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Application of Carbon Nanomaterials in Gene Delivery for Endogenous RNA Interference *In Vitro* and *In Vivo*

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Abstract: Knocking down expression by small interfering RNA (siRNA) has shown high affinity, specificity and potency in silencing target gene sites. For effective endogenous RNA interference (RNAi), proper siRNA delivery vehicles are essential, to take the siRNA inside cells and protect them during the circulation. Carbon nanomaterials (CNMs) have been successfully applied in biomedicine and biosensor based on their ultra-high surface functionalization and nucleic acid molecular loading capacity. Recently, CNMs have drawn considerable research interest and expectation as potential non-viral vectors for siRNA delivery. Here we reviewed the recent application of CNMs in gene delivery for RNAi, mainly about fullerenes, carbon nanotubes (CNTs) and graphene.

Keywords: Carbon nanotube, carbon nanomaterial, fullerene, gene delivery, graphene, RNA interference, small interfering RNA.

1. INTRODUCTION

Along with the fast development of functional genomics, gene therapy has drawn more and more research attention, as a hopeful treatment of various undruggable diseases including cancer, genetic diseases and resistant viral infections. Based on the direct delivery of the nucleic acids into cells, many expressions are regulated/inhibited at transcriptional [1] or translational level [2-4]. Among all the gene therapy technologies, knocking down expression by small interfering RNA (siRNA) has shown excellent advantages like high affinity, specificity and potency to silence target gene sites through endogenous RNA interference (RNAi) pathway within mammalian cells [5].

However, the efficacy of siRNA silencing is still facing challenges of limited cell membrane crossing and the protease degradation, thus proper siRNA delivery vehicles are essential, to take the siRNA inside cells and protect them during the circulation. Several delivery strategies have been developed based on viral or non-viral strategies. Viral vectors have ability to take siRNA into cells with high gene transfer efficiency, like viruses injecting their gene inside the host cells. However they can't avoid the risk of high immune response. Alternatively, the non-viral approaches take polymers, dendrimers [6, 7] or nano materials like silica nanoparticles [8], gluco-nanoparticles [7] or carbon nanomaterials as siRNA delivery non-viral vectors [9, 10].

Carbon nanomaterials (CNMs) including fullerenes, CNTs, graphene, *et al.* have been widely applied in biomedicine, due to their good biocompatibility [11], abundant surface groups and high delivery efficiency, and also, CNMs are capable of special recognition and combination of nucleic acid molecules, which has been successfully proved in so many recent reported biosensors. These excellent advantages make CNMs great potential vehicles for siRNA delivery in target cells. After more than ten years development, CNMs have gained outstanding advances for gene delivery applications both *in vitro* and *in vivo*.

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2. PROPERTIES OF CARBON NANOMATERIALS

The properties of CNMs are mainly determined by the carbon's structural conformation and its hybridization state. Orbital hybridization theory allows carbon atom to hybridize into sp, sp^2, or sp^3 configuration based on bonding relationships, leading to the diversity of CNMs, including zero-dimensional (0D) fullerenes, one-dimensional (1D) CNTs, two-dimensional (2D) graphene, three-dimensional (3D) nanodiamond and so on [12] (Fig. **1**). Each of these classic CNMs has special mechanical, thermal, electrical [13] and optical properties [11], facilitating their more and more outspreading bioapplications.

Fullerenes with unique spherical structures have high hydrophobicity, photosensitivity [17], electrochemical redox property [18] and high chemical reactivity [14]. Surface functionalization of fullerenes has received much attention, to develop water-soluble fullerene derivatives by introducing proper hydrophilic residues, to decrease the toxicity for biological application, and to offer better combination with drugs and biomaterials. In order to develop highly biocompatible and water-soluble fullerenes, different residues including amino, carboxyl [19], polyethylene glycol (PEG) [20], polyethylenimine (PEI) and hydroxyl residues [21] are introduced. In 2005, A complex of fullerene and paclitaxel [22] via an ester bond was developed to realize slow release of the drug, leading to significant anti-cancer activity *in vitro* [23]. Soon afterwards, other different drugs including doxorubicin (DOX) [24, 25], docetaxel (DTX) [26] were successfully combined to fullerene spheres and delivered with well-preserved activity. Recently, a novel fullerene drug delivery system was applied in photodynamic therapy (PDT) [27] with assistant of magnetic targeting: Shi *et al.* [26] introduced iron oxide nanoparticles and PEG onto the surface of fullerene-C60, for the delivery of hematoporphyrin monomethyl ether (HMME). Their results showed effective cancer cell killing both *in vitro* and *in vivo*.

