Dispersibility-Dependent Biodegradation of Graphene Oxide by Myeloperoxidase

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Understanding human health risk associated with the rapidly emerging graphene-based nanomaterials represents a great challenge because of the diversity of applications and the wide range of possible ways of exposure to this type of materials. Herein, the biodegradation of graphene oxide (GO) sheets is reported by using myeloperoxidase (hMPO) derived from human neutrophils in the presence of a low concentration of hydrogen peroxide. The degradation capability of the enzyme on three different GO samples containing different degree of oxidation on their graphenic lattice, leading to a variable dispersibility in aqueous media is compared. hMPO fails in degrading the most aggregated GO, but succeeds to completely metabolize highly dispersed GO samples. The spectroscopy and microscopy analyses provide unambiguous evidence for the key roles played by hydrophilicity, negative surface charge, and colloidal stability of the aqueous GO in their biodegradation by hMPO catalysis.

1. Introduction

In recent times, intensive research efforts have been focused on graphene-based nanomaterials,[1] in particular, graphene oxide (GO) has gathered increasing attention in drug delivery, bioimaging, tissue engineering, biosensors, etc.[2] The largest surface area of GO and its better colloidal stability in aqueous media, have made it as a potential candidate for several biomedical applications as alternative to carbon nanotubes (CNTs).[3] Inevitably, graphene-based materials start to be part of our daily life and of the environment by their manufacturing and eventual disposal.[4] Thus, it is essential and fundamental to evaluate their systematic toxicological effects before their usage in different domains.[5] Similar to in vitro and in vivo cytotoxic studies of CNTs,[6] safety issues related to graphene-based materials have raised many serious concerns about possible adverse effects on the health of humans and other living beings.[3a] Numerous reports have confirmed that GO can cause dose-, time-, thickness-, and size-dependent cytotoxicity including in vivo toxicity, platelet aggregation, pulmonary thromboembolism, granuloma formation, and it can also attenuate immune response.[7] Even similar graphene-based materials synthesized by different methods often exhibit different physicochemical properties[8] leading to contradictory results on toxicity.[7b,8] In view of these results, health impact, biopersistence along with environmental accumulation are considered as key issues for the development of GO in the biomedical
field. In nanomedicine and other related areas, since eventually these factors determine the safety profile from the time of entry into living organisms.\cite{9} Though major research has been focused to develop biocompatible GO, still very little has been reported about its biodegradation. Therefore, it is mandatory to elucidate the key aspects associated with biodegradability of graphene materials for their real translation into possible clinical innovations as well as for their safe disposal in the environment.

Initially, Star and co-workers\cite{9} demonstrated the biodegradation of GO via the catalysis of horseradish peroxidase (HRP). Later, the possibility for biodegradation of graphene by intravenously administration in mice and in vitro on lung macrophages was investigated.\cite{10} However, the data about the mechanisms of clearance and the enzymes involved in this process were not described. In addition, biodegradation of GO was enhanced by conjugating it with a polyethylene glycol (PEG) via a cleavable disulfide bond.\cite{11} Very recently, it has been demonstrated that lignin peroxidase (LiP), secreted by various primary phagocytic cells in the innate immune system has not been reported yet.\cite{12} In particular, MPO is the most abundantly expressed by neutrophils during the first phase of inflammation, which produces potential oxidants such as HOCl and reactive radical intermediates, essential to kill the pathogens or to eliminate endogenous molecules.\cite{13} In this context, we believe that it would be very interesting to study the biodegradation of GO by catalysis of MPO, as GO is completely different in structure, shape, dimensions, and reactivity compared to CNTs.\cite{14}

Herein, we have explored the biodegradation of GO catalyzed by human myeloperoxidase derived from neutrophils in the presence of hydrogen peroxide. The degradation of GO obtained through a modified Hummers’ method (GO 1) was compared to the biodegradability of two commercially available GO samples (GO 2 and GO 3) displaying variable dispersibility in aqueous media. All degradation processes were characterized by UV-Vis-NIR spectroscopy, Raman spectroscopy, transmission electron microscopy, selected area electron diffraction (SAED), atomic force microscopy, and dynamic light scattering (DLS) techniques. The results demonstrated that the degradation of GO by hMPO catalysis strongly depends on its colloidal stability and dispersibility in aqueous solution, since degradation was observed mainly for the highly dispersed GO samples, while negligible changes were observed for one of the commercial samples that resulted more aggregated.

