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# Revealing graphene oxide toxicity mechanisms: A reactive molecular dynamics study

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ABSTRACT

We report the first atomistic-scale study on graphene oxide (GO) toxicity, revealing potentially harmful chemical mechanisms of GO during its interactions with biomolecules. ReaxFF-based reactive molecular dynamics study was utilized to assess the impact of different functional groups on GO biocompatibility. Our study predicts different chemical reactions between the GO sheet and peptides that lead to reactive oxidative species (ROS), acidic or basic pHs and cell surface adhesions. We observe that cell-surface adhesion is a result of strong H-bonding and stable π–π stacking interaction. This stacking can also lead to the disruption of the polypeptide secondary structure.

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## 1. Introduction

Geim and Novoselov referred to graphene as "a rapidly rising star on the horizon of materials science" since every day we are confronted by its new applications in science and industry [1]. Amongst such unique applications, medical applications of graphene have recently been in the center of attention [2–6]. Fullerene, nanodiamond, carbon nanotubes (CNTs), and graphene oxide (GO) all are accounted as a family of carbon based materials and are used widely in places where living tissues are involved [7]. Given considerable applications of carbon based materials in medicine and biotechnology such as biosensors, gene delivery and drug delivery [8–10], a thorough investigation on the possible hazards that it can pose on living organisms seems vital [7,11,12]. Despite various studies on toxicity of different carbon based materials, there are still controversies regarding the amounts of toxicity and the underlying mechanisms [13–15].

Among different graphene based materials, graphene oxide has its own particular applications in medicine because of its hydrophilicity. Graphene oxide has similar structure to graphene but the presence of distinct functional groups enhances GO hydrophilicity. Although today a significant number of reports regarding the number and the range of these functional groups are on hand, one may refer to epoxide (–O–), hydroxyl (–OH) and carboxyl (–COOH) as the main underlying functional groups that form GO family. The density and the distributions of these functional groups vary based on the manufacturing methodologies [16]. While Hofmann and Holst's suggest a model which only consists of epoxide [17], Ruess considers a more complex model by adding hydroxyl groups [18]. Nakajima and Matsuo, on the other hand, changed the hypothetical sheet geometry of GO while keeping the functional groups of Ruess model and thereby, they suggested a lattice structure analogous to $(C_2F)_n$ [19,20]. Other functionalizations are also reported by adding amines [21], polyethylene glycol [22] and polyethylenimine [23].

Given its increasing applications, a systematic analysis of its potential hazards to human health seems necessary. Generally carbon based materials are either biocompatible or have insignificant toxic effect. However, functionalized graphene materials namely GO have been reported to show cytotoxic effects on bacteria [24]. To explore such possibilities, different studies have been performed at cellular level [14,25–32]. Not only are these studies controversial, but they also do not represent the precise mechanisms that bring about the described cytotoxicity. For instance, several studies show that graphene based materials that are functionalized with chitosan, peroxide or PEG have excellent biocompatibility; however, other studies have reported toxic properties for GO because of the production of ROS and different metallic compounds [33]. However, because of the complex interactions between GO and cellular membrane, the final conclusion has often been postponed to

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the future investigations. Moreover, to our knowledge, there is no report available to illustrate the underlying chemical reactions that may cause cytotoxicity at atomic level.

Here, we report the role of different functional groups on GO-peptide interactions at atomic level. We performed a series of ReaxFF-based reactive molecular dynamics simulations to see the explicit interactions between a GO sheet and two peptides in water. First, different functional groups are analyzed separately and their reactivity is studied. Afterwards, a system consisting of all of the previous groups is built and investigated. Our results show epoxide and carboxyl groups have catalytic effects on thiol bond (–CSH) deprotonation reaction as well as aldehyde to carboxyl transition in the presence of ROS. Hydroxyl groups are primarily responsible for secondary structure denaturation. In addition, by forming strong hydrogen bonds with hydrophilic side chains of the peptides, hydroxyl groups can augment cell-surface adhesion as reported by Lee et al. [34]. Moreover, our model describes how π–π stacking interaction between the aromatic rings of different amino acid residues (e.g. Tryptophan) plays a vital role in the experimentally observed peripheral cell-surface adhesion [35]. Our procedure to question GO toxicity can also be employed for further studies on other functional groups on graphene as well as evaluation of toxicity ranking for different metal oxide nanoparticles [49].