CNTs have been intensively researched and widely applied since their discovery in 1991 by Sumio Iijima [28]. CNTs can be divided into single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) depending on the number of concentric graphitic layers. The diameter is typically 1 nm for SWCNTs and 1 to 100 nm for MWCNTs, respectively. The length of CNTs can reach a few centimeters. Thus, the aspect ratio (length

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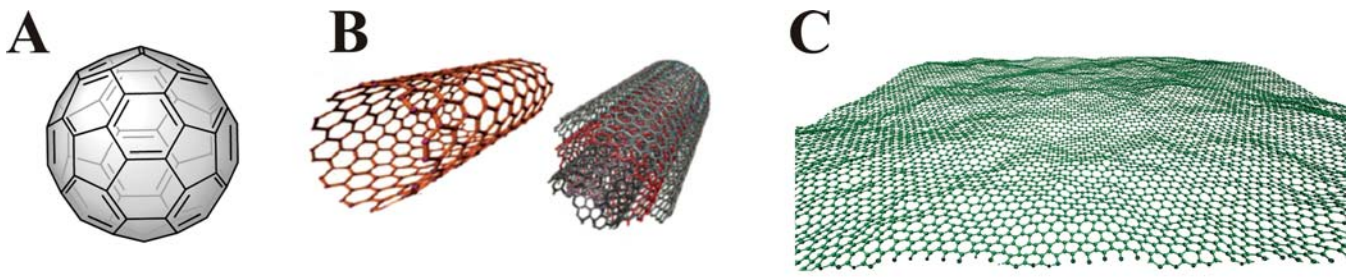

Fig. (1). Classic carbon nanomaterials: **A**) buckminsterfullerene C 60 (fullerene-C60). (Reprinted with permission from [14]). **B**) single-walled carbon nanotube. (Reprinted with permission from [15]). **C**) graphene. (Reprinted with permission from [16]). Copyright (2013) American Chemical Society.

vs diameter) of CNTs can be larger than ~10^6 [29]. Moreover, the surface areas of CNTs are ultra-high up to 2600 m^2/g [30]. CNTs have intrinsic mechanical properties of high mechanical strength, light weight and flexibility, and also possess superior thermal conductivity and stability, high electrical conductivity [31]. Because of the enhanced permeability and retention (EPR) effect, CNMs loaded with drugs can accumulate in tumor tissues, making them ideal drug delivering materials. Recently, successful delivery of anticancer drugs such as DOX [32-37], paclitaxel (PTX) [38-40], docetaxel (DTX) [41] and camptothecin (CPT) [42, 43] using CNMs as carriers has already been demonstrated by tremendous amount of reports. Das *et al.* [36] functionalized MWCNTs with a PEG-linked 17 β-Estradiol (E_2) for DOX delivery to breast cancer cells. The E_2-PEG-MWCNTs facilitated the nuclear targeting and enhanced the anticancer response both *in vitro* and *in vivo*. Dai's group [38] linked paclitaxel (PTX) to the PEG on the surface of SWNTs to form a water-soluble SWNT-PTX complex and successfully achieved higher efficacy in suppressing tumor growth *in vivo*.

