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Ultra-Small Graphene Oxide Functionalized with Polyethylenimine (PEI) for Very Efficient Gene Delivery in Cell and Zebrafish Embryos

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ABSTRACT

Efficient DNA delivery is essential for introducing new genes into living cells. However, effective virus-based systems carry risks and efficient synthetic systems that are non-toxic remain to be discovered. The bottle-neck in synthetic systems is cytotoxicity, caused by the high concentration of DNA-condensing compounds required for efficient uptake of DNA. Here we report a polyethyleneimine (PEI) grafted ultra-small graphene oxide (PEI-g-USGO) for transfection. By removing the free PEI and ensuring a high PEI density on small sized graphene, we obtained very high transfection efficiencies combined with very low cytotoxicity. Plasmid DNA could be transfected into mammalian cell lines with up to 95% efficiency and 90% viability. Transfection in zebrafish embryos was 90%, with high viability, compared to efficiencies of 30% or lower for established transfection technologies. This result suggests a novel approach to the design of synthetic gene delivery vehicles for research and therapy.

KEYWORDS

Gene delivery, graphene, DNA, Gene expression, zebrafish

Gene delivery into cells, in most cases, is used to overexpress transgenes to obtain expression products, assign function to a nucleic acid sequence of interest to achieve on/off regulation of the endogenous genes or repair missing/defective genes for theraputic intervention [1]. Non-viral vectors such as cationic polymers, lipids or peptides show several advantages such as low immunogenicity, target-cell specifity, and relative safety profiles [2]. However, their low transfection efficiency compared to virus based systems combined with their high toxicity, is a big obstacle for their application [1]. Therefore, extensive efforts have

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been devoted to increasing their transfection efficiency while decreasing toxicity. Here we describe an effective, low toxicity gene delivery agent based on PEI-functionalized graphene able to deliver plasmid DNA (pDNA) into cells and zebrafish embryos, which are notoriously difficult in this respect.

Important steps during transfection are cellular association with the vector, cellular uptake, endosomal release, nuclear import of DNA, followed by transgene expression (Fig. 1(c)). The physical chemistry of the transfection vectors influences each of these steps and the rate limiting process is reported to be endosomal escape [3–6]. Polyethylenimine (PEI) is one of the most effective non-viral vectors for gene delivery because of its strong endosomal escape capacity due to the "proton sponge effect" [7, 8], which is considered to be the rate limiting process in the cellular barriers. In PEI based gene transfection, ~86% of the PEI does not condense DNA and hence exists as free polymer [9], which is the major reason for its cytoxicity [5]. However, the free PEI also contributes substantially to the endosomal release, as

