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Toxicity Studies and Biomedical Applications of Graphene Oxide

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11.1 Introduction

Graphene oxide (GO) is a carbon-rich material that is derived from graphene. Similarly to the parent material, GO contains flat regions made of sp^2-hybridized carbon atoms. In contrast to graphene, it also contains non-flat regions and modified edges, which can be formally considered as products of the partial oxidation of the sp^2 system (Figure 11.1). Such non-flat GO sections carry a rich plethora of chemical fragments, including rather abundant epoxides, alcohols, carboxylic acids, carbonyl groups and sulfate esters, as well as a number of less abundant fragments and ions, whose role in GO properties relevant to its biological activity is often poorly understood. The presence of these groups explains the good solubility of GO in aqueous solutions at pH close to 7 and its substantially lower tendency to aggregation than that observed for graphene. Though GO has a lower area of flat, sp^2-hybridized sections, it seems to be sufficient to provide for the efficient interaction with biomolecules of different types, including small molecules and biopolymers such as nucleic acids, as well as with unnatural biologically active compounds, e.g. drugs and fluorescent dyes. Finally, GO exhibits substantial cell membrane permeability and relatively low toxicity both in cellular assays and *in vivo*. This combination of properties, which is rather unusual for carbon-rich materials, makes GO an interesting material for biomedical and medicinal applications.

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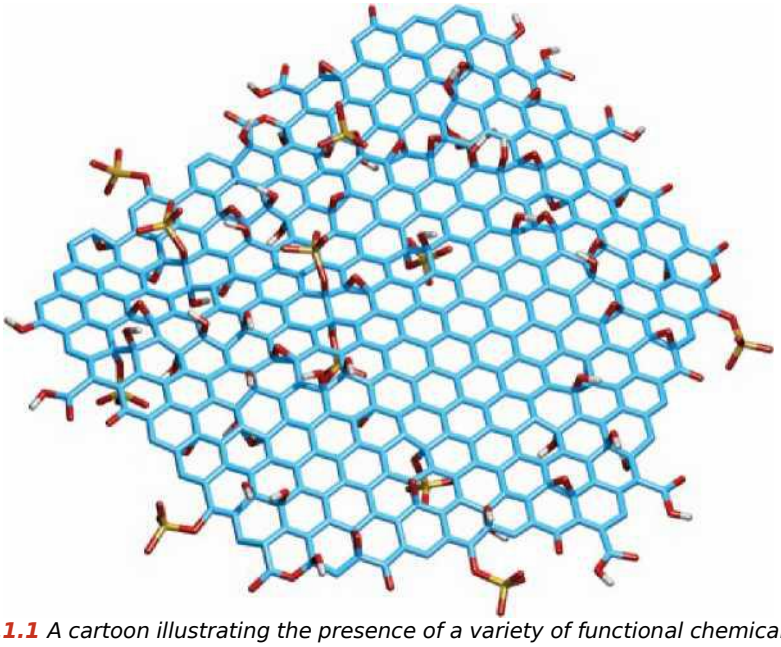

Figure 11.1 *A cartoon illustrating the presence of a variety of functional chemical groups, flat graphene-like regions and non-flat regions on the surface of graphene oxide (GO)*

11.2 Toxicity of Graphene Oxide

Graphene oxide is an amphiphilic material, which has an overall negative charge at physiological conditions. The charge can be reversed by covering GO with polycationic reagents, e.g. polymers or dendrimers. Correspondingly, in cells, GO can potentially interact with hydrophobic, positively charged and negatively charged surfaces, e.g. membranes, proteins and nucleic acids, thereby inducing toxicity. In this section, we will discuss known toxic effects of GO observed in cellular assays *(in vitro)* and *in vivo,* and, where possible, outline reasons for the toxicity. Biological effects of GO and analogous materials, including their cytotoxicity, have been previously reviewed [1-8].

Data on the toxicity of GOs in cellular assays found in the literature are often contradictory [1-9]. This is partially explained by the large number of parameters that have to be controlled to be able to compare the results obtained in different laboratories. In particular, the source of the starting materials as well as the method of synthesis and purification of GO affect the size, the number of sheets in the material, surface charge, oxidative state and the presence of low-molecular-weight impurities and different functional groups on the surface. Substantial efforts have to be invested to account for all of these parameters to obtain standardized GO materials. Unfortunately, this is not yet done routinely. Moreover, GO can interfere with cell viability assays, producing false positive results. For example, Macosko, Haynes and coworkers have observed that methylthiazolyldiphenyl-tetrazolium bromide (MTT), which is used as a reagent in the popular cell viability assay, is

efficiently reduced in the presence of GO with the formation of a blue-colored product [9]. A product of the same color is produced when MTT is reduced in viable cells. Therefore, MTT-based assays will fail to indicate GO cytotoxicity. The same authors have found that another tetrazole-based reagent, the water-soluble tetrazolium salt WST-8, as well as trypan blue exclusion, allow for accurate estimation of the number of viable and dead cells [9].

GO toxicity in cells is usually moderate at low concentrations (<10 pg ml^{-1}). At higher doses, it is dependent upon GO size, aggregation state, oxygen content and surface charge. For example, toxic effects of GO have been observed for:

i. human fibroblast (HDF) cells (>50 pg ml^{-1}) - decreasing cell adhesion, cell apoptosis; GO obtained by Hummers method [10];
ii. human lung carcinoma (A549) cell line - concentration-dependent increase of the amount of reactive oxygen species (ROS); GO obtained by Hummers method and fractionated by size [11];
iii. red blood cells (RBCs) (>25 pg ml^{-1}) - hemolysis; GO obtained by Hummers method and sonicated to obtain GOs of different sizes [9];
iv. human skin fibroblasts (>12.5 pg ml^{-1}) - cell viability decreased; GO obtained by Hummers method and sonicated to obtain GOs of different sizes [9].

