Graphene Oxide

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Endoperoxides Revealed as Origin of the Toxicity of Graphene Oxide

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Abstract: *Potential biomedicinal applications of graphene oxide (GO), for example, as a carrier of biomolecules or a reagent for photothermal therapy and biosensing, are limited by its cytotoxicity and mutagenicity. It is believed that these properties are at least partially caused by GO-induced oxidative stress in cells. However, it is not known which chemical fragments of GO are responsible for this unfavorable effect. We generated four GOs containing variable redox-active groups on the surface, including Mn^{2+}, C-centered radicals, and endoperoxides (EPs). A comparison of the abilities of these materials to generate reactive oxygen species in human cervical cancer cells revealed that EPs play a crucial role in GO-induced oxidative stress. These data could be applied to the rational design of biocompatible nontoxic GOs for biomedical applications.*

Graphene oxide (GO) is a single-layer carbon-based material that can be obtained by oxidation of graphite. It is soluble in water, and permeates cellular membranes. It has been demonstrated that it is an efficient carrier of both high-molecular-weight (nucleic acids, proteins) and low-molecular-weight (anticancer agents, antibiotics) drugs,[1] is suitable for photothermal therapy owing to its strong absorbance in the near-infrared (NIR) spectral region, and can be applied in biosensing.[2] However, GO often exhibits cytotoxicity and mutagenicity, which limits its possible applications in medicine.[3] The latter undesirable properties are caused by the ability of GO to damage cellular membranes, to interact with and to modify genomic DNA, and to increase the concentration of intracellular reactive oxygen species (ROS).[3,4] It has been demonstrated that co-treatment of cells with GO and *N*-acetylcysteine (NAC) alleviates the toxicity of GO.[4b] Since NAC is an ROS scavenger, these data indicate that GO-induced oxidative stress can be considered as one of the key factors defining its toxicity. From a chemist's perspective, it is not yet clear which chemical fragment (or fragments) is responsible for GO-induced oxidative stress. This is an important question, since such knowledge would help in the design of nontoxic, biocompatible GOs suitable for biomedical applications.

GO contains flat regions consisting of a network of sp^2-hybridized C atoms, together with non-flat regions and edges containing epoxide, hydroxy, and carbonyl groups,[5] and sulphate esters.[6] Moreover, several redox-active moieties have been detected or predicted. In particular, GO obtained by oxidation with $KMnO_4$ contains carbon radicals (C$^{\bullet}$)[7] and Mn^{2+} ions,[8] whereas the existence of endoperoxides (EPs) have been predicted based on ab initio calculations[9] and supported by a range of indirect experimental evidence.[10] In this study, we explored the involvement of these redox-active moieties in GO-induced intracellular ROS generation. The presence of C$^{\bullet}$ and Mn^{2+} ions on the GOs was monitored by ESR spectroscopy.[7,8,11] Since no suitable analytical method was available for EPs, we developed an EP-selective fluorogenic probe.

First, we optimized the method for removing Mn^{2+} from GO.[12] In particular, we observed that washing GO with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) solution was sufficient to remove ESR-active Mn^{2+} ions (Figure 1 A). Interestingly, EDTA was found not to react with C$^{\bullet}$, which could be confirmed by following the characteristic peak at $g = 2.003$ in the ESR spectrum. The resulting material was named GO(−Mn), which indicates that it lacks Mn^{2+}. According to AFM analysis GO(−Mn) has a morphology similar to that of GO[12] (Figure 1 B). Furthermore, statistical Raman analysis of the intensities of the D and G peaks (I_D, I_G), as well as the full-widths at half-maximum (Γ) of the 2D peak, in the Raman spectra of the in situ reduced GO(−Mn) revealed that the number of defects remained identical for both the treated and original GO materials: $I_D/I_G = 2.8 \pm 0.2$, $\Gamma_{2D} = 70 \pm 5\ cm^{-1}$ for GO(−Mn) (Figure 1 C) and $I_D/I_G = 3.0 \pm 0.3$, $\Gamma_{2D} = 62 \pm 8\ cm^{-1}$ for GO.[12] These data indicate that EDTA does not harm the carbon framework, which would imply the generation of new functionalized edges. Zhang and co-workers reported that GO is a strong oxidant of I^- ions.[12c] Although we confirmed this effect for GO, GO(−Mn) was found to be unable to oxidize I^- (Figure S1 in the Supporting Information). We can thus conclude that I^- is not simply oxidized by C$^{\bullet}$ present on GO,[7b] but that this is a more complex process that is dependent upon Mn^{2+}.