2. Results and Discussion

2.1. Enzymatic Degradation of Different GO Samples

For this study, we have focused our attention on the capacity of hMPO to degrade three different GO samples. These samples exhibit different degree of dispersibility in water. The GO sample (GO 1) with the highest dispersibility was obtained directly as an aqueous suspension by a modified Hummers’ method,\cite{16} while the other two GO samples, namely, GO 2 and GO 3, are commercially available powders from Grupo Antolin and NanoInnova, respectively. We have initially compared the dispersibility of the three GOs in water. The brownish color of GO 1 and GO 2 dispersions indicated the characteristic color of the graphene oxide and its transparency reveals the presence of highly stable colloidal suspensions in the aqueous media without any aggregation occurring (Figure S1, Supporting Information).\cite{17} In contrast, the pale blackish color of GO 3 accompanied by aggregation supported the presence of few exposed oxygenated hydrophilic groups on its graphenic lattice compared to GO 1 and GO 2, resulting in a weak colloidal stability, leading to complete precipitation in ten minutes. The dispersibility in water followed the trend: GO 1 > GO 2 > GO 3. The zeta potential (\(\xi\)) of the three samples followed the same trend. We measured the values of \(-42.3, -41.8,\) and \(-27.9\) mV for GO 1, GO 2, and GO 3, respectively (Figure S2, Supporting Information). ASTM (American Society for Testing and Materials) defines that colloidal suspension with zeta potential range from 30 to 40 mV (either positive or negative) has moderate stability, while zeta potential higher than 40 mV (either positive or negative) indicates high stability.\cite{18} On this basis, GO 1 and GO 2 have zeta potential below \(-40\) mV forming highly stable colloids, whereas GO 3 stands below the threshold and showed very poor stability. The zeta potential is related to the ionization of the carboxylic acids and, possibly, phenolates that are known to exist on the GO sheets.\cite{19} Thus, the high negative zeta potential values are indicative of a high amount of oxygen-containing functional groups present on the graphenic lattice of GO, that are responsible for the negative charge of GO sheets.\cite{20} Eventually, the zeta potential is in close relation with the colloidal stability of the GO dispersion due to electrostatic repulsion between the individual sheets. Hence, GO 1 was found to be the most stable colloid in pure water without any aggregation and precipitation observed over at least one year, while GO 2 was partially aggregated over 24 h and GO 3 was completely aggregated within 10 min as shown in Figure S1, Supporting Information. In addition, X-ray photoelectron spectroscopy (XPS) analysis of the three GO samples revealed that the percentage of carboxylic groups present on the graphenic lattice of each GO is different. We measured 3.0%,\cite{16b} 1.52%, and 1.06% of carboxylic groups for GO 1, GO 2, and GO 3, respectively (Figure S3, Supporting Information). The XPS results are in line with the trend observed from the zeta potential and dispersibility analysis described above. The aggregation of GO 3 was also supported by the observation of much thicker GO sheets under TEM (Figure S4, Supporting Information). Further, AFM analysis revealed that the thickness of GO 1 varies between 1 to 2 nm (Figure S5A, Supporting Information), allowing to conclude that GO 1 is mainly present as monolayer sheets with thickness \(\approx\)1 nm (Figure S5B, Supporting Information) along with several irregular particles with thickness from 3 to 10 nm. Differently, the thickness of the GO 3 sheets (Figure S5C, Supporting Information) reached 20 nm, confirming the...
presence of aggregated GO sheets. This is likely due to van der Waals or π-π stacking forces because of fewer exposed oxygen functional groups (mainly carboxyl groups) on its graphenic basal plane when compared to GO 1 and GO 2, as revealed by the zeta potential and XPS analysis.

Following the study of colloidal stability, the three solutions of GO were treated with hMPO to prove their degradability induced by this type of intracellular peroxidase. hMPO is a dimeric protein with a molecular weight of ≈146 kDa, which is highly cationic (isoelectric point = 9.2) mainly due to the positively charged arginine residues (i.e., Arg 294, Arg 307, and Arg 507).[^14c][^21] Thus, hMPO is well-known to bind to negatively charged species such as bacterial membranes, endothelial cell surfaces, polyanionic glycosaminoglycan chains like heparan sulfate, and to oxidized CNTs.[^22] Earlier studies reported the strong electrostatic complexation of hMPO with highly negatively charged heparan sulfate and oxidized CNTs.[^21][^22] In addition, it was discussed that the electrostatic interaction of positively charged Arg residues of a protein with the carboxylic groups of CNTs might be a key parameter for stabilizing the binding of oxidized CNTs with many other proteins.[^22b] Similarly, we observed the formation of a strong electrostatic complex mainly at the air/aqueous interface by adding hMPO into GO 1 and GO 2 in phosphate buffer solutions as shown in Figure 1A,B (see vials GO + MPO – 0 h). This type of complex formation was not observed in the case of GO 3 (Figure 1C) because of the poor colloidal stability and lower percentage of carboxylic groups on its graphenic surface that likely lead to an unfavorable interaction with the enzyme. Thus, the binding ability of the GO (because of carboxylic groups on the surface) with cationic hMPO is directly related to its zeta potential, the percentage of carboxylic groups on its surface as well as the colloidal stability.