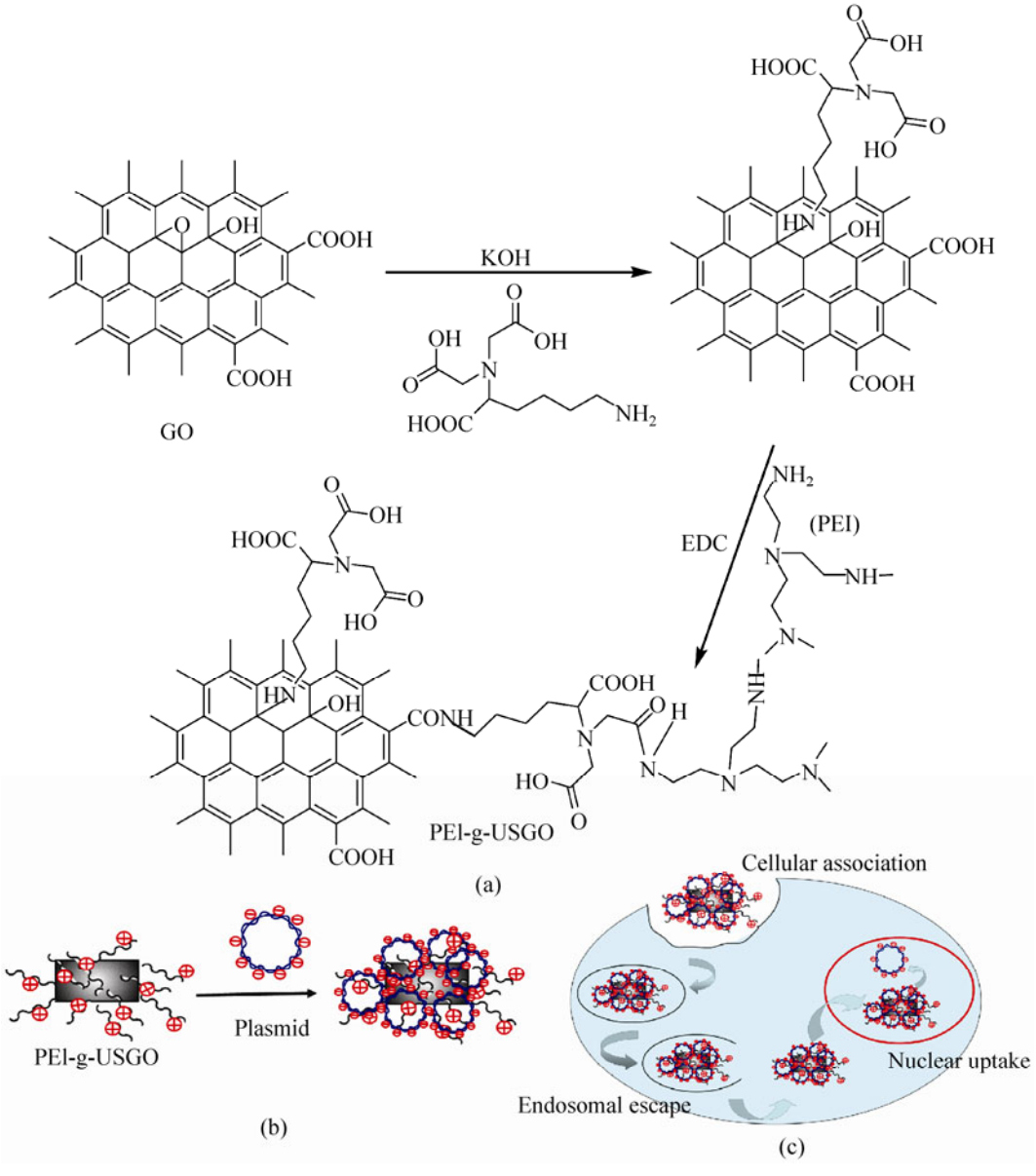

Figure 1 (a) Reaction scheme for preparation of USGO and PEI-g-USGO; (b) schematic representation of plasmid condensation using PEI-g-USGO; (c) schematic representation of the gene transfection pathway

removing the free PEI lowers transfection efficiency [10, 11]. Therefore, apart from other modification methods to decrease the toxicity [12], one possible way for achieving low toxicity and high transfection efficiency is to conjugate PEI into a well-defined nanostructure that is sufficient for the endosomal release, whilst removing the free PEI. It was reported that PEI conjugated onto graphene oxide (GO) can be used for gene transfection, with low cytotoxicity and good efficiency [13–15].

In these reports [13–15], PEI was physically adsorbed on GO or covalently conjugated to GO by a carbodiimide cross-linking reaction between –COOH groups of GO and $–NH_2$ groups of PEI. Introducing PEI onto GO by physical adsorption results in an unstable mixture. For carbodiimide cross-linking, the –COOH groups only reside at the edge of GO, which has a two-dimensional (2D) planar surface of a size of several hundreds of nanometers; therefore, only limited amounts of PEI can be introduced onto GO, which can be detrimental for efficient transfection.

