

Chapter 2

Carbon Nanomaterials for Drug Delivery

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2.1 Introduction

Carbon nanomaterials with unique nanostructure (CMNS) are the most studied materials in nanotechnology, despite some conflicting results about their safety profiles [1, 2]. Although their structures are simple, carbon nanomaterials provide exceptional physical and chemical properties including high electrical and thermal conductivities, unique optical properties, and extreme chemical stability. The field concerning nanostructured carbon materials is the most rapidly developing research field in the past decades due to their potential applications in all fields that might affect our lives [3]. One of the greatest values of carbon nanomaterials will be in the development of new and effective medical treatments, particularly for the therapy of various diseases [4]. The predominately nanostructured carbon materials are graphene (GN, 2-D, sp^2 carbon), carbon nanotubes (CNTs, 1-D, sp^2 carbon), and carbon nanohorns (CNHs, 1-D, sp^2 carbon) and nanodiamonds (NDs, 0-D, sp^3 carbon). GN is a two-dimensional structure, while the CNT is regarded as the GN rolling up into the seamless tubular hollow cylinder in nanoneedle shape. In the case of CNHs, the single GN sheet rolled into a cone. The structures of different materials are shown in Fig. 2.1.

Despite the four types of carbon materials are different in shapes, they are all nanostructured materials with many intriguing features that make them attractive drug delivery carriers [5]. Firstly, the nanostructured carbon materials can disperse in aqueous solutions. Besides from direct translocation through cellular membranes, the CMNS also present the ability to enter cells via energy-dependent endocytic pathways [6]. Secondly, they can enter and accumulate in tumor tissues via the

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M. Zhang et al. (eds.), *Carbon Nanomaterials for Biomedical Applications*,
Springer Series in Biomaterials Science and Engineering 5,
DOI 10.1007/978-3-319-22861-7_2

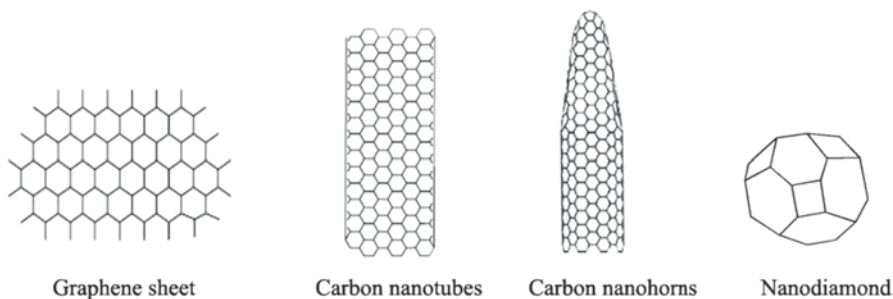


Fig. 2.1 Structures of graphene (GN), carbon nanotubes (CNTs), carbon nanohorns (CNHs), and nanodiamond (ND)

enhanced permeability and retention (EPR) effect [7]. The EPR effect enables the carbon nanomaterials to transport therapeutic agents preferentially to tumor sites. It makes the targeted delivery of antitumor drugs more effectively. Thirdly, the carbon nanomaterials exhibit extraordinary ability for drug loading onto the surface via both covalent and non-covalent interactions owing to their high aspect ratios and surface areas. The drugs can also be packed into the interior cores of CNTs and CNHs [8]. To further augment the efficacy of CMNS-based drug delivery system (DDS), various targeting molecules (such as folic acid (FA) and antibodies) can be incorporated into the drug-loaded CMNS. In addition, imaging tags like radioactive nuclides and fluorescence probes linking with CMNS help to observe their intracellular trafficking and biodistribution in vitro and in vivo easily and noninvasively.

Employing the above-mentioned advantages of the three carbon nanomaterials, many progresses have been achieved for the purpose of drug delivery by using GN, CNTs, and CNHs as carriers. This chapter mainly focuses on the different methods of preparation of drug-loaded carbon nanomaterials including their modification, characterization, and applications as drug delivery carriers.