The electrostatic binding between our GO samples and the enzyme was also evaluated by gel electrophoresis (Figure 1D, top). It revealed that when hMPO was incubated with both GO 1 (Figure 1D, Lane 2) and GO 3 samples (Figure 1D, Lane 4), the enzyme band at ≈150 kDa had a reduced intensity compared to the band relative to the same amount of hMPO alone (Figure 1D, Lane 5). The densitometry analysis (Figure 1D, bottom) provided evidence for some differences between the two GO samples. In fact, it seemed that GO 1 sample was responsible for a major retention of the hMPO enzyme compared to GO 3. This observation suggests that hMPO firmly binds to the GO surface, especially in the case of GO 1 sheets, hampering the migration of the protein into the gel. This strong interaction between the enzyme and the GO sheets likely triggers the enzymatic degradation of the graphenic planes, similar to oxidized CNTs.[^21] The reduced binding tendency of GO 3 to hMPO can be explained in view of the difference in the zeta potential, the quantity of carboxyl groups present on its surface and its poor colloidal stability leading to form the multilayered aggregates compared to GO 1. The morphologies of the complexes between GO and hMPO were also characterized using SEM. In good accordance with AFM imaging, Figure S6, Supporting Information, shows that numerous aggregated GO 3 flakes are visible, while isolated GO sheets are very difficult

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[^14c]: Reference 14c
[^21]: Reference 21
[^22]: Reference 22
to distinguish in the case of GO 1 and GO 2. This is due to the weak complex between GO 3 and hMPO, evidencing the multilayer GO sheets, while the strong complex between the GO 1 or GO 2 and hMPO masks the free GO sheets rendering them invisible and impossible to observe.

The biodegradation of all three GO samples was followed by incubating them with hMPO in the presence of H₂O₂ for 24 h. During the degradation process, 200 × 10⁻⁶ m of H₂O₂ was added every hour and hMPO was renewed every 5 h because of the loss in its enzymatic activity (assessed by the measurement of hMPO activity at different time points, data not shown), as already observed during the degradation of α-SWCNTs. The degradation of the GOs was monitored over the time, and the suspensions of GO 1 and GO 2 became translucent after 24 h treatment as shown in Figure 1A,B, while there was no color change in the case of GO 3 (Figure 1C). The incubation of GO with hMPO alone (without H₂O₂) did not cause degradation (data not shown), while a few holes on the edges of GO 1 sheets were observed by incubating this sample for 24 h with H₂O₂ alone (Figure S7, Supporting Information).

To follow the progress of the degradation on the three samples of GO, we combined the results of complementary analytical, spectroscopic and microscopic techniques. First, we applied the DLS technique to get a qualitative insight into the lateral size distribution of GO samples treated with hMPO/H₂O₂. The DLS size distribution analysis of the GO samples is an approximation because the elaboration of the data is based on a spherical model not completely fitting the 2D structure of GO. However, DLS measurements can be considered as relative information for comparing the size of the three different GO samples and their evolution along the degradation process. The obtained size distributions of all samples, including control GO and after treating with hMPO/H₂O₂ for 15 and 24 h are portrayed in Figure 2. Figure 2A and B provide evidence for an extensive size reduction for GO 1 and GO 2 during the treatment when compared with control GO (untreated) sample. Instead, no decrease in the size was observed for GO 3 (Figure 2C) even after 24 h treatment. The size distribution of GO 1 and GO 2 was exceeding 1 µm in the case of control samples, and was reduced between ≈300 to 700 nm for GO 1 and ≈400 to 700 nm for GO 2 after 15 h treatment, respectively. Finally, after 24 h, the size of the remaining fragments of GO 1, the debris and the protein complexes was around 60 to 100 nm, while for GO 2 the size varied from 100 to 200 nm. The DLS results provide a strong