Graphene, a single-atom thick and two-dimensional (2D) carbon nanomaterial was firstly discovered in 2004 [44]. It consists of a layer with a conjugated structure of six-atom rings, which offers an excellent capability of immobilizing a large number of substances. Moreover, similar to CNTs, it also has a large theoretical specific surface area, high charge carrier mobility as well as excellent thermal conductivity. In addition, it exhibits unique properties such as quantum hall effect at room temperature, significant optical transmittance along with ambipolar electric field effect [45, 46]. Multi-functionalization of graphene improved the specificity of drug delivery, leading to more accurate cancer cell targeting without toxicity to other normal cells [47]. Yang *et al.* [48] constructed a novel molecular-recognition system based on GO and folic acid (FA)-modified β-cyclodextrin (FA-CD), which was capable of recognizing the folic acid receptors in cancer cells, and thus specifically delivered DOX with very low toxicity to normal cells. Kavith *et al.* [42] modified the negatively charged GO with pH-sensitive poly(2-(diethylamino) ethylmethacrylate) (PDEA), and realized the pH controlled CPT release and high-potency cancer cell killing *in vitro*. In a following work of the same group, they designed a novel temperature-responsive drug carrier [43] by covalently conjugating a temperature sensitive poly N-vinyl caprolactam (PVCL) to GO, and obtained superior cancer cell killing ability.

The excellent performance of CNMs in biomedicine has showed their great potential as nanocarriers, taking cargos into cells with excellent biocompatibility and specificity. It is worth to note that, in most of the works mentioned above, low toxicity of CNMs has been demonstrated based on well optimized functionalization. So far, CNMs have already been widely used in biomedicine applications both *in vitro* and *in vivo,* targeting cells including L929 cells [49], HeLa cells [50], human fibroblasts, A549 human lung cancer cells, human hepatoma HepG2 cells [51] *et al.*, with no significant toxic effect reported.

3. THE COMBINATION OF CARBON NANOMATERIALS AND NUCLEIC ACID AND ITS APPLICATION IN BIOSENSING

Research of CNMs in nucleic acid related biological applications revealed that, many of them have special interaction with DNA/RNA molecules. With the fast development of nanotechnology, the complexes of CNMs and nucleic acid have been widely explored in nanomaterial functionalization and biosensing.

Since the interesting interaction between CNTs and nucleic acid was discovered, it has attracted a lot of research interest. It is clearly demonstrated in many reported cases that, the flexible single-stranded nucleic acid molecules could effectively interact with CNTs surface through a parallel orientation. Meanwhile, the nucleic acid wrapping around CNTs are able to enhance the biocompatibility by forming tight helices [52-54], and thus to construct many different biosensor strategies. Many researchers even took DNA as an ideal material for carbon nanomaterials modification. In their strategies, DNA was not only a genetic code but also an excellent biological nanomaterial with no toxicity, which have been used to create various functional structures and devices through the sequence-specific pairing interactions [55, 56]. Zheng *et al.* [57] firstly reported a method to disperse SWCNTs in water by DNA wrapping. In 2009, Qu's group [58] successfully distinguished the single- and multi-walled CNTs with an *i-motif* DNA probe. Li *et al.* [59] developed a method to graft highly hybridizable DNA sequences onto DNA-wrapped SWNTs and realized DNA-hybridization-driven self-assembly of SWNTs. There have been a significant number of publications reporting DNA biosensors based on CNT [60-63]. Many of the reported sensors achieved higher sensitivities or faster electron transfer kinetics based on CNT's advantages including enhanced electronic property, large edge plane/basal plane ratio, and rapid electrode kinetics.

Graphene with special physical and chemical properties has also been paid much attention for nucleic acid combination and its application in biosensing. Interestingly, nucleotide bases in single-stranded DNA (ssDNA) can bind strongly to the graphene surface by π-π stacking, while it is much weaker for double-stranded DNA (dsDNA) [64-68]. More importantly, graphene owns largely dislocated p-electrons, leading to efficient fluorescence quenching by energy transfer with molecules nearby [69], which make it an efficient fluorescence quencher and thus useful in widely explored optical-based detection of biomolecules. He *et al.* [65] utilized the high fluorescence quenching ability of GO and its different affinity toward ss- and ds-DNA, and realized a novel homogeneous sensor for multiplex, sequence-specific DNA detection. Wen *et al.* [64] also utilized the effective fluorescence-quenching ability of graphene, to develop a simple mix-and-detect fluorescence sensor for Ag(I) ions by using a dye-labeled silver-specific oligonucleotide. Moreover, owing to the ultra-high surface area and excellent electron mobility of graphene, they have been used for surface modifi-