Next, we developed an EP-responsive probe. Since EPs are converted into singlet oxygen ($^1O_2$) in a thermolysis reaction,[13] we envisioned that some amount of $^1O_2$ would be present in proximity to sites of surface-bound EPs. To detect this $^1O_2$, we first applied two known $^1O_2$ traps: 4,4′-(isobenzofuran-1,3-diyl)dibenzoic acid (**P1**)[14] and 9,10-di(3-hydroxypropyloxy)-anthracene (**P2**).[15] However, both **P1** and **P2** were found to be unreactive towards GO. To increase

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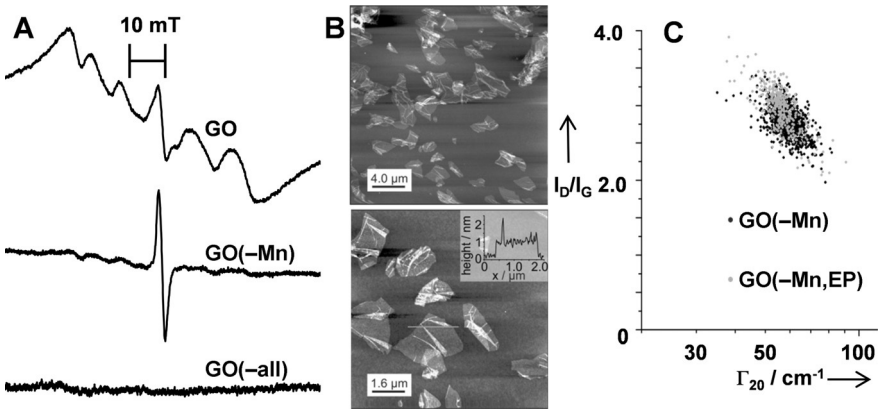

Figure 1. A) ESR spectra of frozen (−78 °C) suspensions of GOs in water (10 µg mL^{-1}). B) Representative AFM images of GO(−Mn). C) Raman spectroscopic data obtained for GO-(−Mn) and GO(−Mn, EP) reduced in situ to graphene as described in the Supporting Information.

the sensitivity of $^1O_2$ detection, we used the probe Fl ~ AN ~ ON (Figure 2 A and the Supporting Information). It contains fluorescein derivative (Fl) as a reporting unit, a 9,10-dialkoxyanthracene linker (AN) as a $^1O_2$-sensitive fragment,[15] and an oligonucleotide (ON) as a GO-binding unit. In contrast to standard $^1O_2$ traps, this compound binds to GO through a strong ON/GO interaction (see the Supporting Information),[16] thereby positioning the reactive moiety (AN) in proximity to EPs on the GO surface, which facilitates the reaction between them. In this probe, the dye Fl exhibits 5-fold lower fluorescence quantum yield than the free Fl. We were pleased to observe that upon addition of freshly prepared GO, the fluorescence of the probe first decreased, thus indicating binding to GO, and then increased as a result of Fl release (Figures S2, S3 in the Supporting Information), as confirmed by HPLC and MALDI-TOF mass spectrometry (Figure S6, trace 4). Interestingly, the reactivity of GO gradually decreases with aging at 4 °C, reaching saturation after 3 weeks (Figure 2 B). In further experiments, GO older than 3 weeks was used to obtain reproducible results. After 80 min incubation of Fl ~ AN ~ ON (100 nM) with GO (10 µg mL^{-1}), the fluorescence reached a value of approximately 15, whereas full cleavage of this probe would produce an equimolar solution of Fl with a fluorescence intensity of 35. Based on these data, we concluded that in the latter reaction, 42 ± 1 nM of the probe was converted, which corresponds to one EP per ca. 2×10^4 C atoms of GO. This estimation is based on the plausible assumption that the fluorescence of Fl ~ AN ~ ON bound to the GO is negligible. For example, we observed that the fluorescence of a model compound Fl ~ ON is fully quenched upon binding to the GO (Figure S2). Addition of more than 100 nM probe to the GO does not substantially facilitate the cleavage reaction, which indicates that at 100 nM, binding of the probe to GO is saturated. Surprisingly, we observed that the rate of probe activation was practically independent of NaN_3 and not affected by substitution of H_2O for D_2O (Figure S4). These data indicate that contrary to our initial expectations, $^1O_2$ is not involved in the reaction.

