Using graphene to protect DNA from cleavage during cellular delivery†

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We have proved that functionalized nanoscale graphene oxide can protect oligonucleotides from enzymatic cleavage and efficiently deliver oligonucleotides into cells.

Genetic engineering holds great promise and opportunities for medicine and biomedical research.[1] For example, RNA interference (RNAi)[2] and antisense (AS)[3] therapies are potentially powerful candidates for clinical treatments of various ailments, including cancer and AIDS. However, these therapeutic oligonucleotides are easily degraded by cellular enzymes or digested by cellular nucleases. Some measures have been taken to protect the oligonucleotides from cleavage and deliver oligonucleotides into cells, such as adding inhibitors in DNA solutions and complexing the nucleic acid with cationic polymers[4] or lipids.[5] Recently, inorganic nanomaterials have been applied as useful molecular transporters due to their unique properties such as large surface area, embedded effect and size effect.[6,7] Some inorganic nanomaterials can protect oligonucleotides from cleavage due to their steric hindrance effect. Up to now, only a few types of nanomaterials have been used in oligonucleotides delivery, such as silica nanoparticles,[8] gold nanoparticles[9,10] and single-walled carbon nanotubes (SWCNTs).[11,12] However, the search for new carriers which can protect oligonucleotides from cleavage and which possess low toxicity, is still highly active.

Graphene, a single layer of carbon atoms in a closely packed honeycomb two-dimensional structure, is a new kind of carbon nanostructure material which was first produced in 2004.[13] It has attracted great attention because of its remarkable electronic, mechanical and thermal properties.[14] Graphene has been exploited in many applications, such as composites,[15] Li-ion batteries,[16] and electrochemical biosensors.[17,18] However, little has been done to explore the use of graphene in the biomedical field. Recently, Dai and co-workers uncovered a unique ability of functionalized graphene in the attachment and delivery of aromatic, water-insoluble drugs.[19] Most recently, we demonstrated the ability of water-soluble graphene oxide as a platform for highly sensitive and selective detection of DNA.[20] However, the application of graphene as a transporter to deliver oligonucleotides for gene detection and therapy have not been reported yet.

Herein, we first report that functionalized nanoscale graphene oxide (NGO) sheets can protect oligonucleotides from cleavage and deliver oligonucleotides into cells (Fig. 1). The model oligonucleotide used in this paper is molecular beacon (MB). MB is a hairpin-shaped DNA with a self-complementary stem that brings a terminal-labeled fluorophore and a quencher into close proximity, causing the fluorescence of the fluorophore to be quenched by energy transfer. When a MB hybridizes with its complementary target, the beacon undergoes a spontaneous conformational reorganization with the opening of the stem, leading to a fluorescence restoration.[21] MBs have widespread use in visualization of mRNA expression in living cells owing to their unique structural property.[22,23]

The MB sequence used in this paper is 5′-Dabcyl-**CGA CGG** AGA AAG GGC TGC CA**C GTC G**-Cy5-3′, the loop of which was designed to incorporate a complementary region for the survivin transcript, a target that has received significant attention due to its potential use in cancer therapeutics and diagnostics.[24]

The functionalized nanoscale graphene oxide (NGO) was synthesized according to the report of Dai and co-workers.[19,25] The sizes of NGO sheets were mostly lower than 100 nm according to atomic force microscopy (AFM) characterization (ESI†). This will increase the transfer efficiency of NGO.

We then investigated the binding of MB to NGO. In a previous paper, we have proved that single-stranded DNA can be adsorbed onto graphene oxide sheets[20] and graphene oxide can efficiently quench the fluorescence of the labeled fluorophore in single-strand DNA. In this work, we found NGO can also adsorb MB and decrease the background fluorescence of MB (Fig. 2(a) and (b)). However, in the presence of target DNA, the fluorescence intensity was significantly enhanced (Fig. 2(c)). This result indicated that the target DNA can

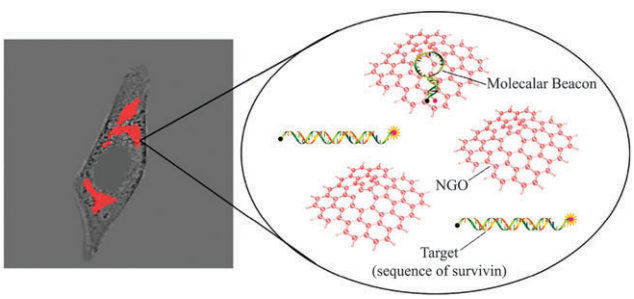

Fig. 1 Schematic representation of NGO delivery of MB into HeLa cells to detect survivin mRNA.