A number of other studies on the toxicity of GO, nano-GO (NGO) and related materials toward various cell lines have appeared recently [1-3, 12-19].

Interestingly, Fiorillo *et al.* have observed that GO inhibits the proliferative expansion of single cancer stem cells in the tumor-sphere assay [19]. The effect has been confirmed for six different cancer types, including breast, pancreatic, prostate, ovarian, lung cancer and glioblastoma. Surprisingly, GO has been found to be only weakly toxic to mature (non-stem) cancer cells. This is a significant result, since cancer stem cells are tumorinitiating cells, which are practically insensitive to conventional chemotherapy and radiation. The survival of a few cells of this type after treatment leads to tumor recurrence and distant metastasis.

Toxicity of GO *in vivo* depends on the experimental settings selected and the parameters investigated. For example, it has been found that NGO at a dose of 25 mg kg^{-1} (injected *via* the tail vein) exhibits practically no toxicity for reproductive function of male mice [20], and GO-derived carrier of Stat3 siRNA is practically non-toxic in mice, as shown in studies with a mouse model of melanoma [21]. However, at ~14mgkg^{-1}, chronic toxicity of GO has been observed for Kunming mice [10], whereas oral exposure to a dose ~0.8mg GO per day per mouse in the lactating period strongly delayed the development of offspring and caused many other negative effects in the development of mice [22]. Furthermore, a systematic study of Li *et al.* on the distribution and toxicity of NGO in C57BL/6 mice for three months after the exposure has revealed that NGO can be retained in the lungs, thereby resulting in acute lung injury and chronic pulmonary fibrosis [23].

11.3 On the Toxicity Mechanism

11.3.1 Membrane as a Target

Graphene is known to enter cells by the edge-first uptake mechanism, which can lead to membrane damage [24]. An analogous mechanism can be assumed for GO and other GO-derived materials, since they have graphene-like regions, including edges, whose extent depends on the C/O ratio and other factors. Other mechanisms of GO-induced membrane damage are possible [1-3]. The current literature on the subject indicates that the effect of GO on outer cellular membranes is strongly dependent upon the cell type. For example, Cao, Wang and coworkers have observed that the incubation of human alveolar adenocarcinoma A549 cells with GO at concentrations of up to 200 pg ml^{-1} does not significantly modulate the level of the extracellular lactate dehydrogenase (LDH) activity, which is a common marker for membrane damage [11]. Similar results have been obtained by Dai, Lu, Liu and coworkers, who studied the effect of GO on eyesight both *in vitro* and *in vivo* [17]. In particular, they have observed that the level of LDH did not exceed 8% in the *in vitro* assay with ARPE-19 cells (a cell line derived from human retinal pigment epithelium) incubated for a variable time (24-72h) with variable GO concentrations (5-100 pg ml^{-1}). For comparison, ~2-3% LDH have been released from the untreated cells. Furthermore, Mullick Chowdhury *et al.* have studied the toxicity of oxidized graphene nanoribbons (O-GNR, width ~125-220nm) stabilized with 1,2-distearoyl-*sn*-glycero-3-phosphoethan- olamine-W[amino(polyethylene glycol) (PEG-DSPE) in several selected cancer cell lines: cervical cancer cells HeLa and breast cancer cells SKBR3 and MCF-7 [25]. Upon the incubation of MCF-7 cells for 24h with 0.4$mgml^{-1}$ of O-GNR-PEG-DSPE (the highest concentration used), the cells release ~55% LDH compared to the LDH activity in the lysed cells. For SKBR3 cells, the effect was comparable. In a negative control experiment (cells not treated with anything), ~40% and ~55% LDH activity was observed for MCF-7 and SKBR3, respectively. These data indicate that the membrane is not significantly affected by the treatment of the breast cancer cells with O-GNR-PEG-DSPE. In contrast, the membrane of HeLa cells has been found to be substantially more sensitive: 95% LDH release in the presence of O-GNR-PEG-DSPE versus ~50% in its absence. Moreover, the membrane of RBCs has been found to be highly sensitive to GO. For example, Jiang and coworkers have investigated the toxic effects of GO and nitrogen-doped graphene quantum dots (N-GODs) on RBCs. By using infrared (IR) spectroscopy in combination with monitoring hemolysis, observing morphological changes and detecting the adenosine triphosphate (ATP) content of RBCs, they have confirmed that the GO materials were first adsorbed on the external part of the lipid bilayer of the RBC membrane, which led to its disintegration, hemolysis and aberrant forms [16]. Haynes and coworkers have found that hemolysis of RBCs was especially pronounced for GOs of small size [9]. In particular, pGO-30 with a hydrodynamic diameter d = 324± 17 nm at 50 pg ml^{-1} induced hemolysis of

>90% RBCs, whereas the usual GO obtained by the Hummers method (d=765 ± 19 nm) applied at the same concentration affected only ~25% RBCs. Finally, the membranes of a variety of bacterial cells have been found to be sensitive to graphene-based materials [26-28].

The interaction of GO with cellular membranes can be further modulated by proteins present in biological fluids, since some of them bind to the GO surface with high affinity. For example, serum albumins (SAs) are present in large quantities in blood and can potentially affect GO toxicity. One example of such an influence has been reported by Ge, Zhou and coworkers. By using electron microscopy, these authors have observed that bovine serum albumin (BSA) reduced the cell membrane permeation of GO, inhibited the cellular damage induced by GO and reduced its cytotoxicity [29]. Based on molecular dynamics studies, they have concluded that the protein-GO interaction weakens the GO-phospholipid interaction due to the reduction of the surface available for binding. In other work, the effect of GO on human serum albumin (HSA) properties has been reported by Ding *et al.* [30]. In particular, they have observed that GO inhibited the interaction of HSA with bilirubin. Thus, GO and serum albumins mutually affect the properties of each other.