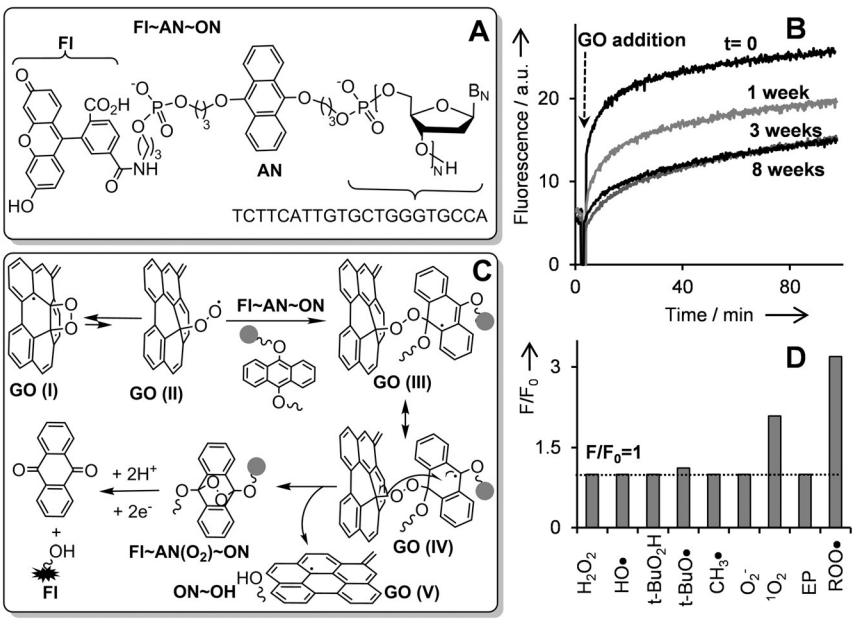

Figure 2. A) Structure of Fl ~ AN ~ ON. B) increase in the fluorescence of this probe (100 nM) upon addition of GO (10 µg mL^{-1}), which after its synthesis was kept at 4 °C for the time shown on the plot. C) A suggested mechanism for the activation of Fl ~ AN ~ ON in the presence of GO. D) Fluorescence increase (F/F_0) observed in solutions of Fl ~ AN ~ ON (100 nM) upon reaction with different ROS; F_0 is the fluorescence of this solution before adding ROS and F is that after adding ROS (further experimental details can be found in the Supporting Information). EP = endoperoxide. ROO$^\bullet$ = $H_2NC(=NH_2^+)C(OO^\bullet)(CH_3)_2$.