2.2 GN for Drug Delivery

2.2.1 GN-Based Inorganic Nanohybrids for Drug Delivery

The GN is hydrophobic, but it still can be dispersed in aqueous solutions by surfactants or hydrophilic polymers. Although it can adsorb some drugs via π - π stacking, cation- π , and van der Waals interactions, the composites are not easy to disperse in blood. In fact, the application of the GN in drug delivery mainly focused on graphene oxide (GO), which can be prepared by oxidation of graphite and followed by exfoliating in the water via Hummers' method [9]. GO is a layered stack of puckered sheets with AB stacking, which completely exfoliates upon the addition of mechanical energy. The exfoliation of multiple layers of graphite is due to the

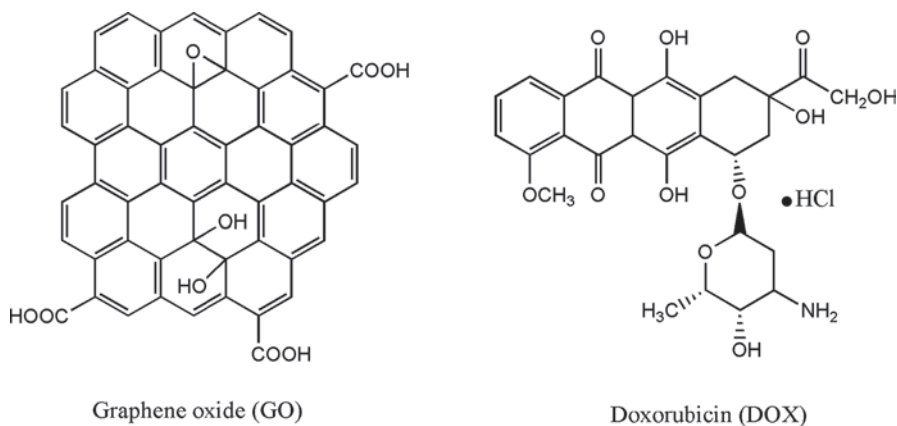


Fig. 2.2 Structures of graphene oxide (GO) and doxorubicin (DOX)

strength of interactions between water and the oxygen-containing (epoxide, hydroxyl, and carboxyl) groups introduced into the basal plane during oxidation. The hydrophilicity introduces water to intercalate between the sheets and disperse them as individuals. The functionalized GO with hydroxyl and carboxylate groups has the ability to covalently bond with various drugs in mild conditions, and it can also adsorb the drugs via non-covalent interactions such as π - π , hydrogen bonding, and hydrophobic force. Compared with covalent modification, non-covalent ones are considered to have less impact on the conjugated structure, and mechanical and electrical properties of GN. Doxorubicin (DOX) and camptothecin (CPT) are the most studied drugs loaded on GO.

The GO-DOX hydrochloride nanohybrid was easily achieved by simply mixing the two compounds (Fig. 2.2) [10]. The loading of DOX onto GO was based on π - π and hydrophobic interactions. An efficient loading of DOX on GO was 2.35 mg/mg. The release of DOX from GO-DOX was strongly pH dependent. At the solution pH of 5, the DOX release rate gradually declined after 5 h and about only 11 % of the total bound DOX was released in 30 h. The release behaviors at basic and acidic conditions indicated that the total releasing amount of DOX within 30 h for these cases was much higher than that at neutral conditions. About 25 and 71 % of the total bound DOX was released at pH of 10 and 2, respectively. It may be due to the partial dissociation of hydrogen bonding that the DOX gets soluble in acidic solutions.

The GO functionalized with Fe₃O₄ or SiO₂ nanoparticles can also be used as drug carriers. They formed multifunctionalized inorganic nanohybrids. The GO complexes with Fe₃O₄ nanoparticles produced superparamagnetic hybrid nanoparticles (GO-Fe₃O₄). After introducing GO to Fe₃O₄, the sedimentation of Fe₃O₄ magnetic nanoparticles was reduced after they were deposited onto GO [11]. About 18.6 wt% of Fe₃O₄ deposited on GO. The loading capacity of DOX on GO-Fe₃O₄ hybrid was 1.08 mg mg⁻¹, which is higher than liposomes (8.9 wt%) [12], for which the loading capacity is always below 1 mg mg⁻¹. After congregating in acidic solution, both the

GO-Fe₃O₄ hybrids before and after loading with the DOX can be moved regularly by the force of an external magnetic field.