11.3.2 Oxidative Stress

A number of reports confirm that GO treatment results in an increase in the amount of ROS in cells. The latter can be detected, for example, by using a variety of commercially available leuco-dyes, including dichlorodihydrofluorescein diacetate or dihydroethidium, in combination with flow cytometry or fluorescence microscopy. In particular, Chang *et al.* [11] have observed that incubation of A549 cells with GO induced a dose-dependent intracellular oxidative stress that leads to a slight loss of cell viability at high concentrations. Moreover, GO toxicity toward human multiple myeloma RPMI 8226 cells has been found to be closely associated with an elevated amount of ROS [14]. A similar effect has been observed by Lammel and Navas [31], who studied the influence of GO and carboxyl graphene (CXYG) on fish hepatoma cell line PLHC-1. For example, they found that graphene materials penetrated spontaneously through the cellular membrane and in the cytosol they interacted with mitochondrial and nuclear membranes. The treated PLHC-1 cells demonstrated significantly reduced mitochondrial membrane potential and increased ROS levels at 16pg ml^{-1} GO and CXYG (72h incubation). Other reports confirming the GO-induced oxidative stress in cellular assays have been reviewed elsewhere [1-3].

The data obtained in *in vitro* assays are supported by *in vivo* data. For example, the effects of prolonged exposure of the roundworm *Caenorhabditis elegans* to GO have been evaluated by Wu *et al.* [32]. *Caenorhabditis elegans* is especially well suited as a model organism for evaluation of the biological effects (including toxicity) of chemical compounds *in vivo,* since this organism is transparent and can be

monitored/studied by using fluorescence imaging. Wu *et al.* [32] have found that prolonged exposure of this organism to 0.5- 100 mg l^{-1} of GO caused a negative effect on the functions of both primary (intestine) and secondary (neuron and reproductive organ) targeted organs. Interestingly, in the intestine, the production of ROS was detected, which correlated with the adverse effects observed. Furthermore, Li *et al.* [23] have proven that NGO-induced acute lung injury (ALI) and chronic pulmonary fibrosis were related to the oxidative stress and could be relieved with dexamethasone treatment, which is a steroid drug with anti-inflammatory properties. In another model organism, zebrafish, GO induced a significant hatching delay and cardiac edema during embryogenesis [33]. Moreover, its treatment led to the excessive production of ROS (e.g. hydroxyl radicals) and changes in the secondary structure of proteins.

The question of why GO induces oxidative stress in cells is currently being actively investigated. For example, Nie's group has reported that the ROS-generating ability of GOs in mouse embryo fibroblasts (MEFs) is dependent upon the oxidation degree of GOs [34]. In particular, the least oxidized GO exhibited the highest ROS-enhancing ability, which was explained by the conversion of less toxic H_2O_2 into highly toxic $HO^{\cdot}$ radicals in cells. The theoretical simulations by the same authors revealed the involvement of carboxyl groups and planar domains of GO in varying the energy barrier of the H_2O_2 reduction reaction. Furthermore, using a fluorogenic, DNA-based probe, Mokhir and colleagues have confirmed that GOs obtained either by the Hummers method or by the milder method first

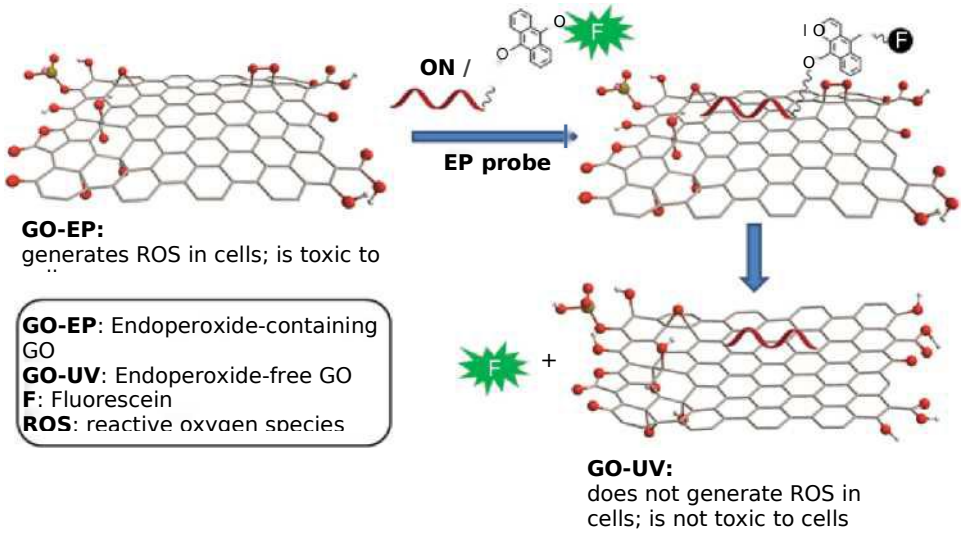

Figure 11.2 *Detection of endoperoxides (EP) on the graphene oxide (GO-EP) surface using fluorogenic probes (EP probe) consisting of an oligonucleotide (ON), which binds strongly to the GO-EP, a reactive moiety (an anthracene derivative) and a fluorescent dye (fluorescein,*

reported by Eigler contained low amounts of surface-bound endoperoxides: one moiety per ~10_4 carbon atoms (Figure 11.2) [35].

These GOs were efficiently taken up by HeLa cells, which was accompanied by an increase in the intracellular ROS concentration and a decrease in cell viability. Interestingly, endoperoxide-free GOs, obtained by irradiation of the GOs with ultraviolet light of low power, were also taken up by the cells, but neither increased the intracellular ROS amount nor affected cell viability. These data allowed the authors to conclude that endoperoxides play an important role in the ROS-generating ability of GOs. Next, Chen and coworkers have investigated the effects of GO on T-lymphocytes and HSA [30]. In particular, they have observed that the treatment of T-lymphocytes with GO led to an increase in ROS generation, damage to DNA, cell apoptosis and limited suppression of the immune response of T-lymphocytes. Based on these data, they suggested that GO interacts directly with protein receptors, which inhibits their ligand binding ability, thereby leading to ROS-dependent passive apoptosis through the B-cell lymphoma-2 (Bcl-2) pathway.