In order to achieve more effective functionalization of PEI we prepared ultra-small GO (USGO, size: 10–20 nm) by a one-pot synthesis, resulting in a compound which has –COOH groups both in the plane and at the edges. The starting GO was prepared by a modified Hummers method [16] from graphite with a lateral size of several hundreds of nanometers and a thickness of ~0.8 nm (Fig. S-1 in the Electronic Supplementary Material (ESM)). The USGO was prepared by an epoxy ring-opening reaction of GO using an NH_2-functionalized nitrilotriacetic acid (NH_2-NTA), as schematically shown in Fig. 1(a). The resulting USGO has a lateral size of ~10–20 nm and a height of ~1.5–2.5 nm (Fig. 2(a) and Fig. S-2 in the ESM). Treating the GO using a strong tip sonification (400 W), or sonificating GO in KOH solution omitting the NTA-NH_2 can reduce the size of GO, but not enough to

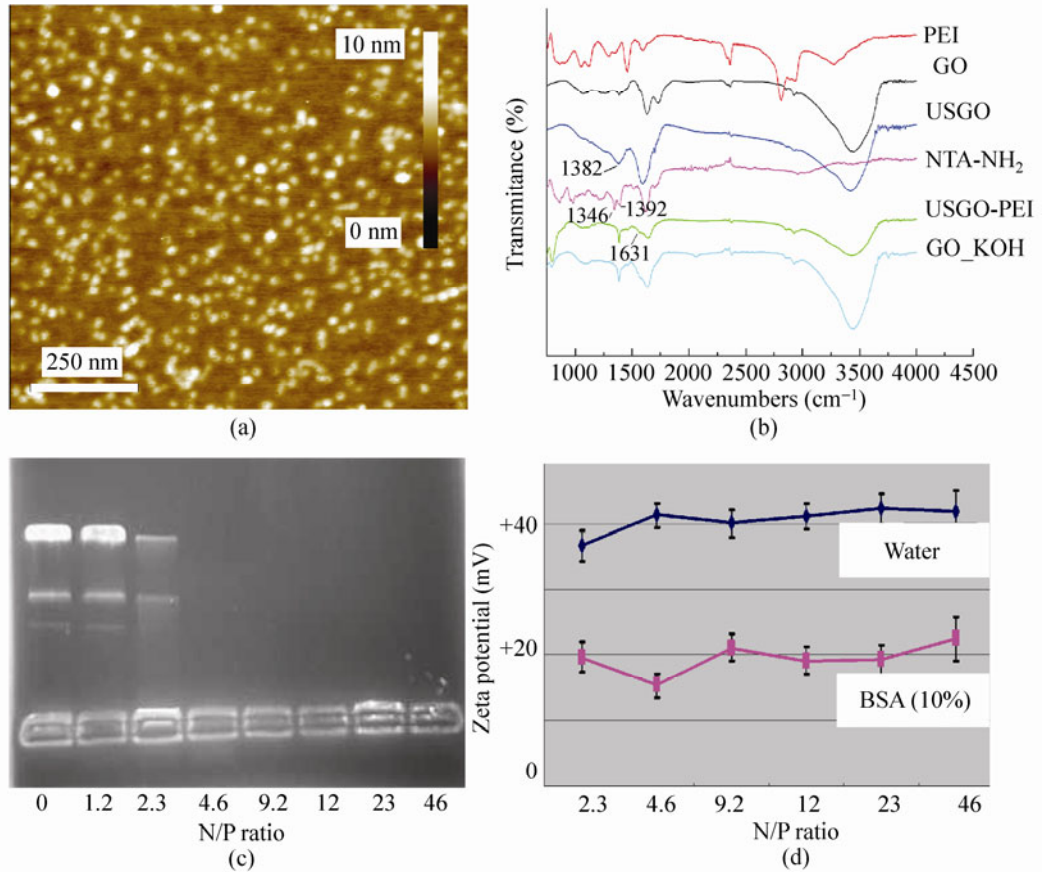

Figure 2 (a) Atomic force microscopy (AFM) image of USGO; (b) FTIR spectra of PEI, GO, USGO, and PEI-g-USGO; (c) band shift assay of pEGFP/PEI-g-USGO with different N/P ratios; (d) zeta potential of pEGFP/PEI-g-USGO with different N/P ratios in water and in BSA (10%)

make it as small as the USGO, as shown in Figs. S-1(b) and S-1(c) (in the ESM). In the literature for preparing graphene of small size, a further separation step such as ultracentrifugation or dialysis is always required after the reaction [17, 18]. Without the need for ultracentrifugation and dialysis, the way presented here will allow the easy preparation of bulk amounts of USGO.

