TriLink Biotechnologies, San Diego, CA, USA) and pDNA (pcDNA3-EGFP, Addgene, Watertown, MA, USA), encoding green fluorescent protein (GFP), were added to various amounts (0, 0.5, 1, 2, 4 μg) of NGQDs in 20 μL of 1× phosphate buffer saline (PBS) solution. The loading process was executed in a 1 mL tube. After 1 h of incubation, the series of complexes were mixed with 4 μL of LoadingSTAR^{TM} (Dyne Bio Inc., Seongnam, Korea) and the mixtures were loaded on a 1% agarose gel. The loading capacity of NGQDs was determined by measuring the intensity of the bands derived from the remaining genes after agarose-gel electrophoresis (Mupid-2plus, ADVANCE, Tokyo, Japan) at 100 V for 30 min.

2.4. Cell Viability Assay

HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells for 24 h before transfection. HeLa cells were then treated with various concentrations of NGQDs in complete media for 24 h. After removal of the media, the cells were washed with 1× PBS solutions and incubated in 90 μL of serum-free media with 10 μL of cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies Inc., Rockville, MD, USA) solution. To evaluate the cell viability of the treated cells, the optical density of formazan salt was measured at 450 nm using a microplate absorbance reader (Synergy Mx, BioTek, Winooski, VT, USA), and the background absorbance of the media was subtracted. Experiments were carried out in triplicate.

2.5. Gene Transfections Efficiency

HeLa cells were seeded in a 24-well plate at a density of 3×10^4 cells. After incubation for 24 h, the cells were treated with 1× PBS (Ctrl), mRNA, Lipofectamine® 2000 (Thermo Fisher Scientific), NGQDs, mRNA with Lipofectamine® 2000, and mRNA with NGQDs in 0.5 mL of serum-free media. For preparation of the complex-containing genes and the NGQDs, 3 μL of mRNA solution (10 μg/mL) and 6 μL of NGQDs solution (0.1 mg/mL) were mixed and 2 μL of 10× PBS solution was added with 9 μL of deionized