11.3.3 Other Factors

New information about the toxicity of GO in relation to gene expression in cells has recently become available in the literature. These data may contribute to further understanding of the mechanism of GO toxicity *in vivo.* In particular, Wu *et al.* [36] have observed that mutations in several genes, including hsp-16.48, gas-1, sod-2, sod-3, aak-2 as well as isp-1 and clk-1, strongly affected translocation of GO into the body of *C. elegans,* its toxicity on both primary and secondary targeted organs compared with wild type, the intestinal permeability and the mean defecation cycle length.

Furthermore, Wang and coworkers have investigated the role of micro-RNAs (miRNAs) in GO toxicity [37]. They have identified 23 up-regulated and eight down-regulated miRNAs in GO-treated *C. elegans,* and provided evidence to suggest that GO may reduce the lifespan of nematodes by affecting insulin/IGF (insulin-like growth factor) signaling, TOR (target of rapamycin) signaling as well as germline signaling pathways. Finally, the same authors have established the role of innate immunity in regulating chronic toxicity of GO in *C. elegans* [38].

11.4 Biomedical Applications of Graphene Oxide

11.4.1 Graphene Oxide in Treatment of Cancer and Bacterial Infections

In general, disease therapy relies on the selective action of a drug on disease-associated cells, biomolecules (e.g. enzymes, nucleic acids) or biochemical states (e.g. inflammation), which ideally occurs without affecting healthy organs and normal cells. The currently applied therapies for cancer treatment, including chemotherapy (using, for example, Pt(ii)-based drugs, bleomycin and 5-fluorouracil) and radiotherapy, are not sufficiently cancer-cell-specific. Therefore, such treatments exhibit characteristic dose-limiting toxici- ties. Moreover, repeat treatments lead to the development of resistance. This partially explains why cancer is still one of the most common causes of death (together with cardiovascular disease) in developed countries. Therefore, the search for new approaches to cancer treatment is warranted. Targeted therapy is an advanced, recently introduced method, in which cancer-specific drugs (or prodrugs) are applied. GO is used in several approaches for cancer targeting, including photothermal and photodynamic therapy and as a nano-sized carrier to improve the cell membrane permeability of drugs and achieve their accumulation in tumors due to the enhanced permeability and retention (EPR) effect [6-8].

11.4.2 Photothermal Therapy

In photothermal therapy (PTT), disease-causing cells, including cancer cells in tumors and bacteria in wounds, are loaded with a reagent that absorbs near-infrared (NIR) light. Then subsequent exposure to NIR light heats up the system, inducing hyperthermia and thereby causing cell death. However, human tissues contain large amounts of hemoglobin and water, which strongly absorb visible and NIR light. To avoid unspecific heating of healthy tissues, for PTT, light is used that is practically not absorbed by the tissues: in the first biological window, 700-980 nm (BW1); and in the second biological window, 1000-1400 nm (BW2) [39]. Such light can penetrate through several centimeters of human tissue [40], whereas deeper located sites can be accessed by delivery of the light *via* optical fibers in combination with endoscopy [41]. Since the light beam can be focused on a specified area (e.g.

tumor location) and its intensity (dose) can be easily controlled, PTT allows surgery- free tumor ablation practically without affecting healthy tissues.

Single-layered GO is suitable for PTT, since, in addition to its excellent water solubility, membrane permeability and stability, this material absorbs light in the NIR range [42-44]. It has been reported that the NIR absorptivity of GO can be improved by optimization of its size. For example, small GOs (less than 300nm in size) absorb NIR light more efficiently than the conventional material. In particular, extinctions at 808 and 1200nm have been found to be between about five- and eight-fold higher for small GOs [45]. The latter material is often called nano-GO (NGO) in the scientific literature.

GO of optimal size accumulates in tumors due to the EPR effect. Additionally, the accumulation can be achieved by decoration of GO with ligands that bind to cancer-specific receptors. These aspects will be discussed in the next subsection. A number of excellent reviews on PTT have been published, which cover the literature on the subject up to 2014 [1, 46, 47]. Here we will discuss only two selected reports, which demonstrate the applicability of GO-based materials as sensitizers for PTT of bacterial infections and cancer.

In 2013 bacterial infections affected over 48 million people in the USA and caused 80 deaths. They are especially dangerous for people with compromised immune systems and patients having extensive wounds following surgery [48]. Moreover, chronic infections are known to develop resistance against conventional organic drugs (antibiotics) and cause cancer: e.g. infection of *Helicobacter pylori* often leads to stomach cancer [49]. Therefore, novel antibacterial drugs are necessary. Wu and coworkers have explored the applicability of conventional GO obtained by the Hummers method in combination with PTT for the treatment of bacterial infection in wounds [50]. This is a rare example demonstrating the biological activity of conventional, chemically unmodified GO *in vivo.* The authors have conducted their investigation on healthy albino mice. Each mouse received three wounds, all of which were infected with *Staphylococcus aureus:* the first wound was left untreated; another one was exposed to the light from a Nd:YAG laser (*2* = 1064 nm, 3 min irradiation, every day for 12 days); and the third wound was treated with GO and exposed to the same light under the same conditions. The healing of the wounds treated with both GO and the laser irradiation was accelerated in comparison to the control wounds. These data indicate that GO combined with PTT can potentially be used as an efficient and cheap alternative to antibiotics.