![Figure 2. Size distribution measurements of the three GO samples starting from untreated GO solution, after 15 and 24 h, obtained from DLS analysis. A) GO 1, B) GO 2, and C) GO 3.](https://www.materialsviews.com/doi/10.1002/smll.201500038)
evidence for the occurrence of the biodegradation of GO 1 and GO 2, as highlighted by the notable reduction in their relative dimensions after treating with hMPO/H₂O₂ compared to the control GO. Conversely, changes in the relative size likely generating bigger aggregates have been monitored for GO 3 confirming that the degradation of these sheets was negligible. However, DLS results were not sufficient to unambiguously prove the complete degradation of GO materials. Thus, we used UV-Vis-NIR spectroscopy to follow the changes in the optical absorbance of GO before and after the treatment with hMPO/H₂O₂. As shown in Figure S8, Supporting Information, for both the GO 1 and GO 2 samples the absorbance was significantly reduced after 24 h compared to their control. The reduction in the absorbance is due to the oxidation or the degradation of GO sheets by MPO treatment. These results are consistent with the data reported in the literature for the enzymatic degradation of SWCNTs or the degradation of GO by photocatalysis.[11,21,26]

Then, we extended our study to Raman spectroscopy in order to analyze the characteristic nature of all three GO samples treated with hMPO/H₂O₂ for 0, 15, and 24 h (Figure 3). The intensity ratio between the characteristic D band (=1350 cm⁻¹) and G band (=1600 cm⁻¹) or \( I_D/I_G \) provides information about the disorder in the graphitic lattice of the GO sheets.[27] For the GO 1 sample, between 0 and 15 h, \( I_D/I_G \) increased from 0.99 to 1.16 as displayed in Figure 3A. The increase in \( I_D/I_G \) could be due to the formation of a number of defect sites on the graphenic lattice of GO 1 as result of MPO catalyzed oxidation, which is similar to the oxidation of GO by HRP reported earlier.[9] GOs were produced using modified Hummers’ method in the both cases. However, the absolute intensity of the characteristic broad D and G bands of GO remarkably diminished after 15 h. Finally, after 24 h of hMPO/H₂O₂ treatment, the D and G bands disappeared confirming the complete degradation of GO 1. For GO 2 sample, the D/G intensity ratio decreased from 0.78 to 0.62 after 15 h. At the end of the experiment (24 h) the D and G bands did not completely disappear. While the D band is almost reaching the baseline, there is still a little peak for G band revealing that the degradation of GO 2 is probably incomplete. Importantly, the decrease \( I_D/I_G \) in the case of 15 and 24 h treated GO 2 sample shows that there could be the formation of more amorphous carbon structures or sp² noncrystalline carbon phase due to high defect density as reported previously.[28,29] The similar type of low \( I_D/I_G \) ratio was reported for the graphene quantum dots (GQDs) synthesized through photo-Fenton’s reaction, involving the generation of hydroxyl radicals[30] and graphene nanomeshes formed by treating with nitric acid.[31] In addition, the incomplete degradation was supported by observing a few tiny residual particles in the pale brown color at the bottom of the vial after 24 h as shown in Figure 1B. The two different D/G ratio trends as observed in the case of GO 1 and GO 2 can be attributed to the different synthetic methods and the difference in the structure of GO sheets. For GO 3, intensity ratio of D/G increased from 0.94 to 0.99 at 15 h, and it further increased to 1.0 after 24 h (Figure 3C). The results obtained for GO 3 are in contrast with the other two highly dispersed GOs, revealing that there was no degradation of GO 3 even after 24 h treatment. The slight increase in the D/G ratio may be due to the oxidation of some GO 3 sheets by hMPO in the presence of H₂O₂ (less aggregated sheets) leading to the formation of a few defects on the graphitic lattice of the sheets.