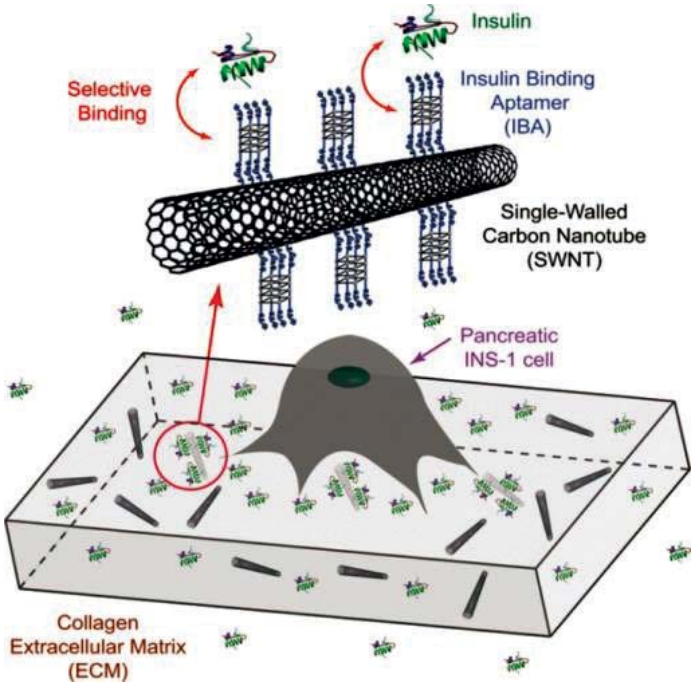

Fig. (2). Schematic illustration of optical nanosensor based on CNTs, to measure insulin in pancreatic INS-1 cells *in situ* and in real time. Reprinted with permission from [77]. Copyright (2011) American Chemical Society.

cation for electrochemical biosensing of various biomolecules, including proteins and microRNAs with high sensitivities [70-73].

Enzyme degradation is known to be an important challenge for nucleic acid probe based biosensors. In recent reports, CNMs were proved to be capable of protecting DNA probes, plasmid DNA (pDNA), aptamers and siRNA from enzymatic cleavage [74-76], thus CNMs were thought to be capable of propelling the development of more robust DNA biosensors facing biological samples. In 2011, Cha *et al.* [77] developed an optical biosensor based on a complex of DNA aptamer and SWNTs, and successfully performed sensitive *in situ*, real-time analysis of insulin which is an important cell-signaling molecule (Fig. **2**). Wang *et al.* [74] designed an aptamer-carboxy fluorescein (FAM)/GO nanosheet (GO-nS) nanocomplex and investigated its ability of molecular probing in living cells. The results suggested that GO nanosheet possessed dramatic delivery, protection, and sensing capabilities in living cells, indicating the great potential of CNMs as a potential robust candidate for biosensing and gene delivering.

4. APPLICATION OF CNMs IN GENE THERAPY

4.1. Functionalized Fullerenes

To date, many different biocompatible water-soluble functionalized fullerene derivatives have been developed by introducing amino, carboxyl, and hydroxyl [21], towards potential application in nuclear acid delivery [78, 79]. In order to overcome the low efficiency and the acute immune response during conventional lipid-based gene delivery, Nakamura's group pioneered a novel field of fullerene functionalization towards reliable non-viral gene delivery vectors since 2000 [80]. They firstly reported a cationic tetraaminofullerene (Compound **1** in Fig. **3**) with two diamine sidechains which are capable of combining with the phosphate backbones of DNA strands. Compound **1** demonstrated its high transfection efficiency by successfully delivery a GFP plasmid into COS-1 cells, probably benefiting from two main advantages: 1) The fullerene derivate is very small and could condense with DNA very tightly to form a spherical molecule, which is highly penetrable to the cell membrane, 2) Compound **1** has hydrophobic nature to form stable complex with DNA and protect it from degradation. In their following work [81], Compound **1** even showed higher transfection efficiency and stronger nuclease resistance than commercial lipofectin.

As a milestone of *in vivo* gene delivery using fullerene derivatives for potential clinical application, Nakamura's group developed a water-soluble tetra(piperazino) fullerene epoxide (TPFE) molecule with four positive charges [80]. They for the first time utilized TPFE to deliver enhanced green fluorescent protein (EGFP) gene efficiently into the liver and spleen without any acute toxicity, while lipofectin induced liver and kidney injury. Finally, insulin-2 gene was taken into female C57/BL6 mice by TPFE, leading to an increased plasma insulin level, which demonstrated the fullerene derivatives as potential nanocarriers for gene delivery *in vivo*.