PTT in combination with GO or other NIR-absorbing nanomaterials has the potential to improve the side effects that occur during conventional chemotherapy. Moreover, when applied together, PTT and chemotherapy have been shown to exhibit synergistic effects [51]. For example, Guo and coworkers have prepared a hybrid material NGO-PEG-DOX containing NGO covalently modified with polyethylene glycol (PEG) residues and doxorubicin

(DOX) [52]. The PEG fragment stabilizes NGO in the serum-containing media, enabling the application of this material *in vivo*. DOX is an anthracycline antitumor drug, which exhibits its activity due to binding of genomic DNA *via* intercalation. This fragment binds the NGO-PEG *via* non-covalent n-n interactions. The antitumor activity of NGO-PEG-DOX *in vivo* has been studied on a xenograft tumor mouse model, balb/c female mice, which carried tumors derived from murine mammary tumor cell line EMT6. In particular, the solution of the NGO construct was injected intravenously and the tumor was irradiated for 5 min (24 h post-injection) by a laser with 2_{em} = 808 nm (2 W cm^{-2}) focused on a 6 x 8 mm spot. The authors have reported a strong synergistic antitumor effect of DOX and PTT: the tumors were completely destroyed 30 days after the beginning of treatment. Interestingly, DOX alone exhibited substantially stronger side effects than the nano-sized construct NGO-PEG-DOX. These data demonstrate that PTT combined with chemotherapy can be a feasible approach for the improvement of current methods of cancer treatment.

11.4.3 Graphene Oxide as a Drug Carrier

In GO, every atom is exposed to the surface. Therefore, its surface area is very large (for graphene, 2600 m^2 g^{-1}). Correspondingly, one can densely load GO with cargo, e.g. drugs, cell-surface-directing fragments, nucleic acids and proteins. Though GO is soluble in

water, it can aggregate in the presence of salts and components of serum. Therefore, this material is often chemically modified to improve its bioavailability, which includes either non-covalent (electrostatic or n-n interactions) or covalent modification [53]. For example, mixing GO with poly(ethyleneimine) (PEI) of different sizes (1.2-10 kDa) leads to facile formation of hybrid GO-PEI materials, which, in contrast to GO, are retained in the physiological solution and the serum-containing medium in the monomeric state. Moreover, such constructs have lower toxicity than free PEI [54]. In this case, the electrostatic interaction at neutral pH between positively charged PEI and negatively charged GO is the driving force for the GO-PEI formation. Furthermore, n-n interactions can be applied to modify GO and reduced GO (RGO). For example, planar aromatic molecules, such as porphyrins, pyrenes, perylenes and coronenes, have been used as anchors for attaching different functionalities to GO and RGO [55]. Since GO contains a variety of reactive functional groups, one often applies covalent chemistry to modify the GO surface. The most popular reactions include the formation of amide bonds, which typically occurs between a GO-bound -COOH group and a modifier-bound -NH_2 group [42, 53]. For example, the attachment of polymers like PEG, poly-L-lysine and polyacrylamide (PAA) to GO has been conducted by using such reactions.

11.4.3.1 Low-Molecular-Weight Drugs as a Cargo

Organic drugs containing extended n systems are common. For example, such compounds can act as intercalators of genomic DNA (e.g. anthracycline antibiotics), inhibit specific kinases (e.g. imatinib) and act as antimetabolites (e.g. methotrexate). Many such drugs are not well soluble in water. This problem can be solved by loading them onto well soluble GO-based materials. Moreover, GO-drug hybrids often enter the cells *via* pathways that are different from those of the free drugs. The same is the case for the removal of the drugs from the cell: hybrids may be retained in cells longer. Therefore, the activity of hybrids is often higher and they can be used to overcome the resistance of cells to particular drugs. Resistance is an important problem in the chemotherapy of cancer. It is developed as a result of repeated treatments with the same drug.

A number of reports were devoted to the transport of DOX and its analogs with the help of graphene-based carriers, as reviewed elsewhere [45-47, 53]. One such example has already been described in section 11.4.2 devoted to PTT [52]. Despite being a potent anticancer drug, DOX has an extended aromatic polycyclic structure and can, therefore, interact with sp^2-hybridized regions of GO due to n-n stacking interactions. This interaction is so strong that it is sufficient for the immobilization of DOX onto GO and no additional covalent attachment is usually required. Moreover, DOX is a fluorescent molecule that allows easy monitoring of its loading into cells by using either fluorescence microscopy or flow cytometry. In particular, Wang, Zhang and colleagues have used as a carrier NGO with sheet size below 100 nm and

thickness between 0.8 and 1.5 nm, suggesting single- and double-layered structure [56]. By simple incubation of the components and removal of excess drug by centrifugation, high cargo loading could be achieved, 0.468 g DOX per 1 g NGO. Interestingly, the interaction strength between the NGO and DOX was strongly modulated by the pH of the solution. In particular, at neutral (pH7.2) and basic (pH 9.0) conditions, less than 6.5% of the drug was released after standing for over 40 h in the correspondingly buffered phosphate saline (PBS) solutions. In contrast, at slightly

acidic conditions (pH 5.0) ~15% of DOX was released within the same time. This effect has been attributed to the presence of hydrogen bonding between the functional groups on the NGO (-OH, -COOH) and those of DOX (-OH, $-NH_2$) in addition to the n-n stacking. The former bonds were expected to be destabilized under the acidic conditions. Since the microenvironment in tumors is often acidic, this property of NGO-DOX can be used for the selective release of DOX at the cancer-specific conditions, which should improve the therapeutic index of the nano-drug. Zhang and colleagues have explored whether NGO- DOX can reverse the DOX resistance of cells that overexpress multi-drug-resistant (MDR) gene [56]. In particular, they have tested the cytotoxicity of NGO-DOX toward DOX- sensitive MCF-7 and DOX-resistant MCF-7/ADR cell lines and compared the data obtained with the effects of free DOX. They have observed that NGO-DOX exhibited a toxicity toward MCF-7 cells (~1 pg ml^{-1}) that was comparable to DOX, but was substantially more toxic toward MCF-7/ADR than DOX: IC_{50} ~ 1 and 14 pg ml^{-1} respectively. These data indicate that application of the NGO-based hybrid drug allows reversal of the resistance of MCF-7/ADR cells toward DOX. Other examples of graphene-based hybrids containing organic and metal-containing anticancer drugs, photosensitizers for photodynamic therapy (PDT) and receptor-targeting fragments include PEGylated reduced NGO carrying natural phenol resveratrol [57], unmodified NGO carrying bioactive flavonoid quercetin [58], GO-PEG loaded with chlorin e6 (Ce6) [59], RGD-motif-containing reduced NGO [60] and others [45-48, 53].