To complement the results of Raman spectroscopy, the significant changes in the morphology and structure of the GO 1 and GO 2 samples treated with hMPO/H₂O₂ were also followed by TEM all along the progress of degradation (Figure 4). TEM images of GO 1 and GO 2 treated for 15 h are shown in Figure 4B,E. The characteristic sheet shape and flat morphology of GO, as observed in the control GO samples (Figure 4A,D) started to significantly disappear. Numerous damaged parts throughout the graphenic lattice of GO are visible, forming perforated graphene oxide, where most of the holes were connected to each other. This morphology is very similar to the “holey graphene oxide” obtained through hydroxyl radicals attack generated via HRP mediated catalysis[9] and gold nanoparticles mediated catalysis.[32] After 24 h, only a few visual fields showed evidence of residual carbonaceous material, while all GO 1 sheets completely degraded (Figure 4C) and the large GO sheets were no longer present. The holey GO 1 sheets (15 h treated) lost entirely their holey morphology and we could hardly find a few amounts of debris of irregular size varying between ≈50 and 200 nm, in good accordance with the DLS data (Figure 2A). In the case of GO 2 after 24 h, the large GO sheets were also extensively degraded into several small particles of size ranging from ≈100 to 200 nm (Figure 4F). In addition, residual carbonaceous materials containing few fragments, tiny nanoparticles, including rod-shaped structures are visible (Figure 4F and Figure S9A, Supporting Information). Similar types of rod-shaped morphologies were also observed in the control GO 2 sample (Figure 4D and Figure S9B, Supporting Information). These nanoparticles similar to fibers derive from the starting material used for the production of this GO.
and cannot be completely removed from the final manufactured product. They are relatively thick and they remained almost intact even after 24 h treatment. In contrast to GO 1 and GO 2, there were no signs of degradation or changes in the structure of GO 3 after 15 and 24 h. Thick and large sheets of GO 3, similar to the control sample (Figure 4G), are clearly visible in Figure 4H,I. However, a few signs of degradation or changes in the morphology of GO 3 sheets were observed in the 24 h treated samples as pointed by the black dotted arrow in Figure 4I. In addition, a few degraded or heavily damaged GO 3 sheets, which appeared to be thinner or nonaggregated are shown in Figure S10, Supporting Information, along with aggregated sheets. Overall, TEM observations confirm the DLS data for all three GO samples.

Finally, high-resolution TEM along with SAED analyses were employed to gain additional information about the crystalline patterns and the drastic changes in the graphenic structure of GO samples and the residual carbonaceous fragments during the enzymatic oxidation (Figure 5), as observed in the TEM images. The SAED pattern of control GO 1 shows a typical ring like pattern along with a few diffraction spots indicating its polycrystalline nature (Figure 5A). The polycrystalline nature is mainly due to merging of diffraction spots of GO few-layer formation. After the treatment with hMPO/H₂O₂ for 15 h, large holes in the graphenic lattice of GO 1 were clearly observed as shown in Figure 5B, where a few holes of more than 100 nm size were present. The SAED patterns of GO 1 in the various regions indicate differences in the crystallinity of the GO samples based on the incubation time with hMPO/H₂O₂. GO 1 remaining sheets after 15 h treatment have two distinct regions. One area consists of a large number of holes found to be nearly amorphous as indicated by the diffuse diffraction pattern shown in the inset in Figure 5B1. The diffraction pattern at the place without holes was instead crystalline as shown in Figure 5B2. The holes in the graphenic lattice are likely deriving from the formation of the defects via cleavage of the carbon atoms connected to oxygenated groups by potent oxidants such as hMPO intermediate radicals and hypochlorite. Furthermore, the SAED analysis of the few residual carbonaceous material after 24 h treatment with hMPO/H₂O₂ revealed that the remaining species or fragments have large amorphous regions.

Figure 4. TEM images of hMPO-mediated degradation of GO at three time points: control (t = 0), t = 15 h, and t = 24 h; images A–C) correspond to GO 1, D–F) correspond to GO 2, and G–I) correspond to GO 3. Scale bar represents 500 nm. Black arrows (B and E) point to holes formed on GO 1 and GO 2 after 15 h; white arrows in (D) and (F) mark rod-shaped particles in GO 2 sample before and after the degradation, and dotted black arrow in (I) indicates degraded GO 3 sheets.
along with poor or disordered crystalline areas (Figure 5C). However, the carbonaceous residues after 24 h treatment of GO 1 were found to consist of extended amorphous regions alternated with small crystalline domains (Figure 5). Similar types of crystalline as well as amorphous regions were also visible in the case of residual carbonaceous fragments of GO 2 after 24 h treatment with hMPO/H$_2$O$_2$ (Figure S11, Supporting Information).

In addition to HRTEM analysis, we also employed AFM to acquire more details about the topography and thickness of the residual carbonaceous fragments of GO 1 and GO 2 after 24 h. The AFM images of the residual GO 1 fragments (Figure S12A, Supporting Information) show highly porous sheets without their characteristic smooth surface along with several large holes or patches on the entire surface similar to HRTEM images (Figure 5C). However, the thickness of these residual fragments varied between ≈2 and 15 nm, likely due to the presence of salts, enzyme, and residual particles. In addition, AFM images of residual fragments of GO 2 (Figure S12B, Supporting Information) show several tiny particles, including a few big aggregated and rod-shaped structures as we observed earlier in the TEM images (Figure 4F and Figure S9A, Supporting Information) with thickness ranging from 10 to 50 nm. Moreover, the mass spectra analysis of these carbonaceous residues after 24 h treatment allowed to detect numerous peaks between m/z 100 and 1000 Da (Figure S13, Supporting Information). The position of the peaks are similar to the oxygenated products or fragments obtained after chemical degradation of graphene oxide by photo-Fenton’s reaction via generation of hydroxyl radicals. Thus, we believe that the oxygenated products or the fragments formed after 24 h treatment may consist of condensed or conjugated aromatic rings with oxygenated groups. Analysis of the remaining fragments to assess their chemical composition is important but beyond the scope of this work. The degradation was instead not observed in the case of GO 3. This is certainly due to stacking problems of this type of GO layers forming aggregated sheets or unexfoliated graphitic oxides.