Initially, TPFE was designed to form a complex with dsDNA with a particle size about 50-100 nm, which is proper for endocytosis. Most recently, the complex of TPFE-siRNA was found to further agglutinate with plasma proteins in the bloodstream to form micrometer-sized particles which would accumulate in the narrow lung capillaries, and release the siRNA into lung cells. A lung-specific delivery of siRNA strategy was thus developed using the interestingly controllable dimension change of TPFE-siRNA complexes [83], showing 62% knockdown in the lung, without any significant knockdown in other tissues. Most importantly, the TPFE was quickly cleared from the lung about 12 h after the silencing. Finally, TLR4-targeted siRNA (siTLR4) was delivered with assistance of TPFE system, and suppressed the neutrophil accumulation in the lung.

4.2. Carbon Nanotubes

CNTs have already been extensively explored in biological applications like biomedicine, bioimaging and biosensing. Recently they attracted considerable research attention as potential nanocarriers to deliver siRNA directly into target cells. CNTs offer various possibilities of functionalization to facilitate the solubility, to decrease the toxicity and to improve the combination of DNA/siRNA. Non-covalent functionalization with amine [84] or cationic polymers [85] *et al.* and covalent functionalization with polymers like PEI [86] have been developed for siRNA delivery. More impor-

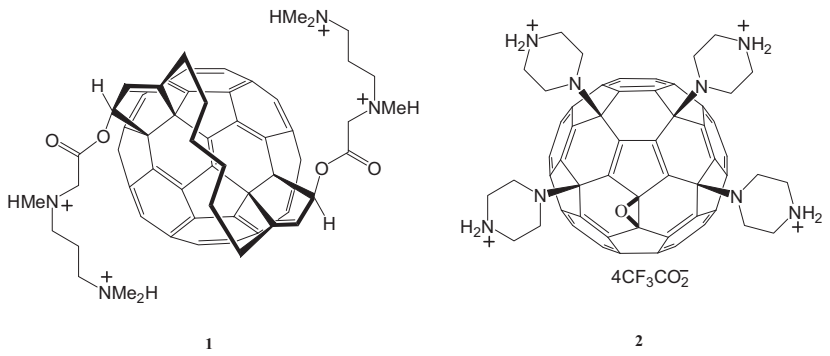

Fig. (3). Molecular structures of **A**) a tetraaminofullerene (compound **1**). Reprinted with permission from [80].Copy right (2000) WILEY-VCH, **B**) TPFE (compound **2**). Reprinted with permission from [82] Copyright (2010) National Academy of Sciences.

tantly, CNT conjugates exhibit negligible cytotoxicity [87] during siRNA delivery even lower than conventional Lipofectamine [88], and CNTs provide a protection effect against the enzyme degration [89].

The first research of DNA delivery using CNTs as gene nanocarriers was reported in 2004 [90], which utilized ammonium-functionalized CNTs to deliver plasmid DNAs into mammalian cells. In their work, 10 times higher expression level than pDNA alone was achieved. The high efficient delivery of pDNA [91] indicated great potential of CNTs as novel nanocarriers for gene therapy. A following work strongly supported the feasibility of CNT transfection by delivering a fluorescently labeled non-coding siRNA into mammalian cells, avoiding the protease degradation [92]. Then, RNAi was performed by CNT *in vitro*. In 2005, Dai's group [93] functionalized SWNTs by the non-covalent adsorption of phospholipid molecules with poly(ethylene glycol) chains and amine terminals (PL-PEG-NH_2). The PEG molecular offered stable dispersibility in solution and the cleavable disulfide bonds were used to conjugate with siRNA, providing highly controllable release of siRNA. In 2007, the same group [87, 94] developed a PL-PEG-NH_2 carrier system for the delivery of siRNA into T cells and primary cells, and successfully silenced the HIV-specific cell-surface receptors CD4 and coreceptor CXCR4/CCR5 at a 90% silencing efficiency. Many other covalently modified CNTs [92] were applied for siRNA delivery and gene knockdown *in vitro* [95]. For example, PEI-CNT was used to deliver a siRNA to silence luciferase gene expression [96]. Hexamethylenediamine (HMDA) and poly(diallyldimethylammonium) chloride (PDDA) functionalized SWNT was used to carry a siRNA into cardiomyocytes, leading to a 75% silencing of ERK1 and ERK2 gene [85]. SWNT functionalized with -CONH-$(CH_2)_6$-$NH_3^+Cl^-$ was utilized to deliver a telomerase reverse transcriptase (TERT) siRNA into murine tumor cell, leading to a suppressed tumor growth [97]. Ladeira *et al.* developed a highly efficient siRNA delivery system by coiling siRNA into carboxyl-functionalized SWNT. Their results showed 96% of InsP3RII expression was silenced in cardiomyocytes without any nonspecific gene silencing or toxicity [96].