11.4.3.2 Oligonucleotide-Based Drugs as a Cargo

Nucleic acids play a central role in the storage of genetic information, protein synthesis and regulation. Due to the recent scientific and technological advancements in the field of genome wide sequencing, knowledge of the role of nucleic acids in cellular biology is rapidly expanding. For example, apart from messenger RNAs (mRNAs), ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs), which have been known for a long time, many new RNA types have recently been discovered, and further discoveries of this type are certainly under way. They include micro-RNAs (miRNAs), pseudo-genes, circular RNAs, long noncoding RNAs and others. These biomolecules are termed non-coding RNAs (ncRNAs). Synthesis, processing, mode of action and targets of miRNAs are mostly well understood. They participate in the regulation of gene expression, regulation of protein synthesis and are often overexpressed or down-regulated in diseases (e.g. cancer) with respect to the normal state. However, functions of other ncRNAs are less well understood and are still actively being investigated.

Binders of intracellular RNAs can inhibit their biological activity (*via* antisense effect or RNA interference), which can help in the elucidation of the functions of newly discovered ncRNAs. Moreover, binders of the RNAs, which are fluorescently labelled with dyes and are responsive to the hybridization state (e.g. molecular beacons, MBs), can be used to monitor

ncRNAs and mRNAs directly in cells. Oligonucleotides (ONs) are highly specific and strong binders of complementary RNA sequences. However, due to their polyanionic character, these reagents are not permeable through the cellular membrane. Moreover, they are not stable in cells due to their efficient cleavage by abundant intracellular endo- and exonucleases. To improve these properties, a number of chemically modified oligonucleotides have been prepared. They include phosphorothioate DNAs (PTOs), 2'-OMe RNAs, peptide nucleic acids (PNAs) and others. These compounds are more stable to nucleases than unmodified ONs. However, with few rare exceptions (e.g. PTOs), ON analogs are not cell-membrane-permeable.

Apart from inhibiting the RNA function, it can be required to upregulate (increase) the RNA amount in cells. This goal can be achieved by the introduction of circular, doublestranded nucleic acids (plasmids). The genes within the plasmids are expressed, producing the corresponding mRNAs and proteins, which can be used for determination of gene functions and design of cellular reporters, e.g. fluorescent proteins or luciferases. Moreover, required RNAs can also be introduced directly. However, in both cases the problem of cell membrane permeability exists. Typically, nucleic acids (plasmids, RNAs) and nucleic acid inhibitors (ONs, analogs of ONs, small interfering RNAs, siRNA) are brought into cells by reversible membrane permeabilization using streptolysin O (SLO), electroporation, transfection with positively charged oligomers or dendrimers and direct microinjection. These approaches are toxic or damage cells to some extent, are not applicable to all cell types and their possible applications *in vivo* are limited. Therefore, studies of new approaches for improving cell membrane permeation of nucleic acids are warranted.

GO-based materials can potentially evolve as true alternatives to the usually used transfection reagents. For example, it has already been demonstrated that they are applicable for transfection of plasmids and siRNAs into cells. In particular, Liu and coworkers have modified GO covalently with cationic PEI polymers of different sizes: 1.2 and 10 kDa [54]. They have found that both GO-PEI-1.2 kDa and GO-PEI-10 kDa induce the efficient transfection of a plasmid carrying enhanced green fluorescent protein (EGFP), which could be followed by monitoring the EGFP expression *via* fluorescence microscopy. In contrast, PEI-1.2 kDa itself is not functional. Though PEI-10 kDa is an efficient transfection agent, it has been found to be toxic. In contrast, the toxicity of the hybrid GO-PEI-10kDa was reduced.

Zhang *et al.* [61] have designed a hybrid consisting of GO that was covalently modified with PEI-25 kDa and contained an siRNA targeting an mRNA of the Bcl-2 gene. The latter reagent was adsorbed onto the GO-PEI-25 kDa due to electrostatic interactions. The authors have confirmed that, in human cervical cancer cell line (HeLa cells) incubated with the GO-PEI-25 kDa-siRNA hybrid, which had the optimal ratio of nitrogen (proportional to the PEI) to phosphorus (proportional to the RNA) of 20, the expression of the Bcl-2 gene was suppressed down to ~30%. The inhibition observed using the PEI-25 kDa-siRNA associate under the same conditions was significantly

weaker, ~60%. Additionally, the toxicity of the GO-PEI-25 kDa has been found to be negligible up to a concentration of 4 ng l^{-1}, whereas only ~50% of cells remained viable after their incubation with the same amount of PEI-25 kDa. Switching off the Bcl-2 gene was expected to overcome the MDR system of cancer cells and make them more sensitive to chemotherapeutic agents. To prove this hypothesis, the authors treated HeLa cells first with GO-PEI-25 kDa-siRNA, which was followed by DOX. In a control experiment, they used the hybrid containing a scrambled siRNA, which was not targeting any gene in the cells. The authors observed that the cells become more sensitive to DOX after the inhibition of the Bcl-2 gene by the siRNAcontaining hybrid. These data confirm the applicability of GO as a carrier for the transport of siRNAs into cells. Moreover, Yin *et al.* [21] have demonstrated the delivery of a plasmidbased Stat3 siRNA in a mouse model of melanoma, which resulted in the significant inhibition of tumor growth without any toxicity.