The conjugation of the nitrilotriacetic acid (NTA) moiety to the USGO is also confirmed by the Fourier transform infrared spectroscopy (FTIR) spectra shown in Fig. 2(b). The NTA moiety shows two peaks at 1346 and 1392 cm^{-1}; after conjugation of NTA to the USGO, these two peaks combined together and exist as a broadened peak at 1383 cm^{-1}. In our method, USGO of homogeneous size can be obtained directly after the reaction. The increased height of USGO compared to the starting GO (~0.8 nm) can be attributed to the presence of the NTA moieties. One epoxy addition reaction introduces three –COOH groups into USGO, therefore the –COOH groups should exist both in the plane and at the edge of USGO.

PEI (M_w = 60 kDa) was covalently conjugated to USGO via the carbodiimide cross-linking reaction between –COOH of USGO (Fig. 1(a)), and the free PEI was removed by filtration. The product is referred to as PEI-g-USGO below. The experimental details are listed in S-9 in the ESM. The functionalization of PEI onto USGO was confirmed by the new peak at 1631 cm^{-1} in the FTIR spectra shown in Fig. 2(b), corresponding to the stretching mode of the amide bond. Elemental analysis gave 13.5% N, 55.8% C, 8.6% H, and 22.1% O. The zeta potential of PEI-g-USGO was +44 mV, which is similar to that of typical PEI-g-GO [13–15]. For comparison, we fabricated PEI-grafted GO (PEI-g-GO) via carbodiimide cross-linking (Fig. S-3(a) in the ESM), using GO which had a surface size of several hundreds of nanometers (Fig. S-3(c) in the ESM). Elemental analysis of PEI-g-GO gave a composition of 11.3% N, 45% C, 7.5% H, and 36.2% O. Compared to PEI-g-GO, the PEI-g-USGO not only had a significantly smaller size, but also had a higher nitrogen content, indicating a higher loading of PEI.

The cationic surface of PEI-g-USGO facilitates DNA condensation, as schematically shown in Fig. 1(b). Upon mixing PEI-g-USGO with an enhanced green fluorescent protein plasmid (pEGFP) reporter gene, the pEGFP spontaneously condensed onto PEI-g-USGO. High affinity was confirmed with a band shift assay (Fig. 2(c)). By measuring the brightness using Quantity One software (details are shown in S-9 in the ESM), we calculated ~4.4 μg of pEGFP were condensed per μg of PEI-g-USGO, almost three times the capacity that we found for PEI-g-GO (~1.4 μg of pEGFP per μg of PEI-g-USGO, as shown in S-9 in the ESM).

The positive charge of the pEGFP/PEI-g-USGO nanoparticles is important for the association with the negatively charged cell membrane and subsequent uptake. Therefore, we characterized the surface charge of DNA/PEI-g-USGO nanoparticles by measuring their zeta potentials at N/P ratios (the molar ratio of nitrogen in PEI-g-USGO to phosphate in DNA) from 2.3 to 46 (Fig. 2(d)). In water, all the nanoparticles had a positive charge, ranging from +35 mV to +42 mV. However, the cell culture medium always contained 10% serum, with a high concentration of negatively charged proteins. Therefore, we also determined the zeta potential of pEGFP/PEI-g-USGO nanoparticles in bovine serum albumin (BSA, 10%) (Fig. 2(d)). As expected, the zeta potentials of the nanoparticles formed in BSA solution were lower than those in H_2O: ~+20 mV. The nanoparticles formed by pEGFP/PEI-g-GO showed a negative zeta potential for N/P ratios of 4.6 and 9.2 in a 10% BSA solution, as shown in Fig. S-4 in the ESM. This indicates that the nanoparticles formed by pEGFP/PEI-g-GO are negatively charged, which is caused by the positive charge of PEI-g-GO being insufficient to render the whole nanoparticle positively charged in the presence of negatively charged BSA. This confirmed that the PEI-g-USGO has a higher positive charge and consequently a higher DNA condensation capacity than PEI-g-GO.