Encouragingly, several works have already realized *in vivo* application of CNT delivery of siRNA towards efficient cancer therapy. Podesta *et al.* [84] reported the silencing effect of MWCNTs-siRNA conjugates in human lung cancer cell line H12991, which significantly suppressed the tumor volume and prolonged the survival of cancer cell bearing animals. Bartholomeusz *et al.* [98] prepared a complex of SWNT-siRNA (targeting hypoxia-inducible factor 1 alpha (HIF-1α)) simply by sonication. Surprisingly, the non-covalently adsorbed siRNA showed high efficient both for SWNT dispersing and gene silencing *in vivo*. Their significant suppression of tumor cells in mice implied a great value of SWNT-siRNA as therapeutic agents.

4.3. Graphene

Graphene has properties of facile synthesis, easy surface functionalization, high specific surface area and good biocompatibility. Unmodified graphene is not colloidally stable, but GO and polymer-modified GO can be stably dispersed in aqueous solution, and have been widely used in gene delivery.

Liu's group [99] developed GO-PEI complexes with different PEI molecular weight, and applied them to deliver EGFP gene into HeLa cells, their results showed low cell toxicity and high EGFP expression. Other functionalization strategies of GO [100, 101] have also been utilized for efficient pDNA transfection. In 2011, Bao *et al.* [102] developed a chitosan-GO complex via amidation process, and studied its application as drug and pDNA nanocarrier. In 2012, Ren *et al.* [103] introduced a nuclear localized signals (NLS) peptide-PKKKRKV (PV7) into the GO-PEI delivery system, and achieved higher transfection efficiency and lower cell toxicity than GO-PEI alone. Recently, Yao's group [104] synthesized a graphene-oleate-polyamidoamine (PAMAM) dendrimer complex by the nucleic acid adsorption and covalent linkage of PAMAM dendrimer with good dispersity and stability in aqueous solutions. When utilized as a novel non-viral vector for pEGFP transfection in HeLa and MG-63 cells, it was demonstrated to have better biocompatibility and gene transfection capacity.

Some of graphene related gene vectors have been successfully applied in gene knockdown research [105]. In 2011, almost at the same time with Liu' group [99], Zhang *et al.* [106] reported a similar GO-PEI complex and firstly applied it to siRNA delivery, resulting in a higher knockdown efficiency. Before long, Liu's group [107] improved their own GO functionalization and developed a dual-polymer-functionalized nano-GO by covalent conjugation of PEG and PEI (nGO-PEG-PEI). For the first time, they utilized a low power NIR laser irradiation to enhance the transfection of the nanocarrier, and achieved remarkably increased transfection efficiency. Their results showed a great potential of GO based nanocarriers in photocontrollable gene therapy [108].

5. CONCLUSION AND PERSPECTIVES

Gene therapy offers advantages over traditional pharmaceutical drugs to treat various genetic and acquired diseases, and great hope has been placed on RNAi and siRNA. However, the development of siRNA delivery is still hindered by the incapability of crossing cellular membranes and its instability in cytoplasm. CNMs have highly developed surface modification and nucleic acid molecular loading capacity which have been successfully applied in biomedicine and biosensing, and thus draw considerable research interest and expectation as potential non-viral vectors for siRNA delivery.