Since GO has a large surface area, many different components can be introduced onto it simultaneously to obtain multi-functional drugs or prodrugs. A demonstration of this possibility has been described in the publication of Yang, Xiang, Chen and coworkers [62]. In particular, they have prepared PEGylated GO, which carried at the terminus of each PEG residue one folic acid (FA) fragment. FA was attached to direct (target) the hybrid to cancer cells overexpressing the FA receptor. Next, 1-pyrenemethylamine was adsorbed *via* strong non-covalent n-n interactions with flat regions of GO that provided an overall positive charge for the resulting construct. Finally, siRNA targeting a human telomerase reverse transcriptase (hTERT) gene was attached *via* electrostatic interactions. The authors have demonstrated that the obtained hybrid is not toxic to HeLa cells, but acts as a strong inhibitor of hTERT expression, as evidenced by monitoring the corresponding transcript and the protein.

Both plasmids and siRNAs act by catalytic mechanisms. Therefore, one molecule of the plasmid can generate many equivalents of mRNAs, whereas one molecule of siRNA can induce cleavage of many equivalents of mRNAs. Thus, delivery of even small amounts of these reagents in cells will cause dramatic changes of the concentration of the targeted nucleic acids. Correspondingly, experiments that rely on detection of gene expression from plasmids or target inhibition using siRNAs allow one to answer the question whether the carriers transport their cargo through the cell membrane or not. However, they do not provide an accurate estimate of how much cargo crosses the membrane and stays in the active form in the cell. It should be mentioned that the large proportion of ONs that cross the cellular membrane are trapped in intracellular compartments and remain inactive. A more accurate experiment for determination of the delivery efficiency would be the transport of a labelled ON, e.g. molecular beacons (MBs) or another hybridization-sensitive probe, which binds to its intracellular target and causes changes of the fluorescence of the probe. The latter reaction is stoichiometric and the fluorescence intensity is expected to correlate with

the concentration of the target in the cell. Chen, Yang and coworkers have reported on the transport of a DNA-based MB containing Dabcyl as a quencher and Cy5 as a reporter fluo- rophore [63]. This probe was designed to bind a survivin mRNA. The authors have observed that the NGO protected the MB from nucleases and substantially reduced the background signal of the MB in the absence of its target. Moreover, the NGO brought this MB into cells where it bound the survivin mRNA, as indicated by the increase of the fluorescence intensity in cells. A construct containing NGO and control MB has been found to generate a 2.4-fold lower fluorescence signal in cells. These results confirm some selectivity of the survivin MB, which is, however, still not sufficient for practical applications. Therefore, further optimizations of the delivery of MBs or other hybridization-sensitive probes are warranted.

Aptamers are short ON sequences (RNA or DNA) that bind specifically small molecules. These reagents can be applied as drugs and for imaging of biomolecules both in cell-free conditions and directly in cells. For example, Li, Lin and coworkers have explored the possibility for the delivery of an ATP-aptamer (ON) to cells using GO [64]. To be able to monitor the reagent delivery as well as its binding to intracellular ATP, the authors have labelled the aptamer with a fluorescent dye (Fl) to obtain ON-Fl. The GO-bound ON-Fl remained weakly fluorescent due to strong quenching of the Fl with the GO. However, upon binding of ATP, the aptamer was folded with the formation of the structure, which does not have the high affinity to GO. Therefore, the aptamer was released from the GO in the presence of ATP, which was reflected in the dequenching of the fluorescence of the ON-Fl. Thus, the fluorescence of ON-Fl/GO correlates with the concentration of ATP in solution. Li, Lin and coworkers have observed this behavior both in cell-free assays and in cells, thereby confirming that GO is a suitable carrier of aptamers.

11.5 Bioanalytical Applications

Graphene oxide has been used in a variety of electrochemical and optical assays for the detection of biomolecules and xenobiotics, as reviewed elsewhere [65]. In this section we will concentrate on the detection of nucleic acids by using fluorescence-based approaches, which is mainly due to the current research interests of the authors of this chapter.

Oligonucleotides (ONs) interact strongly with GO. In particular, Maheshwari, Liu and coworkers have studied in detail the binding of fluorescein-labeled oligonucleotides (Fl-ONs) of different length with GO [66]. The GO used in that work was obtained by a modified Hummers method, including oxidation with potassium persulfate and phosphorus pentoxide at 90 °C. The binding was studied by fluorescence spectroscopy monitoring the fluorescence quenching upon the addition of the GO to the Fl-ONs. The authors observed that the binding efficiency decreased with increasing ON length (12- to 32-mers have been investigated). Moreover, the kinetics of this interaction has been found to be substantially faster for shorter strands

(12- to 24-mers) than for the longer one (36-mer). Since both the nucleobases of ONs and the flat regions of the GO are planar aromatics, they are able to get engaged in n-n interactions with each other analogously to those found between singlewalled nanotubes (SWNTs) and nucleic acids [67]. However, this does not seem to be the only factor defining the affinity of the GO to ONs. For example, it has been observed that the binding of Fl-ONs to GO was strongly salt-dependent, which allowed the formation of salt bridges $GO^{\cdots}$-Na^{+}-ON^{m} between the reacting partners to be suggested. Furthermore, the GO-ON interaction is highly sensitive to the hybridization state of the nucleic acids. For example, single-stranded nucleic acids bind GO efficiently, whereas folded ones, including double-stranded nucleic acids, quadruplexes [68] or aptamers [64] bound to their target molecules, do not bind GO. This property of GO has been used in a number of bioanalytical applications for detection of nucleic acids and, in combination with aptamers, for the detection of small molecules.

In early assays, a single-stranded ON containing a fluorophore was loaded onto GO or NGO, leading to fluorophore quenching (Figure 11.3). Adding a complementary nucleic acid to the resulting mixture caused the formation of dsDNA, its release from the GO surface and, consequently, fluorophore dequenching (pathway A, Figure 11.3).