Although the GO-Fe₃O₄ hybrid can target the cells by external magnetic field, it tends to aggregate in acidic solution and serums. Thus, attached small signal molecules would improve its solubility, stability, and target tumor more effectively. They were attached with different target molecules such as FA. A dual-targeting drug delivery and pH-sensitive drug release GO system was developed by linking Fe₃O₄ with FA (Fig. 2.3) [13]. DOX as an antitumor drug was loaded onto the surface of the multifunctionalized GO via a simple mixture and adsorbed by π - π stacking and hydrophobic interactions. The maximum capacity of this nanocomposite is 0.387 mg mg⁻¹ (38.7%). Many targeted tumor cells had overexpress folate receptors. The DOX-loaded multifunctionalized GO can be quickly and effectively delivered into SK3 cells (a human breast cancer cell) and significantly inhibited its growth. The HeLa cell lines is a type of human cervical carcinoma cell, which is

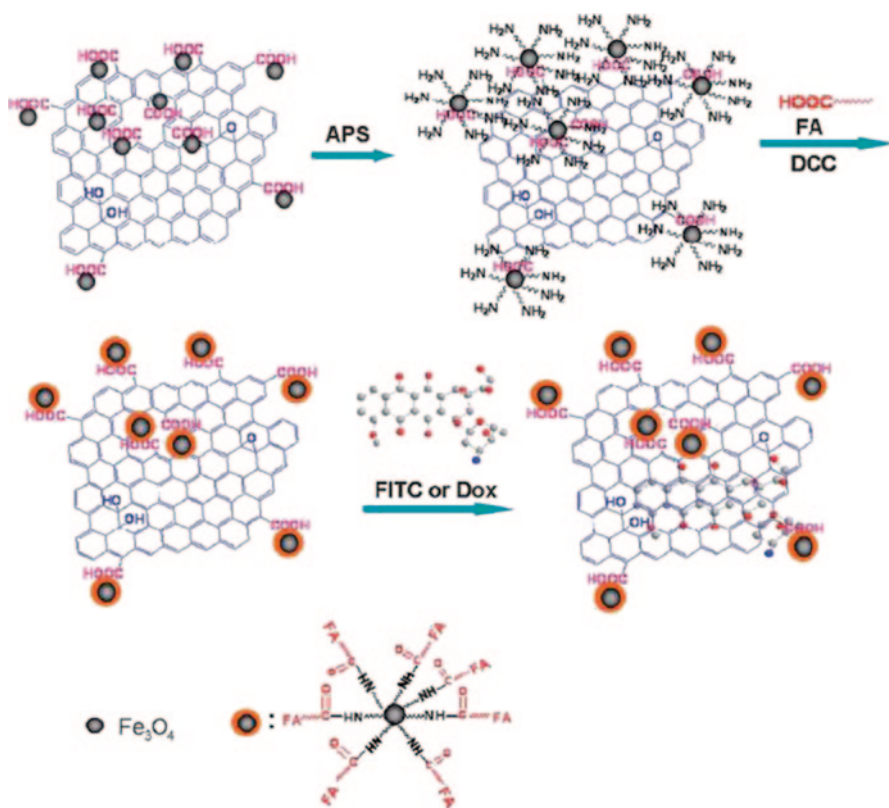


Fig. 2.3 The preparation of the multifunctionalized GO-based anticancer drug carrier with dual-targeting function and pH sensitivity. *APS* 3-aminopropyl triethoxysilane, *DOX* doxorubicin hydrochloride, *DCC* *N,N'*-dicyclohexylcarbodiimide, *NHS* *N*-hydroxysuccinimide, *FITC* fluorescein isothiocyanate

widely used as a model for folate receptor-positive cells. The cytotoxicity of DOX-loaded GO-Fe₃O₄ nanohybrid was much higher than the pure GO-Fe₃O₄ nanohybrid without DOX, but lower than that of GO-Fe₃O₄-FA-DOX under the same drug concentration in HeLa cell. The release of DOX from the nanohybrid at neutral and basic conditions was very slow. Only about 7.5 and 11 % of the total bound DOX was released for 80 h under neutral conditions (pH 7) and basic conditions (pH 9), respectively. However, in acidic conditions, DOX was released very quickly in the early stage, but the release rate gradually declined after 5 h and about 24 % of the total bound DOX was released from the nanohybrid in the first 80 h. It was due to the enhancement of the solubility of DOX and the protonated amine groups to release more loaded drugs.