2.2. Mechanism of GO Degradation

During the inflammation process, neutrophils are rapidly recruited at the sites of pathogens or infections and secrete MPO. MPO catalyses the reaction with chloride ions along with H$_2$O$_2$ to produce strong oxidants such as HOCl and reactive radical intermediates of the MPO against exogenous materials. The enzymatic cycle of hMPO involves reaction of H$_2$O$_2$ with native hMPO (Fe-III state) forming Compound I [Fe (V) species] (Figure S14, Supporting Information). Compound I can undergo either two electron reduction with halide or pseudo halide ions to form hypohalous acid (i.e., halogenation cycle) or undergo two sequential one-electron reductions via Compound II with consequent radical formation (i.e., peroxidase cycle). The generated potent oxidants, responsible for the antimicrobial activity of neutrophils, were also shown to cause the biodegradation of implants made of poly(ester)urea-urethane, extracellular matrix such as heparan or chondroitin sulfate, and of oxidized SWCNTs, PEGylated carbon nanotubes including nitrogen-doped CNT cups. hMPO could oxidize the multiple substrates because of the high redox potentials of Compound I, Compound II, and hypochlorite were mostly responsible for the significant degradation of CNTs. hMPO could oxidize the multiple substrates because of the high redox potentials of Compound I/native enzyme (1.16 V), Compound I/Compound II (1.35), Compound II/native enzyme couples and HOCl (1.48 V) via halogenation and peroxidase cycles. Thus, the high redox potentials of these species are also likely playing a key role in the degradation of planar GO sheets similar to the degradation of oxidized CNTs. On the basis of GO degradation by
hydroxyl radicals generated via photo-Fenton’s reaction,[30] HRP catalysis[9] as well as photocatalysis,[26] we assume that the degradation of GO by hMPO catalysis likely starts at the level of carbon atoms connected with oxygen containing groups in the graphenic lattice. This was supported by the Raman analysis, where the characteristic G and D bands in 15 h treated samples of GO 1 and GO 2 clearly diminished. The reactive radical intermediates of hMPO can cleave the carbon–oxygen bonds of epoxides and tertiary hydroxyl groups introduced in the lattice of GO by the initial oxidation of H$_2$SO$_4$ and KMnO$_4$ used in its preparation.[9] In addition, hMPO is known to catalyze the epoxidation as well as the cleavage of carbon–carbon bonds in the presence of hydrogen peroxide.[41] Furthermore, the previous degradation studies of CNTs by hMPO catalysis emphasized the importance of the strong oxidant HOCl in the degradation process deriving from a synergistic effect along with reactive intermediates of hMPO.[14b,21,42] When compared with larger reactive intermediates of hMPO, smaller oxidant like HOCl has the ability to diffuse from the active sites and oxidize CNTs to yield carboxyl and hydroxyl groups on the surface.[43] In addition, HOCl is also known to react with the double bonds of lipids to give chlorohydrin and eventually yield epoxides.[22a] Therefore, HOCl could significantly damage the GO sheets during the degradation process by reacting with C=C bonds on the graphenic lattice yielding to new carboxyl, hydroxyl, and epoxide groups. Subsequently, these new oxygen-containing groups are cleaved by the hMPO reactive radical intermediates. It has been previously reported that the surface charge seems also to play a key role in the biodegradation of ox-SWCNTs by hMPO catalysis. It was demonstrated that the biodegradation was more effective on negatively charged materials such as oxidized CNTs[14a,21,43] and graphene oxide[9] compared to pristine CNTs or rGO, where peroxidase intermediates (from MPO, HRP, and EPO) were in strong interaction with negatively charged materials[40a] As a consequence, the positively charged amino acid residues of the proteins can directly bind to the opposite charged surfaces of CNTs, driving the catalytic enzymatic process.[21,40a,46] As hMPO is a highly cationic protein, which can strongly interact with negatively charged species,[45] binding between the highly negatively charged GO 1 and GO 2 and the enzyme was clearly demonstrated through gel electrophoresis as well as SEM analysis (Figure 1 and Figure S6, Supporting Information). Similar to oxidized CNTs, hMPO may bind to highly negatively charged surfaces of GO via two putative binding sites located each side of the protein.[21] On the basis of the previous reports on oxidized CNTs,[21,46] the current results on degradation of GO are in good agreement. Indeed, we have not observed a significant degradation in the less negatively charged and aggregated GO 3 sheets when compared with high negatively charged GO 1 and GO 2 by hMPO/H$_2$O$_2$. The complete degradation of GO 1 and significant degradation of GO 2 by hMPO is very encouraging, keeping in mind that neutrophil immune response to pathogens or to nanomaterials is short-lived.[46] The subsequent long-lived inflammatory response induced by the macrophages via engulfing worn-out neutrophils, including the foreign particles[47] may further degrade any residual carbonaceous materials similarly to the degradation of SWCNTs by secretion of highly expressed NADPH oxidase (superoxide) and iNOS (peroxynitrite).[10,40a]

