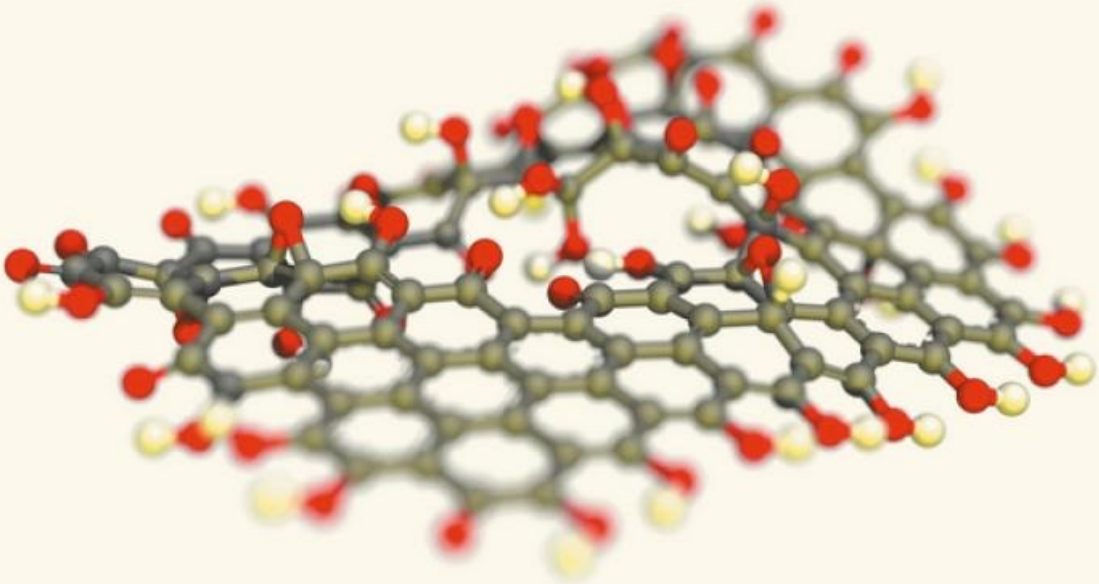
Edited by
Ayrat M. Dimiev
Siegfried Eigler



Graphene Oxide

Fundamentals and Applications

WILEY

Graphene Oxide

Fundamentals and Applications

Edited by

AYRAT M. DIMIEV

Laboratory of Advanced Carbon Nanostructures, Kazan Federal University, Kazan, Russian Federation

SIEGFRIED EIGLER

Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Sweden

WILEY

List of Contributors

Seyed Hamed Aboutalebi Condensed Matter National Laboratory, Institute for Research in Fundamental Sciences (IPM), Tehran, Iran

Young Hoon Cho Department of Energy Engineering, Hanyang University, Seoul, Republic of Korea

Ayrat M. Dimiev Laboratory of Advanced Carbon Nanostructures, Kazan Federal University, Kazan, Russian Federation

Siegfried Eigler Department of Chemistry and Chemical Engineering, Chalmers University of Technology, Gothenburg, Sweden

Mohsen Moazzami Gudarzi Department of Inorganic and Analytical Chemistry, University of Geneva, Geneva, Switzerland

Cary Michael Hayner Chief Technology Officer, SiNode Systems Inc., Chicago, IL, USA

Larisa Kovbasyuk Department of Chemistry and Pharmacy, Inorganic Chemistry II, Friedrich-Alexander-Universität, Erlangen-Nürnberg (FAU), Erlangen, Germany

Anton Lerf Emeritus Walther-Meissner-Institut der Bayerischen Akademie der Wissenschaften, Garching, Germany

Sean E. Lowe Department of Materials Science and Engineering, Monash University, Clayton, Australia

Andriy Mokhir Department of Chemistry and Pharmacy, Inorganic Chemistry II, Friedrich-Alexander-Universität, Erlangen-Nürnberg (FAU), Erlangen, Germany

Anton V. Naumov Department of Physics and Astronomy, Texas Christian University, Fort Worth, USA

Ho Bum Park Department of Energy Engineering, Hanyang University, Seoul, Republic of Korea

Ioannis V. Pavlidis Department of Biochemistry, University of Kassel, Kassel, Germany

11

Toxicity Studies and Biomedical Applications of Graphene Oxide

Larisa Kovbasyuk and Andriy Mokhir

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.

Graphene Oxide: Fundamentals and Applications, First Edition. Edited by Ayrat M. Dimiev and Siegfried Eigler.