In order to observe the target ability of GO, it was modified with CdTe-Fe₃O₄/SiO₂ microspheres [14]. The CdTe quantum dots (QDs) made the possibility of visualization of particle distributions in cells. Firstly, the amino functionalized CdTe-Fe₃O₄/SiO₂ nanoparticles were prepared by reverse microemulsion method [15]. As a consequence, the CdTe-Fe₃O₄/SiO₂ particles were surrounded by -NH₂ groups, and they can react with the carboxylate groups on GO. The formed GO-based nanocomposite was magnetic fluorescent. Then the DOX was attached to GO nanohybrid by π - π stacking and hydrophobic interaction. The capacity of the nanocomposite increased with the increase of the initial concentration of DOX. The maximum DOX loading capacity was 919 mg g⁻¹ (or 91.9 % in weight); however, it was lower than that of the original GO. Such a value was still higher than that of some common drug carriers, such as liposomes, for which the loading capacity was usually below 10 % [16]. The reasons affecting the maximum capacity were (i) the active surface areas on the GO-based magnetic fluorescent hybrids and (ii) the ratio of GO in the hybrids in weight. The DOX release was pH dependent. Under neutral and basic conditions, the DOX was released slowly, while low pH value was beneficial to more release amount and quicker release rate. At pH 7.4 and 8.5, after 6 days, about 49 and 46 % of DOX were released, respectively. In the solution of pH value of 3, 80 % of loading DOX was released in 1 day. There was no obvious cytotoxicity (>80 % cell viability) observed for the GO-based nanocomposite at a higher concentration of 10 mg L⁻¹ for HepG2 cells (liver hepatocellular cells). An obvious decrease in cell viability occurred when the cells were incubated with DOX loading hybrids for 24 h even at a concentration as low as 1 mg L⁻¹. The GO-based composite entered the tumor cells through the endocytosis mechanism, which resulted in the strong fluorescence in HepG2 cells.

The gold nanocluster (GNC) attached GO also presented luminescent property, and the hybrid can be used as a drug delivery carrier [17]. The GNC was firstly modified with dodecanethiol-cetyltrimethylammonium bromide (CTAB). Then the dodecanethiol-CTAB-capped GNC simply mixed with the GO solution to produce the GNC-GO nanohybrid. It displayed two emission peaks at 606 and 705 nm. The loading of DOX did not change the fluorescence. The drug delivery to the HepG2 cells could be imaged by fluorescence. DOX-loaded GNC-GO inhibited HepG2 cell growth more strongly than DOX and GNC-GO alone. It indicated that the DOX-GNC-GO is more effectively transported into the cell cytoplasm.

2.2.2 Polymer-Modified GN for Drug Delivery

The GO was soluble and stable in water but readily aggregated in phosphate-buffered saline (PBS), cell growth medium, and fetal bovine serum (FBS). This might be the electrostatic and salt effect that broke the equilibrium state of GO in solutions [18]. To solve the problem, water soluble and biocompatible polymers were employed to stabilize the GO. Such modification made the GO–drug system dispersing in aqueous solution and blood stability, so the polymer–GO was regarded as a promising drug nanocarrier.

The polyethylene glycol (PEG)ylate–GO composite was studied as a polymer–GO carrier [19]. The ultrasmall nano-GO with lateral dimensions down to <10 nm was preferred for DOX loading to deliver to tumor cells. To enhance its stability in aqueous solutions, PEG was grafted to the small GO. Even when the GO is linked with PEG, the GO sheets still contained some smaller aromatic domains, which favored the DOX adsorbing to GO by π – π interaction. The GO–PEG (>100 mg/L) showed harmless for Raji cells, while the DOX-loaded nanoscale GO (NGO)–PEG exhibited high cytotoxicity to Raji cells as well as the DOX behaviors. If the rituxan (CD20+ antibody) is conjugated to GO–PEG, the nanocomposite would selectively target specific cancer cells for killing. Enhanced DOX delivery and cell killing had increased above 20% with Raji cells treated with Rituxan–GO–PEG–DOX at lower concentration at $2 \mu\text{mol L}^{-1}$. Drug release was dependent on the chemical environment. In acidic solutions, the DOX can be protonated and dissolved slowly. About 40% of DOX loaded on GO–PEG was released over 1 day in a solution with pH of 5.5. The release rate was reduced when the solution was neutral, only 15% of loading DOX released after 2 days.