Transfection into H293T and U_2Os cell lines was tested using the pEGFP reporter gene. Transfection efficiency was characterized qualitatively by fluorescence microscopy (Fig. S-5 in the ESM) and quantitatively by fluorescence-activated cell sorting (FACS) (Fig. 3(a)). The cytotoxicity was measured with a trypan blue assay (Fig. 3(b)). Transfection by lipofectamine 2000 and PEI (60 kDa) were used as

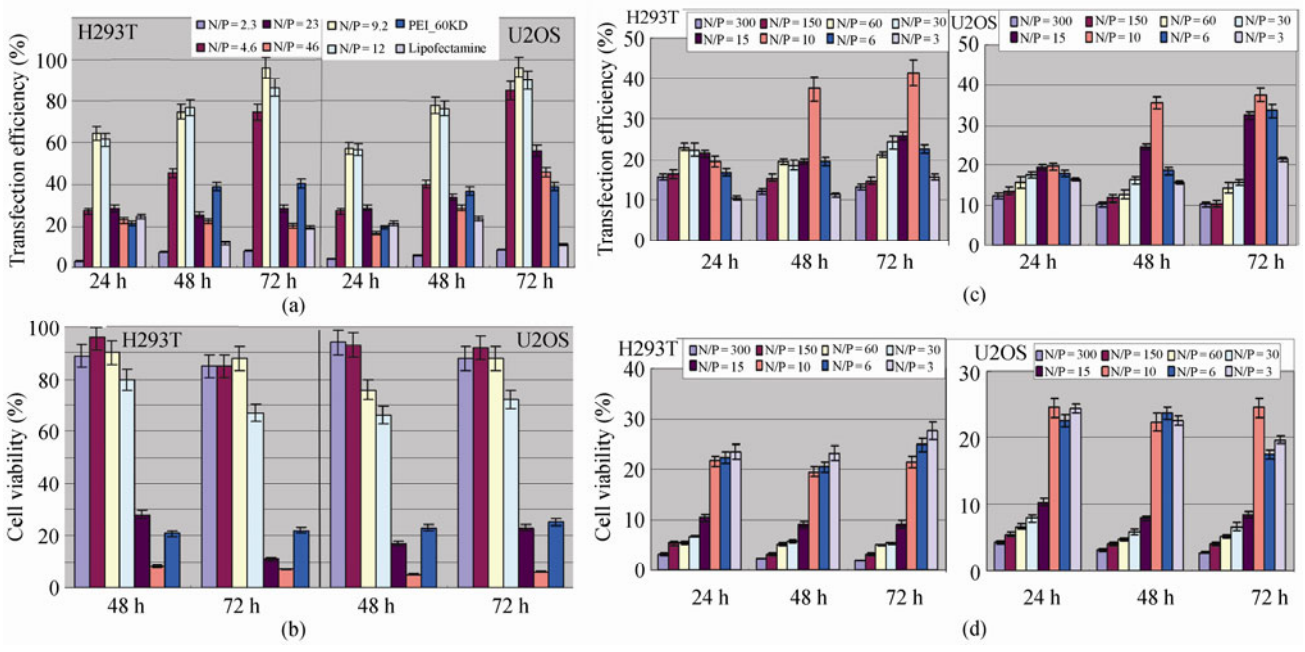

Figure 3 Transfection efficiency of a pEGFP reporter gene (a) and cell viability (b) of PEI-g-USGO in H293T and U2Os cell lines at different post-transfection times, using 60 kDa PEI and lipofectamine as controls; transfection efficiency of a pEGFP reporter gene (c) and cell viability (d) of PEI (60 kDa) in H293T and U2Os cell lines at different post-transfection times

positive controls, which is at the optimized feed ratio in our experiment (lipofectamine 2000 was optimized according to the protocol, and the PEI was optimized the same way as PEI-g-USGO, as shown in Figs. 3(c) and 3(d)).