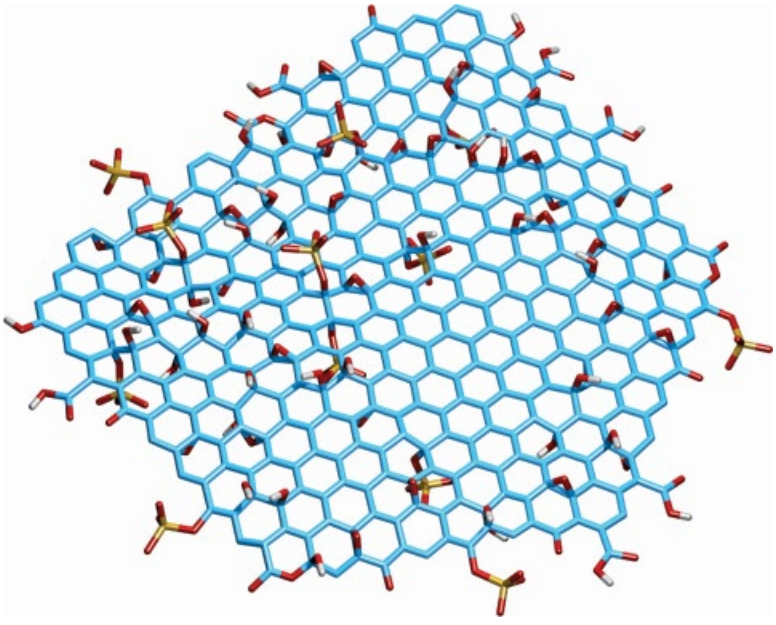


Figure 11.1 *A cartoon illustrating the presence of a variety of functional chemical groups, flat graphene-like regions and non-flat regions on the surface of graphene oxide (GO)*

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

!! Graphene oxide exerts its toxicity on CELL MEMBRANES, PROTEINS AND NUCLEIC ACIDS (i.e on every organ in the human body)

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 μg 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 μg 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 μg ml^{-1}) – hemolysis; GO obtained by Hummers method and sonicated to obtain GOs of different sizes [9];
iv. human skin fibroblasts (≥12.5 μg 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 tumor-initiating 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 ~14 mg kg^{-1}, chronic toxicity of GO has been observed for Kunming mice [10], whereas oral exposure to a dose ~0.8 mg 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].

!! Graphene oxide causes lung injury and chronic lung fibrosis.

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

!! Graphene oxide DESTROYS :- 1. Red blood cells which carry OXYGEN to the whole body, 2. Human Fibroblast cells necessary for structural integrity of tissue & collagen formation, 3. Skin fibroblasts necessary for generating connective tissue and allowing the skin to recover from injury.

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 μg 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 (24–72 h) with variable GO concentrations (5–100 μg 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 ~125–220 nm) stabilized with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[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 24 h with 0.4 mg ml^{-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 $d = 324 \pm 17$ nm at 50 μg ml^{-1} induced hemolysis of >90% RBCs, whereas the usual GO obtained by the Hummers method ($d = 765 \pm 19$ 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 16 μg ml^{-1} GO and CXYG (72 h 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$^{\bullet}$ 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

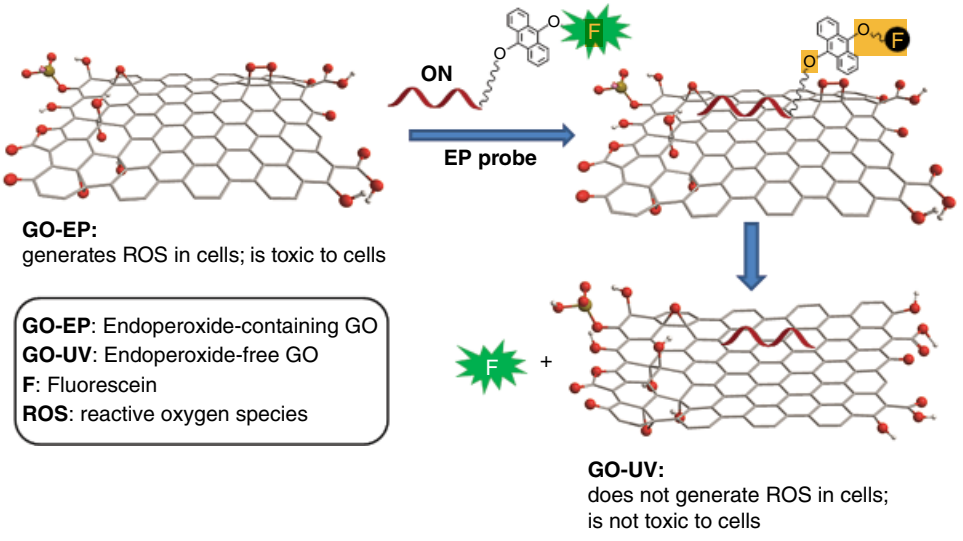


Figure 11.2 *Detection of endoperoxides (EP) on the graphene oxide (GO–EP) surface using fluorogenic probes (EP probe) consisting of an oligonucleotide (ON), which binds strongly to the GO–EP, a reactive moiety (an anthracene derivative) and a fluorescent dye (fluorescein, F) [35]*

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

!! Graphene oxide causes DAMAGE to DNA, MUTATION of genes, SUPPRESSION of body's natural immune response and EARLY CELL DEATH!

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 toxicities. 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 300 nm in size) absorb NIR light more efficiently than the conventional material. In particular, extinctions at 808 and 1200 nm 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.

!! Exposure to graphene oxide may REDUCE the LIFESPAN (THINK POPULATION CONTROL!) 💀