A CPT analogue SN38 can also be stable in aqueous solution after loading on the PEGylate–GO (Fig. 2.4) [20]. Their interaction is based on noncovalent van der Waals force. A total of 1 g of GO–PEG loaded about 0.1 g of SN38. SN38 is a water insoluble aromatic molecule, and the GO–PEG–SN38 complexes were water soluble at concentrations up to 1 mg/mL, which improved the drug delivery. The release of SN38 from the nanocomposite was controllable. The release of SN38 from GO–PEG is about 30% in serum in 3 days. The drug-loaded nanocomposite exhibited high potency with inhibition concentration (IC_{50}) values of about 6 nM for HCT-116 cells (colon cancer cells). It is about 1000-fold more potent than CPT-11 (CPT-11, a Food and Drug Administration (FDA)-approved SN38 prodrug for colon cancer treatment) and similar to that of free SN38 dissolved in dimethyl sulfoxide (DMSO).

The GO–PEG nanosheets can also load the photosensitizer chlorin e6 (Ce6) and DOX simultaneously [21]. The Ce6- and DOX-loaded GO nanocomposite (Ce6–DOX–GO–PEG) were 148.0–180.0 nm in size. DOX and Ce6 with a molar ratio of 1:2 provided the highest synergism. GO–PEG nanosheets increased the cellular uptake and tumor tissue accumulation of Ce6 compared to treatment with free drugs. Combining DOX and chlorin e6, Ce6–DOX–GO–PEG could accumulate in tumor tissues over 3 days. Moreover, in squamous cell carcinoma tumor-bearing mice, the