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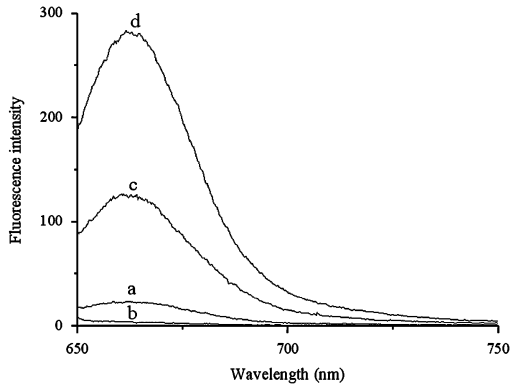

Fig. 2 Fluorescence emission spectra of MB (50 nM) at different conditions: (a) MB in Tris-HCl buffer; (b) MB + NGO; (c) MB + NGO + target DNA (300 nM); and (d) MB + target DNA (300 nM). Fluorescence measurements of (c) and (d) were performed after incubation with target DNA for 1 h.

Fig. 3 Image of gel electrophoresis of MB and MB/NGO with and without DNAse I treatment. Lane 1: MB only; lanes 2 and 3: MB treated with DNAse I for 3 and 15 min, respectively; lane 4: MB/NGO; lanes 5 and 6: MB/NGO treated with DNAse I for 3 and 15 min, respectively.

hybridize with MB on the NGO surface followed by the release of MB from NGO. The beacon undergoes a spontaneous conformational reorganization with the opening of the stem, leading to a fluorescence restoration of the fluorophore.[21] As anisotropy measurements are commonly used to probe molecular interactions, the fluorescence anisotropy of MB and the MB/NGO complex was also measured for evidence of the adsorption of MB on NGO. In the absence of NGO, the fluorescence anisotropy of free MB is 0.07, whereas that for the MB/NGO complex is 0.134, indicating that the MB is adsorbed on NGO.

Another important observation is that after non-covalent adsorption on NGO, the MB is protected from enzymatic cleavage (Fig. 3). We monitored the digestion of MB by DNase I, which can nonspecifically cleave ss-DNA and ds-DNA. As shown in Fig. 3, free MB was mostly digested after a 3 min incubation with DNase I, and after 15 min, the enzymatic hydrolysis was complete. However, there was no obvious hydrolysis of the MB in the presence of NGO after 15 min. These experiments demonstrate that MB is protected from DNase I cleavage after adsorption on the NGO. The protection of MB may be due to a steric hindrance effect that prevents DNase I from binding to the MB.[12]

We further used melting-temperature measurements to study the stability of the MB/NGO complex. At lower temperatures, the MB is in a closed state and weakly fluoresces. However, at high temperatures, the helical order of the stem gives way to a random-coil conformation, separating the fluorophore from the quencher and restoring a higher degree of fluorescence. As to MB/NGO complex, we found that the complex shows excellent thermostability even at high temperatures (ESI†). This result implied that there was exceptionally strong affinity between MB and NGO. Such tight binding can provide a stable MB/NGO complex during the cell transport.

After proving the protective property and high stability of MB/NGO complex, its ability to enter cells and detect mRNA targets was investigated. The HeLa cell, which expresses survivin transcripts, was used as a model. As a control, another MB probe containing a noncomplementary sequence was used (control MB sequence is 5′-Dabcyl-**CGA CGT** CGC GTA CAA TCT GCC **GTC G**-Cy5-3′). We hypothesized that the NGO can translocate across the cell membrane and deliver MB into cells. Upon encountering with target mRNA, MB will hybridize with the target mRNA and release from NGO, leading to a fluorescence restoration which could be detected by confocal microscopy.