## 2. Methods

ReaxFF based reactive molecular dynamics is utilized to assess the cytotoxicity of graphene oxide based materials. First, different systems consisting of distinct functional groups are created separately and their interactions with a peptide are studied. Afterwards, a unified model is built that contains all the previously analyzed groups and their combined effects are investigated. Also the possible pathways that peptide-graphene adhesion may arise from are studied.

The objective of this study is to provide a detailed analysis of the chemical interactions between graphene oxide functional groups and different amino acids at atomic scale to clarify the amount of cytotoxicity of GO and the underlying mechanisms of such interactions. Computational limitations hinder larger scale investigations such as full atomistic study on GO-protein system to further analyze tertiary structure interactions, allosteric effects, etc., which would be more descriptive of GO toxicity. However, our results can be generalized for larger systems consisting of significant number of amino acids.

In practice, it is hardly possible to purify GO in order to obtain specific functional groups on GO. Hence, using simulation is an obvious way to perform such studies. Additionally, by employing molecular dynamics techniques, a time dependent picture can be obtained that allows one to see atom-by-atom interactions, which can ultimately describe the toxicity of the material at the femtosecond level. In contrast to experimental approaches, our molecular dynamics method is able to probe chemical reactions in a time dependent manner for each of functional groups that facilitates drawing conclusion about the relevance of each of these groups.

It is essential to mimic the biological environment in our simulations so that the obtained results are trustable. As a first step towards that goal, we consider a 12-residues alpha helix to represent the biomaterial. This peptide is designed to cover most of the significant activities of amino acid side chains such as ionic interactions, H-bonds, hydrophobic interactions and π–π stacking interaction. In order to show that the presented results are not sequence specific, a mutated version of this peptide sequence is also studied (AHAGADACAMWA → ARAGADACAMGA). Note that the peptides are capped by aldehyde to prevent fast denaturation [48].

Graphene with periodic boundary conditions (29.55882 by 34.15774 Å) is built for all studies unless otherwise mentioned. Subsequently, different functional groups are added to graphene. Chemical formula of $C_{96}O_7$, $C_{96}(OH)_7$ and $C_{96}(COOH)_7$ are used for epoxide, hydroxyl and carboxyl, respectively. Fig. 1 shows these different systems schematically. Obviously, such high density of functional groups usually is not used for practical applications; however, we study the extreme case in our model to magnify any possible phenomena. The GO sheet and the peptides are then placed in the vicinity (with an initial 3 Å gap) and then the simulation box