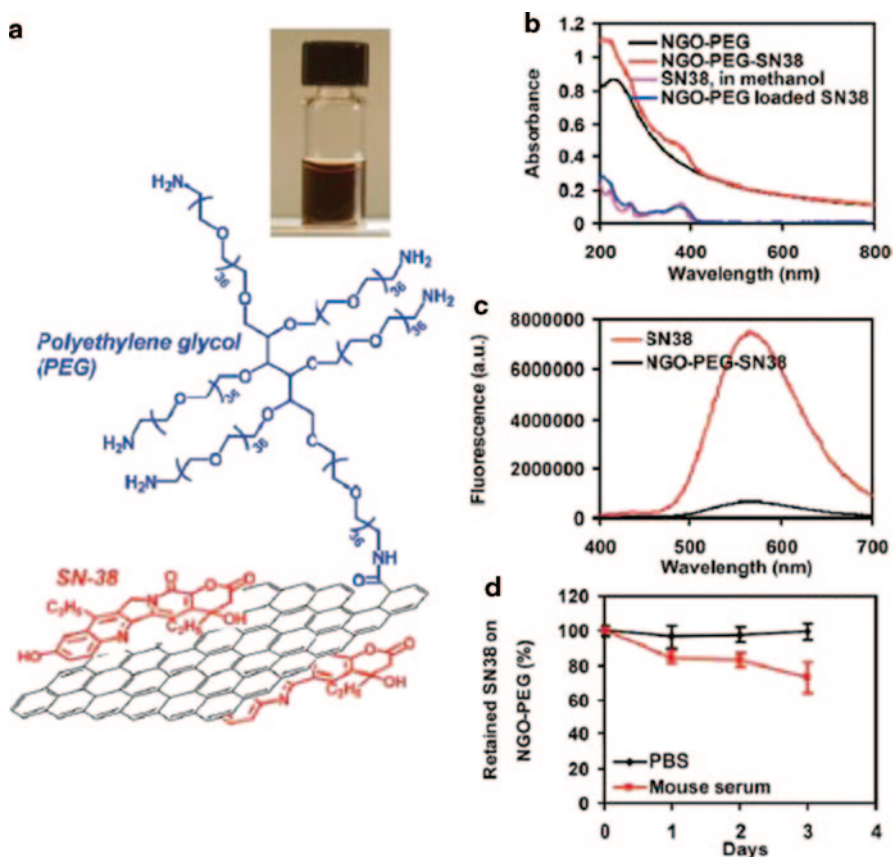


Fig. 2.4 SN38 loading on NGO-PEG: **a** schematic draw of SN38-loaded NGO-PEG; (*inset*) a photo of NGO-PEG-SN38 water solution. **b** UV-visible absorption spectra of NGO-PEG, NGO-PEG-SN38, SN38 in methanol, and difference spectrum of NGO-PEG and NGO-PEG-SN38. The SN38 absorbance at 380 nm was used to determine the loading. **c** Fluorescence spectra of SN38 and NGO-PEG-SN38 at [SN38] = 1 μ M. Significant fluorescence quenching was observed for SN38 adsorbed on NGO. **d** Retained SN38 on NGO-PEG over time incubated in PBS and serum, respectively. SN38 loaded on NGO-PEG was stable in PBS and released slowly in serum. Error bars were based on triplet samples. *NGO* nanoscale graphene oxide, *PBS* phosphate-buffered saline

photodynamic anticancer effects of Ce6-DOX-GO-PEG were higher than those of Ce6-GO-PEG or DOX-GO-PEG.

The PEGylate-GO further linked the branched polyethylenimine (BPEI) resulting in PEG-BPEI-GO ternary nanocomposite [22]. The PEG-BPEI was covalently conjugated with the carboxylic acid group of GO using *N*-Ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) chemistry, and the resulting PEG-BPEI-GO has been reduced by hydrazine monohydrate. It exhibited good colloidal stability and remained stable as dispersion after loading with DOX via π - π stacking and hydrophobic interactions. Photothermally

controlled drug release via endosomal disruption had finally induced higher cancer cell death. The nanocomposite had a higher near-infrared (NIR) absorbance, and it can trigger the release of loaded DOX after photothermal treatment. Under NIR irradiation (808 nm, 6 W/cm², 30 min), cell viability remained above 90% for PEG-BPEI-GO. However, cell viability of PEG-BPEI-GO-DOX-treated cells was about 20% decreased in the NIR irradiation condition as compared with the dark condition. These phenomena clearly show the enhanced and controllable release of drug molecules from PEG-BPEI-GO by the photothermal effect. This DOX-loaded nanocomposite can effectively inhibit the growth of HeLa and human prostate carcinoma PC-3 cells.

Another PEGylated GO was linked by $\text{-NH}_2\text{-S-S-}$ disulfide group (Fig. 2.5) [23]. It can respond to the tumor relevant molecule, glutathione (GSH). A sheddable methoxy PEG (mPEG) shell with a disulfide linkage attaching to GO made up of the drug carrier. It can respond to GSH changes in the tumor cells for intracellular