At an N/P ratio of 9.2, we observed a strikingly high transfection efficiency of PEI-g-USGO for both cell lines with a low toxicity (only ~10% cell death). Because of the low toxicity, we could incubate cells with pEGFP/PEI-g-USGO for up to 72 h without substantial cell death. We observed strong expression at 24 h, which increased with time until 72 h post-transfection (up to ~95% efficiency). This behavior is different from standard transfection using PEI. It is reported that transgene expression peaks at ~24 h post-transfection and does not further increase with time [19]. In our control experiments, the transfection efficiency of PEI (60 kDa) plateaus at 48 h (~40%), with high cytotoxicity (~80% cell death). The transfection efficiency of PEI-g-USGO (~95%) is strikingly high when compared to lipofectamine 2000 (less than 30%), which is currently one of the most widely used synthetic transfection reagents. At the optimized N/P ratio for the H293T cell line, PEI-g-USGO showed much better transfection efficiency (N/P = 9.4, ~60% at 24 h, and ~95% at 72 h,) than PEI-g-GO at the optimized N/P ratio (N/P = 46, ~40% at 24 h, and ~80% at 72 h, Fig. S-6 in the ESM), with similar cytotoxicity (~90% cell viability for both samples). Similar results were obtained in U_2Os cell line. Moreover, the N/P ratio required for the optimized transfection of PEI-g-USGO (9.2) was much smaller than that of PEI-g-GO (46), indicating that the transfection with PEI-g-USGO is much more effective than with PEI-g-GO.

In recent years, zebrafish have proven to be an excellent model organism in molecular biology, vertebrate developmental biology and cryobiology due to the relatively low-cost maintenance of fish stock, the short generation time, and translucent features facilitating *in vivo* imaging [20]. Therefore, we tested the transfection capacity of PEI-g-USGO and pEGFP mixtures in zebrafish embryos. Micro-injection inside the embryo interlayer in the first cell stage led to green fluorescent protein (GFP) overexpression ~6 h post-injection, as shown in Fig. 4.

By counting ~200 embryos, we observed ~90% pEGFP positive transfection (~90% embryos with some EGFP positive cells), with viability of ~90%. High