For example, in this way Yang and coworkers have detected a DNA with an HIV1 sequence [69]. Ai and coworkers have recently optimized this assay for detection of single mismatches in nucleic acids [70]. However, since GO binding to single-stranded nucleic acids is in general strong and weakly sequence-specific, an alternative activation mechanism can occur. In particular, a mismatched nucleic acid can replace the probe from the GO surface by an unspecific interaction with the GO that leads to a strong background signal (false positive, pathway B in Figure 11.3). For example, Yang and coworkers have observed [69] that an MHIV1 target, containing a single mismatch, also enhances the fluorescence of the solution containing the probe-GO construct. In the latter case, the signal was only about half that observed in the presence of the fully matching target. Recently, Liu and coworkers have provided experimental evidence that the unspecific probe displacement

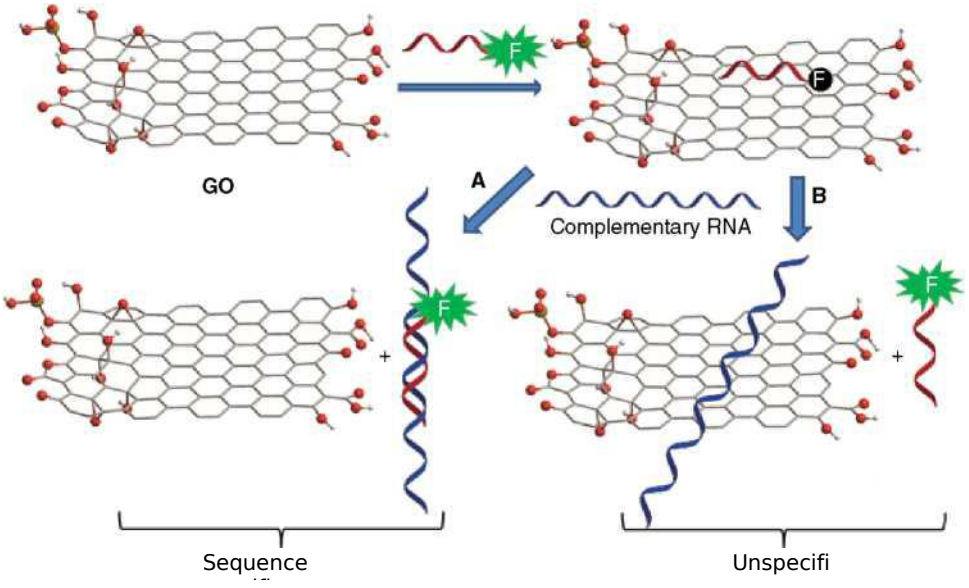

Figure 11.3 *An approach for detection of nucleic acids by making use of quenching and ON-binding properties of GO. Binding of the probe* via *pathway A leads to the sequencespecific fluorescence increase, whereas when pathway B is realized the fluorescence increase is not specific [66, 69, 70]*

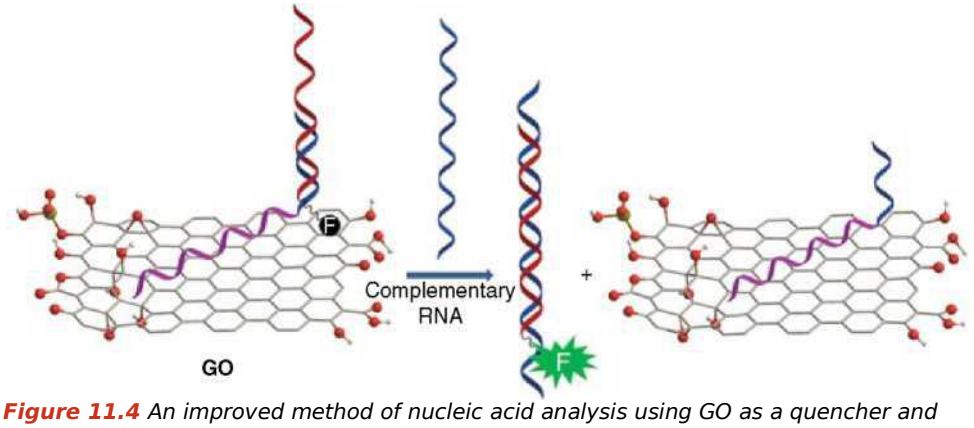

Figure 11.4 *An improved method of nucleic acid analysis using GO as a quencher and probe binder. A toehold sequence of the probe was selected [72]*

from the GO followed by the hybridization of the probe with the target in solution is a major pathway of activation of the probe-GO sensors. They have also confirmed that only a small portion of the target is hybridized to the probe, whereas the majority of it remains bound to the GO [71]. The solution to this problem has been suggested by the research groups of Kitamura and Ihara, who separated a target-binding DNA sequence from the GO-binding element (Figure 11.4) [72]. The latter can in principle be any GO-binding chemical moiety that has a substantially stronger affinity toward GO than any analyte present in solution. In the original work, DNA dA_{20} (magenta

colored in Figure 11.4) has been selected as a GO-binding sequence and a toehold construct, containing a doublestranded part covalently connected to the dA_{20} and a single-stranded DNA overhang for anchoring the target. The reporter fluorophore in the resulting sensor has been placed in the proximity to the GO and was, therefore, strongly quenched. In this case the target cannot displace the sensor (dA_{20}-toehold sequence) from the GO. Rather, it hybridizes with one of the strands of the toehold sequence, thereby replacing another strand and leading to the fluorescence dequenching. In this system, the target-induced fluorescence enhancement with respect to that obtained in the presence of a mismatched target has been found to be about 7-fold higher than the same parameter for the parent system with a simple, singlestranded probe.

Another important factor to account for in GO-based DNA detection assays is the level of oxidation of GO (C/O ratio). This factor has not been considered in earlier reports. For example, Nguyen and coworkers have reported that the C/O ratio strongly affects the fluorescence quenching ability of GO as well as its affinity toward singlestranded ONs [73].

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