Overall, we proved the total degradation process of GO through hMPO catalysis. The strong oxidants, reactive radical intermediates and hypochlorite generated through hMPO catalysis likely cause further oxidation of highly stable GO colloids by cleaving the hydroxyl and epoxide groups along with the cleavage of adjacent C–C and C=C bonds likely leading to the formation of several holey regions with the amorphous nature in the graphenic lattice after 15 h treatment. By 24 h treatment, the strong oxidants caused a further cleavage of holey regions in the graphenic lattice, leading to the eventual degradation of the whole GO only leaving few residual carbonaceous fragments.

3. Conclusion

In summary, we have demonstrated that hMPO could catalyze the degradation of GO in the presence of H$_2$O$_2$. By comparing the biodegradability of three GO samples with different dispersibility levels, we assessed the capacity of the MPO to completely degrade the most stable GO 1 dispersion, significantly degrade the moderately stable GO 2 dispersion and failed to degrade the mostly aggregated GO 3 dispersion. Overall, the degradation of the various GO dispersions was proportional to the percentage of their carboxylic groups and to their aqueous colloidal stability. As a consequence, we propose that the hydrophilic nature of graphene oxide and its colloidal stability in the aqueous media are crucial factors for its biodegradation by peroxidases. In addition, we suggest that high stable GO colloid may pose less in vivo toxicity risks to the human cells compared with less stable GO colloids. Along with the control of the size of the GO sheets, the aqueous colloidal stability may also be considered while designing the GO materials for biomedical applications. Further functionalization of GO to improve its dispersibility may enhance its biodegradability by peroxidases. However, further investigation on the biodegradability of the GO in vitro and in vivo need to be explored to assess in detail the real biodegradation mechanisms of graphene oxide and the effects of the degradation (by)-products generated during this process. We believe that our finding will also help to guide development of future biomedical applications using graphene oxide by designing biodegradable carriers for delivery of drugs similar to CNTs.

4. Experimental Section

Materials: Graphene oxide sample GO 2 was purchased from Grupo Antolin (Spain) and GO 3 sample (batch: GO.M.60-8) was obtained from Nanolinnova (Spain). hMPO derived from human neutrophils (Athens Research and Technology, USA) with an activity of 180–220 U mg$^{-1}$. Diethylenetriamine pentaacetic acid (DTPA), hydrogen peroxide (30% aqueous solution), NaCl, NaH$_2$PO$_4$, 2H$_2$O, and Na$_2$HPO$_4$·2H$_2$O were purchased from Alfa Aesar and used directly without any further purification.
Degradation of GO by hMPO/H₂O₂: Mixtures of 160 µg of each GO sample (GO 1, GO 2, and GO 3) and 100 µg of hMPO were suspended in 1 mL of 50 × 10⁻³ m phosphate buffer containing 140 × 10⁻³ m NaCl and 100 × 10⁻⁶ m DTPA. H₂O₂ was added at a rate 200 × 10⁻⁶ m h⁻¹ for 5 h. hMPO was renewed every 5 h (5 additions: at 0, 5, 10, 15, and 20 h). The reaction mixture was maintained at 37 °C for 24 h. The control experiments for assessing the degradation of GO samples only by adding the H₂O₂ or hMPO were also performed using the same protocol.

Raman Spectroscopy: Raman analysis of all the GO samples was performed using Raman spectra Renishaw inVia micro-Raman equipped with 514 nm laser and a Leica microscope. All the spectra were recorded with 5% laser power using ×50 objective lens. All samples for Raman analysis were prepared by drop-casting 10 µL of the respective samples on Si window (ThorLabs) and dried for 24 h at room temperature.