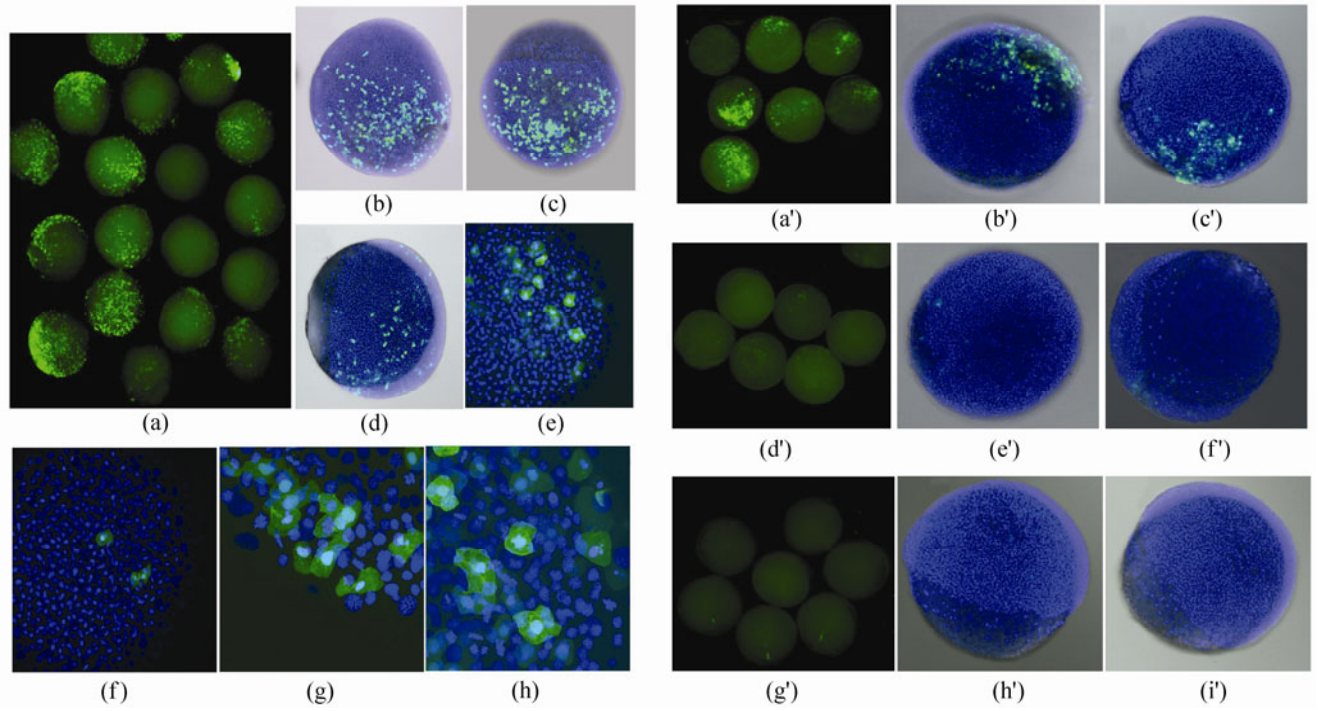

Figure 4 Fluorescence images of PEI-g-USGO in zebrafish using a pEGFP reporter gene 6 h after injection. (a) Overview image, (b)–(e) confocal images at low resolution, (f)–(h) confocal images at high resolution; fluorescence images of pEGFP- transfection into zebrafish embryos, using PEI-g-USGO (a′)–(c′), lipofectamine (d′)–(f′), and PEI (60 kDa) (g′)–(i′) as the transfection agent, injected in the interlayer (6 h post-transfection), (a′,d′,g′: overview image; b′,c′,e′,f′,h′,i′: confocal images)

resolution laser confocal fluorescence microscopy using paraformaldehyde (PFA) fixed embryos confirmed cytosolic and nuclear GFP expression inside the zebrafish embryo cells (Figs. 4(f)–4(h)). Transgene delivery efficiency was compared with the fluorescent signal from Hoechst 33342. The transfection efficiency using PEI-g-USGO was much higher than bare DNA injection (~30%, Fig. S-8 in the ESM), and similar to that with PEI-g-GO (~80%, Fig. S-7 in the ESM). However, the transfection using PEI-g-USGO gave a much stronger pEGFP signal than PEI-g-GO (Fig. S-7 in the ESM) and PEI (60 kDa) and lipofectamine 2000 (Figs. 4(a')–4(i')), indicating that PEI-g-USGO is also a very promising vehicle for gene transfection in zebrafish embryos.

We conclude that highly efficient gene delivery can be achieved using a PEI-grafted ultra-small graphene oxide (PEI-g-USGO) with low cytotoxicity. By removing the free PEI from our transfection system, which we assume to be a major factor in determining its low cytotoxicity compared to other PEI-based systems, PEI-g-USGO allowed gene transfection protocols to last for up to 72 h without increase of cell death. Transfection using PEI-g-USGO in zebrafish embryos had a relatively high efficiency and bright GFP signal was observed upon micro-injection. Compared to the PEI-g-GO prepared by simple carbodiimide cross-linking, the PEI-g-USGO showed a strikingly effective transfection. The above features suggest PEI-g-USGO is a safer and more efficient vector compared to current PEI-based gene delivery systems. The versatility of the system may even be extended to multifunctional applications, such as targeted delivery and intra-cellular sensors.

More detailed studies are on-going, such as the detailed mechanism of the low toxicity, the fraction of bound/free USGO-PEI with DNA, the size influence of the USGO-PEI with DNA, and the influence of PEI molecular weight.

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Electronic Supplementary Material: Supplementary material including the atomic force microscopy (AFM) of GO, size and height distribution of USGO, information of gene transfection using PEI-g-GO, gene transfection in zebrafish using PEI-g-GO and H_2O is available in the online version of this article at http://dx.doi.org/10.1007/s12274-012-0254-x.

References

[1] Mastrobattista, E.; van der Aa, M. A. E. M.; Hennink, W. E.; Crommelin, D. J. A. Artificial viruses: A nanotechnological approach to gene delivery. *Nat. Rev. Drug Discov.* **2006**, *5*, 115–121.