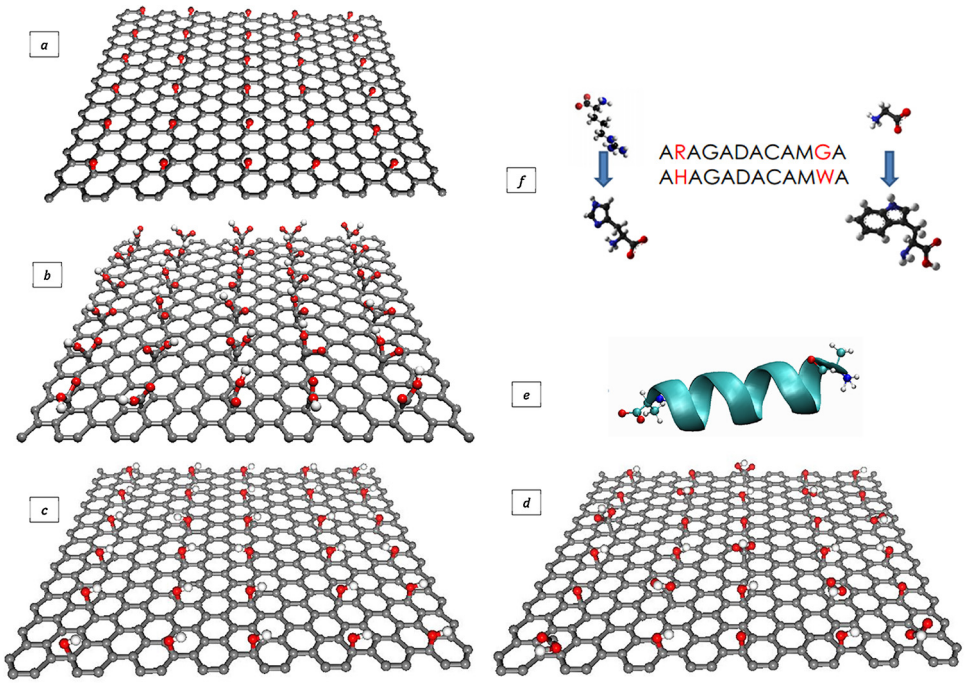
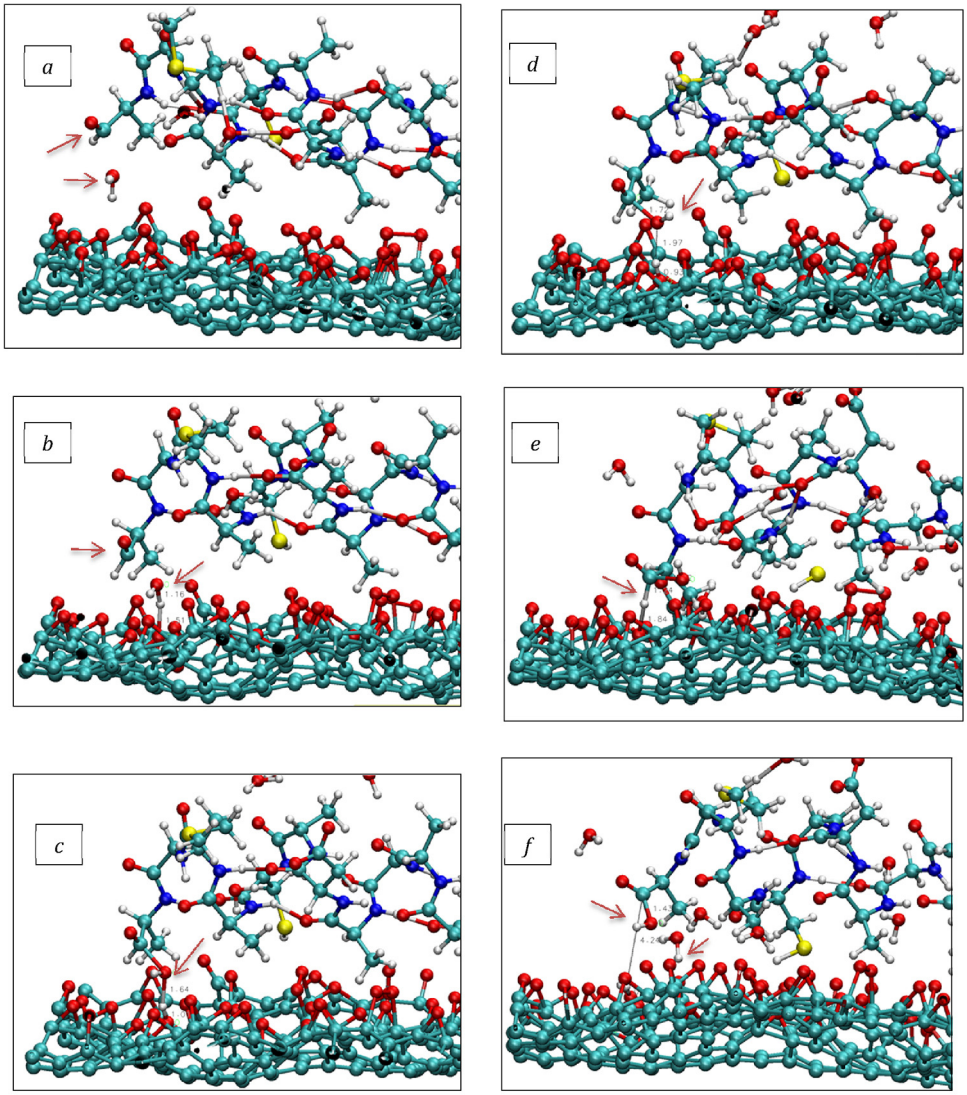



**Fig. 1.** Graphene oxide in the presence of (a) epoxide, (b) carboxyl, (c) hydroxyl, and (d) combination of all functional groups. (e) polypeptide; (f) R→H and G→W mutation.