Dynamic Light Scattering and Zeta Potential: Samples of GO 1, GO 2, and GO 3 (untreated GO, t=15 h, and t=24 h) were sonicated for 5 min before the DLS size distribution measurements and all the measurements were performed using Zetasizer Nano S (Malvern Instruments) spectrometer operating under 633 nm laser irradiation. All zeta potentials of GO dispersions were measured at pH 7.

Transmission Electron Microscopy: For TEM characterization, 6 µL of each suspension of GO were deposited on a carbon coated copper grids and dried for 24 h before the analysis. All the samples were analyzed by a Hitachi H7500 microscope (Tokyo, Japan) with an accelerating voltage of 80 kV, equipped with an AMT Hamamatsu camera (Tokyo, Japan). HR-TEM and SAED analyses were performed with a JEOL 2100F TEM/STEM electron microscope operating at 200 kV.

Scanning Electron Microscopy: For SEM characterization, 10 µL of each suspension of GO and GO-hMPO samples and MPO alone were deposited on micro cover slips or thin glass slides kept over aluminum stub and dried for 24 h. A thin layer of gold was sputtered on the samples before the analysis.

Agarose Gel Electrophoresis: The immobilization of hMPO on the surface of GO sheets was evaluated by incubating hMPO with the different GO samples during 24 h at room temperature. Briefly, GO and hMPO were allowed to interact by mixing together 10 µL of GO 1 or GO 3 (1 mg mL⁻¹ in milli-Q water at pH 7.4) and 5 µL of hMPO (1 µg µL⁻¹ in phosphate buffer). The final volume of 20 µL was reached by adding phosphate buffer to the GO/hMPO interaction mixture. At the end of the incubation time, Laemmli buffer was added to the mixture and samples (25 µL) were loaded on a SDS-PAGE 4%–15% precasted gel (BioRad). The control GO 1 and GO 3 samples without hMPO were prepared by adding 10 µL of phosphate buffer to 10 µL of GO dispersion (1 mg mL⁻¹ in milli-Q water at pH 7.4).

Gel electrophoresis was then run under nonreducing conditions to evaluate the stable electrostatic interaction between hMPO and GO using a Mini-PROTEAN II apparatus (BioRad) and applying a 150 V during 60 min. After electrophoresis, gels were stained with Coomassie Blue (overnight at room temperature) and dried for 24 h. The bands were revealed after applying a 150 V during 40 min. After electrophoresis, gels were stained with Coomassie Blue (overnight at room temperature) and dried for 24 h. The control experiments for assessing the degradation of GO samples only by adding the H₂O₂ or hMPO were also performed using the same protocol.

Atomic Force Microscopy: AFM measurements of all GO samples were performed under ambient conditions using a Veeco Dimension 3100 AFM equipped with a Nanoscope IV controller. TESP-200V2 tips with a nominal force constant of 42 N m⁻¹ and a nominal tip radius of 8 nm were used in the intermittent-contact (tapping) mode. All samples for the AFM analysis were prepared by drop-casting 20 µL of each sample on freshly cleaved mica sheet and allowed to dry completely before to the analysis.

UV-Vis-NIR Spectroscopy: UV-Vis-NIR spectra of both the GO 1 and GO 2 samples (before and after the degradation) were recorded using a Varian Cary 5000 UV-Vis-NIR spectrophotometer from 400 to 800 nm wavelength. For GO 1, 200 µL of original sample (160 µg mL⁻¹) was diluted with 800 µL PBS (which was used for the degradation experiments) and for GO 2 sample, 200 µL of original sample (160 µg mL⁻¹) was diluted with 600 µL PBS. As hMPO induces an initial aggregation of GO, instead of GO-hMPO 0 h samples, we prepared fresh GO solutions at the same concentration (160 µg mL⁻¹) used for degradation experiments. Then spectra were recorded after dilution as mentioned above.

Liquid Chromatography–Mass Spectrometry (LC/MS): LC/MS analyses were performed on ThermoFisher Finnigan LCQ Advantage Max instrument. An aliquot of 100 µL of degraded GO 1 and GO 2 obtained after 24 h treatment with hMPO/H₂O₂ was injected into a Macherney-Nagel column EC100/2 Nucleodur 100-3 C18 ec (2 mm inner diameter, 100 mm length). The column was eluted with a gradient solvent from A:B (10:90) to A:B (0:100) at a flow rate of 0.30 mL min⁻¹, where A is deionized water with 0.1% (v/v) formic acid and B is acetonitrile.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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