Significant advantages of many classic CNMs have been demonstrated in gene delivery: Fullerenes have excellent sphere configuration and abundant sidechains. Through high controllable self-

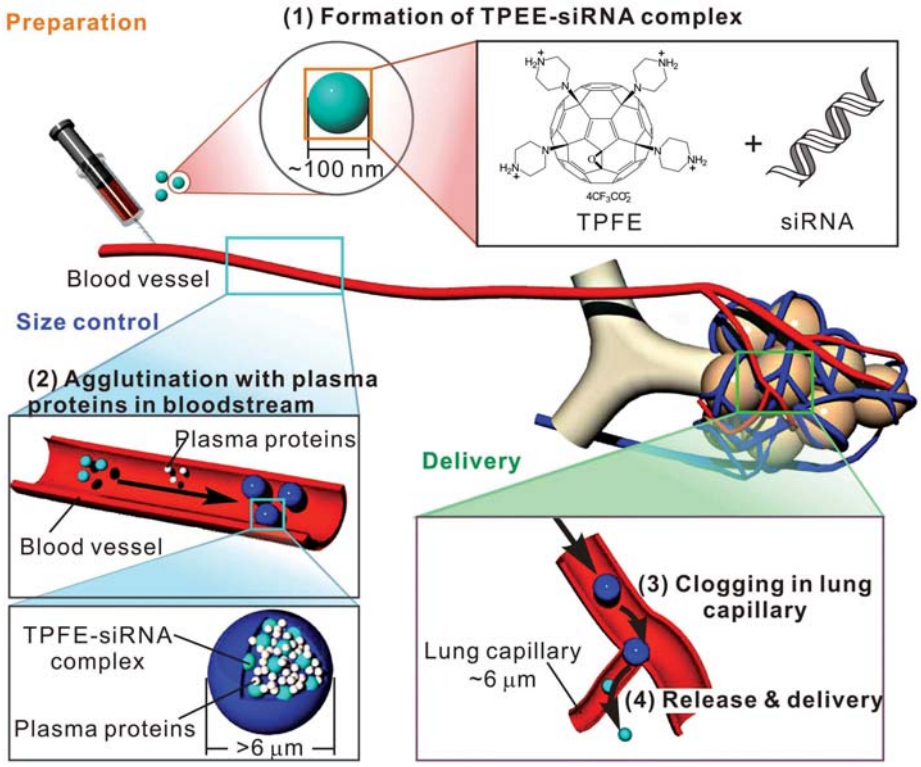

Fig. (4). The rationale for lung-specific delivery of siRNA mediated by TPFE. Reprinted with permission from [83]. Copyright (2014) Nature Publishing Group.

assembling, functionalized fullerene was capable of agglutinating and accumulating in lung, and is then cleared after the siRNA release, which showed its outstanding potential for clinical applications. CNTs have unique 2D structure with high surface-to-volume ratio and deeply developed surface functionalization. CNTs with disulfide bonds realized controlled release of thiol-modified siRNAs, leading to a highly advanced specificity for gene therapy. More importantly, CNTs have been reported to show nonendocytotic pathways to enter cell membrane, which also could help to improve the cellular delivery. GO has been demonstrate to be an advanced carrier for DNA, siRNA, anticancer drug and other nanoparticles, endowing GO wider applications in gene therapy and biosensing. Moreover, graphene can be used as photothermal agent for *in vivo* tumor, due to its high NIR absorbance.

Since so many CNMs-based gene delivery strategies have realized successful gene silencing *in vivo*, we strongly believe that CNMs have great potential for endogenous RNA interference towards future clinical therapies for cancer, genetic diseases and viral infections. However, for more efficient clinical application, more research energy is still needed to focus on two issues: 1) Better multi-functionalization of CNMs is essential to not only improve the loading of siRNA, but also to realize more specific cell targeting. In addition, more efficient surface modification of CNMs is needed to decrease the toxicity. Based on the reported successful modifications of CNMs, advanced biocompatibility can be achieved by combination of polymers like PEG, PEI and hydroxyl residues. We believe the novel polymers and biological materials like DNA origami can greatly improve the clinical application of CNMs. 2) More *in vivo* research is needed to clearly examine the biological toxicity and gene silencing efficiency. Especially, no *in vivo* siRNA delivery by GO was reported so far.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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