[2] Putnam, D. Polymers for gene delivery across length scales. *Nat. Mater.* **2006**, *5*, 439–451.

[3] Kunath, K.; von Harpe, A.; Fischer, D.; Peterson, H.; Bickel, U.; Voigt, K.; Kissel, T. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: Comparison of physicochemical properties, transfection efficiency and *in vivo* distribution with high-molecular-weight polyethylenimine. *J. Control. Release* **2003**, *89*, 113–125.

[4] Ogris, M.; Steinlein, P.; Kursa, M.; Mechtler, K.; Kircheis, R.; Wagner, E. The size of DNA/transferrin–PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther.* **1998**, *5*, 1425–1433.

[5] Lungwitz, U.; Breunig, M.; Blunk, T.; Göpferich, A. Polyethylenimine-based non-viral gene delivery systems. *Eur. J. Pharm. Biopharm.* **2005**, *60*, 247–266.

[6] Godbey, W. T.; Wu, K. K.; Mikos, A. G. Size matters: Molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* **1999**, *45*, 268–275.

[7] Behr, J. P. The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia* **1997**, *51*, 34–36.

[8] Sonawane, N. D.; Szoka, F. C.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine–DNA polyplexes. *J. Biol. Chem.* **2003**, *278*, 44826–44831.

[9] Clamme, J. P.; Azoulay, J.; Mely, Y. Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy. *Biophys. J.* **2003**, *84*, 1960–1968.

[10] Boeckle, S.; von Gersdorff, K.; van der Piepen, S.; Culmsee, C.; Wagner, E.; Ogris, M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J. Gene Med.* **2004**, *6*, 1102–1111.

[11] Boeckle, S.; Fahrmeir, J.; Roedl, W.; Ogris, M.; Wagner, E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Control. Release* **2006**, *112*, 240–248.

[12] Zintchenko, A.; Philipp, A.; Dehshahri, A.; Wagner, E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjugate Chem.* **2008**, *19*, 1448–1455.

[13] Zhang, L. M.; Lu, Z. X.; Zhao, Q. H.; Huang, J.; Shen, H.; Zhang, Z. J. Enhanced chemotherapy efficacy by sequential delivery of siRNA and anticancer drugs using PEI-grafted graphene oxide. *Small* **2011**, *7*, 460–464.

[14] Yang, K.; Wan, J. M.; Zhang, S. A.; Zhang, Y. J.; Lee, S. T.; Liu, Z. A. *In vivo* pharmacokinetics, long-term biodistribution, and toxicology of PEGylated graphene in mice. *ACS Nano* **2011**, *5*, 516–522.

[15] Chen, B. A.; Liu, M.; Zhang, L. M.; Huang, J.; Yao, J. L.; Zhang, Z. J. Polyethylenimine-functionalized graphene oxide as an efficient gene delivery vector. *J. Mater. Chem.* **2011**, *21*, 7736–7741.

[16] Hummers, W. S.; Offeman, R. E. Preparation of graphitic oxide. *J. Am. Chem. Soc.* **1958**, *80*, 1339–1339.

[17] Pan, D. Y.; Zhang, J. C.; Li, Z.; Wu, M. H. Hydrothermal route for cutting graphene sheets into blue-luminescent graphene quantum dots. *Adv. Mater.* **2010**, *22*, 734–738.

[18] Robinson, J. T.; Tabakman, S. M.; Liang, Y. Y.; Wang, H. L.; Casalongue, H. S.; Vinh, D.; Dai, H. J. Ultrasmall reduced graphene oxide with high near-infrared absorbance for photothermal therapy. *J. Am. Chem. Soc.* **2011**, *133*, 6825–6831.

[19] Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. A two-stage poly(ethylenimine)-mediated cytotoxicity: Implications for gene transfer/therapy. *Mol. Ther.* **2005**, *11*, 990–995.

[20] Gerlai, R. Zebrafish: An uncharted behavior genetic model. *Behav. Genet.* **2003**, *33*, 461–468.