As shown in Fig. 4A and B, the fluorescence of Hela cells treated with the free MB probe was almost undetected by confocal microscopy. This might be the result of MB digestion in media and inefficient self-delivery into the cells after a long incubation time.[12] In contrast, when the cells were treated with the MB/NGO complex, a high fluorescence signal was observed for most of the cells (Fig. 4C and D). For comparison, another control MB/NGO complex was tested under the same conditions, and there was a significantly lower fluorescence signal (Fig. 4E and F). These results prove that NGO can protect MB from cleavage and efficiently deliver MB into cells to detect the target mRNA.

In order to quantify the intracellular signaling of the MB/NGO, we examined the cells using flow cytometry (Fig. 5). Flow cytometry can collect the fluorescence data one-by-one for a large population of cells. The result of the flow cytometry revealed that the cells treated with the MB/NGO complex were highly fluorescent and about 2.4 times more fluorescent than the cells treated with control MB/NGO complex. The signal-to-background ratio of the MB/NGO is close to that of the oligonucleotide-modified gold nanoparticle complex.[10] The great signaling ability and low background of MB/NGO may be due to the high fluorescence quenching efficiency of NGO and the high stability of MB/NGO in intracellular circumstances.

Finally, the cytotoxicity of the NGO was measured. The relative cell viability data was close to 100% even when the NGO concentration reached 100 mg L^{-1} (ESI†). This result suggests that the NGO is of low cytotoxicity.

In summery, We have proved that nanoscale graphene oxide can protect oligonucleotides from enzymatic cleavage and

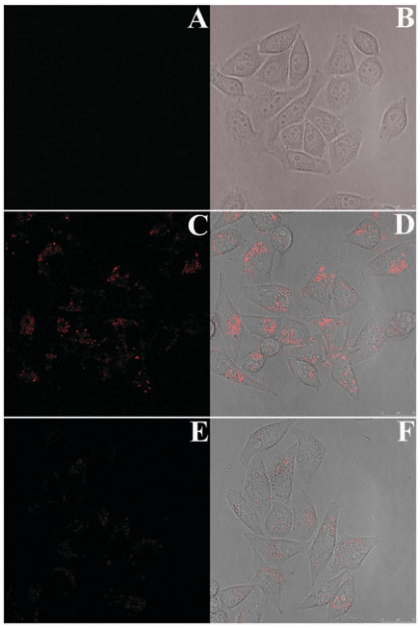

Fig. 4 Intracellular testing of free MB or MB/NGO complex. Confocal fluorescence microscopy of HeLa cells treated with free MB (A and B), survivin MB/NGO (C and D) and control MB/NGO (E and F): Cy5 fluorescence field (left) and bright field Cy5 fluorescence overlay (right) are shown.

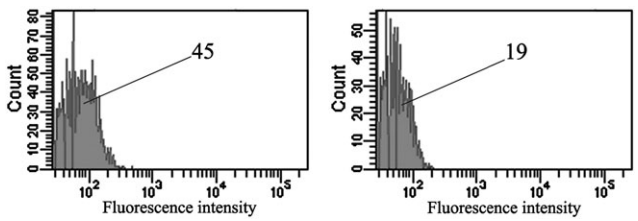

Fig. 5 Flow cytometry data. Left: Cells treated with MB/NGO. Right: Cells treated with control MB/NGO. The bold numbers to the right of the histogram are the total mean fluorescence of the cell populations.

efficiently deliver oligonucleotides into cells. The use of NGO in oligonucleotides delivery system has several advantages. First, the strong adsorption of the oligonucleotides on NGO can protect oligonucleotides from cleavage in intracellular environments. Moreover, the high stability of MB/NGO complex can prevent the release of MB during the cell transporting. Second, NGO can improve the transfection efficiency of oligonucleotides as a carrier. Third, NGO is a less cytotoxic transport agent compared with SWCNTs (SWCNTs showed obvious cytoxicity when their concentration was 20 mg mL^{-1}). Fourth, the low cost and large production scale of the NGO makes it a promising biomaterial. For these advantages, NGO may have potential use in genetic engineering, such as cloning and gene therapy. Currently, the application of NGO in delivery of therapeutic oligonucleotides such as antisense DNA and siRNA is being conducted in our laboratory and will be communicated in due course.

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