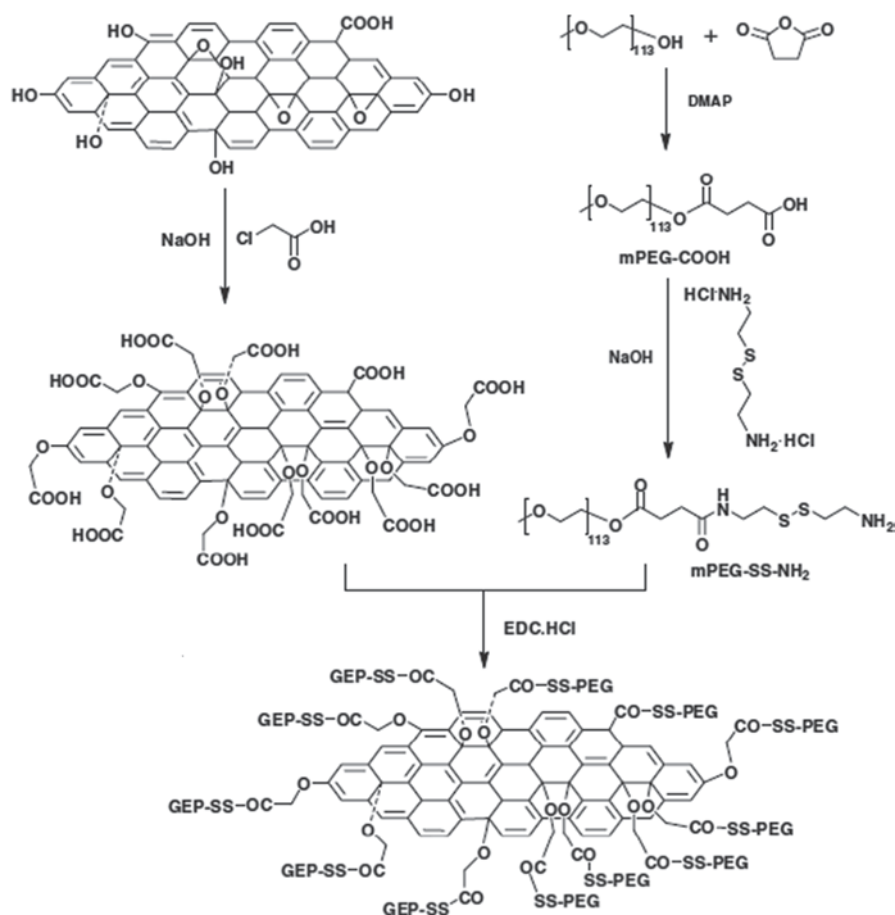


Fig. 2.5 Synthesis pathway of disulfide-linked NGO-SS-mPEG. PEG polyethylene glycol

drug delivery. It has been reported that the cytosolic GSH level in some tumor cells has been found to be at least four fold higher compared with normal cells [24]. The GSH can quickly cleave the disulfide linkage by reduction reaction in tumor site, which leads to drug release. The antitumor drug DOX hydrochloride was loaded to the carrier also by π - π stacking and hydrophobic interactions between DOX and GO. The pharmaceutical system was also pH-dependent similar to the above PEG-GO system. The GSH-mediated drug release was carried out in HeLa cells in terms of GSH concentration and tumor cell viability. The carrier itself did not show significantly inhabitation of the growth of Hela cells even up to a concentration of 1 mg mL^{-1} . Either free DOX or DOX-loaded carrier had effectively reduced the viability of HeLa cells in a dose-dependent fashion. The maximum efficacy of about 90% growth inhibition after 24 h was achieved at the concentrations of equivalent DOX dose $>20 \text{ mg L}^{-1}$. At higher DOX concentrations, the passive uptake of DOX-carrier nanocomposite was high so that tumor cells were killed with or without the presence of GSH. However, the addition of GSH-reduced ethyl ester to cell culture media potentiates the inhibitory effect of DOX-loaded nanocomposite, particularly at the concentrations of 0.25 and 0.125 mg mL^{-1} . Upon exposure to 10 mM GSH, reductive cleavage of the disulfide-linked PEG shell initiated rapid release of encapsulated DOX.

The transferrin-modified PEGylate-GO (Tf-PEG-GO) became glioma-targeted drug delivery carrier [25]. The PEG used here was the amine terminated six-armed PEG to form amide linkage with GO and transferrin. The DOX and Tf-PEG-GO is also simply mixed and adsorbed by non-covalent force. The DOX loading ratio (the weight ratio of loaded drug to carriers) was estimated to be 115.4% of Tf-PEG-GO, closing to that of PEG-GO (127%). The DOX-PEG-GO and DOX-Tf-PEG-GO were used to evaluate their cytotoxicities to C6 glioma cells. The IC_{50} values (50% glioma inhibition drug concentration) of DOX-PEG-GO, DOX-Tf-PEG-GO, and free DOX were 13.61, 63.01, and $28.43 \text{ }\mu\text{g/mL}$, respectively. It indicated that Tf-PEG-GO-DOX presented a 4.63 intensification of cell inhibition compared to PEG-GO-DOX. Because TfR was overexpressed at the surface of glioblastoma cells, it would compete with Tf-PEG-GO-DOX to bind TfR on the cell surface after adding free Tf into the culture medium. Within 12 h, the cellular uptake index of Tf-PEG-GO-DOX was much higher than that of PEG-GO-DOX. The Tf-PEG-GO-DOX and PEG-GO-DOX were also performed to the brain glioma bearing rats. As a result, the DOX distribution in other tissues displayed that DOX was accumulated mostly in both the spleen and the liver. Interestingly, the concentrations of Tf-PEG-GO-DOX and PEG-GO-DOX in the spleen and the liver were significantly lower than that of the free DOX group. By the log-rank test, the median survival time of Tf-PEG-GO-DOX was significantly prolonged compared with that of the saline control, DOX, or PEG-GO-DOX, which reached nearly 41.7, 31.6, and 19.0% life span extension, respectively. The targeted delivery of DOX to the glioma tissue is achieved.