Since GO (I, Figure 2 C) contains EPs and C$^\bullet$, it is sensible to assume that peroxy radicals (II) can be formed. II can interact with the probe with formation of the peroxo-bridged species III, which is stabilized by delocalization (IV). Finally, IV can be converted into Fl ~ AN(O_2) ~ ON and decomposed with the formation of Fl. This hypothesis is supported by the results of an experiment in which we confirmed that Fl ~ AN ~ ON is efficiently cleaved by peroxy radicals ROO$^\bullet$ generated from α,α′-azodiisobutyramidine dihydrochloride (AAPH; Figure S6, trace 2). By contrast, this probe was found to be insensitive to other redox-active species that can be potentially present on the GO surface (Figure 2 D). Moreover, the model compound 9,10-di(3-hydroxypropyloxy)anthracene was

found to react with AAPH-derived ROO• with formation of anthraquinone and 1,3-propanediol (Figure S7).

We observed that the reactivity of GO(−Mn) and GO with Fl ~ AN ~ ON is practically identical (Figure S8), which indicates that treatment of GO with EDTA does not affect EPs. Since it is well known that EPs decompose under UV irradiation,[13] we obtained GO(−Mn, EP) through irradiation of GO(−Mn) with UV light. We observed that GO(−Mn, EP) exhibits substantially lower reactivity towards Fl ~ AN ~ ON, which confirms the removal of the surface-bound EPs (Figure S9). These results also validate the applicability of the probe. Interestingly, the intensity of the C•-specific ESR signal was even slightly increased upon UV irradiation. GO(−Mn, EP) thus lacks both Mn^{2+} ions and EPs, but still contains C•. Furthermore, we observed that treatment of GO with basic aqueous solution leads to the removal of all reactive groups, including C•. This material was named GO(−all).

Finally, the ability of all of the prepared GOs to generate ROS in HeLa cells was explored by using 2,7-dichlorofluorescin diacetate (DCFH-DA) in combination with flow cytometry (Figure 3). We observed that despite its high cell-membrane permeability, GO(−all) had practically no effect on the intracellular ROS amount at concentrations of up to 10 μg mL^{-1}. By contrast, the approximately 2-fold less permeable GO and GO(−Mn) increased the ROS amount in a concentration-dependent fashion in the range between 2.5 and 5 μg mL^{-1}. These data indicate that Mn^{2+} is not important for ROS generation in cells. In contrast to GO and GO(−Mn), GO(−Mn, EP) causes practically no increase in ROS in cells at up to 10 μg mL^{-1}. Therefore, we conclude that surface-bound EPs rather than Mn^{2+} or C• define the ability of GO to increase oxidative stress in live cells. As expected, these data correlate with the toxicity of the GOs (Figure 3).

In summary, we confirmed that surface-bound endoperoxide groups are responsible for GO-induced oxidative stress and GO cytotoxicity in HeLa cells. We suggest a simple means of alleviating this undesired effect through irradiation of GO with UV light or treatment with aqueous base. The results of this study will facilitate future biomedical applications of graphene oxide.

GOs	C•/ Mn^{2+}/ EP	Rel. Permeability[a]	Cell viability (%)[b]
GO	+/+/+	1	72±3
GO(–Mn)	+/-/+	1.3±0.0	55±8
GO(–Mn,EP)	+/-/-	1.5±0.2	87±10
GO(–all)	-/-/-	2.4+0.4	99±7

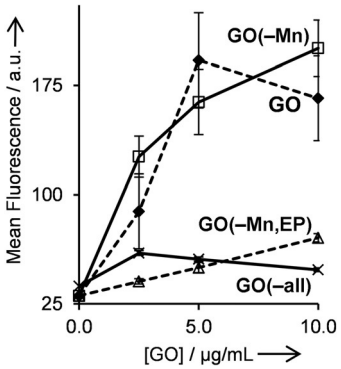

Figure 3. Table: Cellular permeability of GOs and effects of GOs on cell viability. [a] The permeability of GO was taken as a reference. [b] HeLa cells were incubated with GOs (20 μg mL^{-1}) for 48 h. Plot: GO-induced increase in ROS concentration (proportional to mean fluorescence) in HeLa cells; further experimental details are provided in the Supporting Information.

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