Chitosan (CHI) can graft to GO via a facile amidation process [26]. The CHI-grafted GO GO-CHI has maximum amount of CHI up to 64%, which guaranteed the nanocomposite good aqueous solubility and biocompatible. The GO-CHI can

load CPT via π - π stacking and hydrophobic interactions. GO-CHI possessed 20% of weight-loading capacity for CPT. In the study on the cytotoxicity to HepG2 and HeLa cells, the GO-CHI-CPT exhibited a potency with a 50% growth inhibition concentration (IC_{50}) of $\sim 29 \mu M$, while free CPT displays a $\sim 20\%$ growth inhibition near the same concentration. It meant that the GO-based nanocomposite can improve the drug efficacy without increasing the drug dose. The cell membrane was negatively charged, and the GO-CHI-CPT was easy to be protonated and became the positive charged particles. This course made the GO-CHI-CPT to enter the cells much easier through an efficient endocytic process. Interestingly, the GO-CHI was also able to condense plasmid DNA into stable, nanosized complexes. Such DNA hybrids showed reasonable transfection efficiency in HeLa cells at a nitrogen/phosphate mixture ratio of four. The nanoparticles in the diameter range of 100–200 nm with a narrow polydispersity were preferred.

CHI is viable polymeric material to enhance the biocompatibility of the matrix for controlled release of therapeutic molecules, while FA is a ligand for targeting cell membrane and promoting endocytosis via the folate receptor (Fig. 2.6) [27]. The preparation process of the nanocomposite has three steps. Firstly, the FA conjugated to CHI via the amide groups. Secondly, the DOX loaded to GO by non-covalent forces. Thirdly, the DOX-GO was encapsulated with the folate-CHI to produce the nanocomposite. The release of DOX was similar to the PEG-GO at different pHs. The loading and release of DOX depended upon the hydrogen bonding interaction with GO and was a function of pH.

CHI-modified GO composites as nanocarriers for anticancer drugs (e.g., CPT) and genes via π - π stacking and hydrophobic interactions have been realized. However, the covalent between antitumor drug and GO is not well studied. Incorporation of GO with the CHI-xanthone schiff base could provide additional pH sensitivity which is useful for controlled release of the as-designed drugs [28]. As can be seen from the Fig. 2.7, a novel nanostructured antitumor drugs from GO-CHI-xanthone (GCS) base compound was designed, in which CHI-xanthone (CS) schiff base acted as the tumor inhibitor while GO worked as the drug delivery carrier. The pH-triggered simultaneous release of the CS from the GCS was achieved by changing the solution from acidic to base conditions. The as-synthesized GCS showed less cytotoxicity against the normal human retina cells when a concentration of 10 nM was applied. In the same concentration, the GCS induced nearly 50% HeLa cells apoptosis. It exhibited super antitumor activity when compared with xanthone and paclitaxel (PTX) under the same conditions.

Except PEG, other polymers were employed to covalently bond with GO for drug delivery. The GO sheets grafted with a thermo-responsive polymer would produce desirable DDS [29]. The poly(*N*-isopropylacrylamide) (PNIPAM) is a thermal responsive polymer. PNIPAM was incorporated onto GO by applying click chemistry. The PNIPAM-grafted GN sheets (PNIPAM-GS) consisted of about 50% polymer, which formed a long-term stable dispersion. The PNIPAM-GS sheets had good solubility and stability in physiological solutions and PBS solutions. It had a hydrophilic to hydrophobic phase transition at 33 °C, which was relatively lower than that of a PNIPAM homopolymer due to introduced nanoGO as shown

Carbon Nanomaterials for Biomedical Applications

Zhang, M.; Naik, R.R.; Dai, L. (Eds.)

2016, XI, 576 p. 232 illus., 154 illus. in color., Hardcover

ISBN: 978-3-319-22860-0