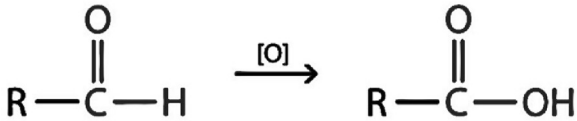




**Fig. 2.** Effects of epoxy functional group on the peptide, mechanisms of oxidative stress production and aldehyde to carboxyl transformation. (a) Water molecule approaches the graphene sheet and (b) form H-bond with the epoxy on the surface; (c and d) one proton transfers to epoxy and results in two hydroxyls. One hydroxyl remains on the surface and the other one attacks the aldehyde-capping end; (e) hydroxyl binds to aldehyde and deprotonates it. Then the proton moves back to the hydroxyl on the surface and (f) forms a water molecule. Finally, water molecule dissociates from the surface and diffuses into the solution.

is filled with water at the density of 1 g/cm$^3$. Here we highlight two facts about initial structures in Fig. 1. First, it is reported [36] that carbonyl groups are usually formed on the edges and since an infinite sheet of GO is considered, no carbonyl groups should be observed. To pinpoint this property, carbonyl groups (=O) are initially placed on the surface (as is shown in Fig. 1); however, after energy minimization epoxide (–O–) are the only present groups on the graphene surface, which is in agreement with experimental observations [37]. Hence, hereinafter, we refer to structure Fig. 1a as graphene with epoxide. Second, carboxylic groups are placed on the surface of the graphene sheet for the sake of comparison with other groups, which inevitably bring about many defects on the graphene sheet. A more realistic structure consists of peripheral carboxyls instead which is studied later.

GO, the peptides and water are minimized at 1 K and equilibrate at 310 K for 10 ps before mixing. Afterwards, series of MD simulations are performed on each system for 200 ps with a time step of 0.1 fs. NVT ensemble is used for all simulations with Verlet-Berendsen thermostat and a damping of 100 fs.

Among different choices of simulations, reactive molecular dynamics seems the most reasonable option. Non-reactive force fields are not able to capture the reactivity of GO because of their inability to take bond breaking and bond formation into account and thus, they cannot show the reaction mechanisms that can give toxicity to GO. Even though Sun et al. [38] found exciting results using Amber99SB force field for toxicity of graphene sheets, they only could analyze hydrogen bonds energies and Van Der Waals forces. On the other hand, ab initio techniques are too

computationally expensive to follow the reaction dynamics for the relatively large systems that are used here. As such, the ReaxFF protein force field, as developed by Monti et al. [39] is recruited in this study.

ReaxFF force field has been used extensively so far to tackle numerous vital problems such that it has been widely accepted to be a powerful tool to predict atomic behavior [40–42]. Several simulations have been performed using ReaxFF force field and verified by DFT calculations and experimental results so that it can be counted as a promising tool in scientific literature [41–43,50]. Amongst, one may refer to our previous work in investigation of alpha helix to random coil transition [48], or Goverapet Srinivasan et al. [42] and Bagri et al. [41] who recruited ReaxFF to investigate graphene oxide chemistry. Furthermore, Compton et al. [43] emplyed ReaxFF to study graphene oxide behavior and provided good agreement between ReaxFF and experimental results.

## 3. Results and discussion

As discussed earlier, to quantify the toxicity of graphene oxide based materials, we assessed the direct interactions between graphene functional groups and the peptides in the helical conformation. First, we studied the influences of each functional group in separate models. Here, any change in the conformation or composition of the peptides is defined as toxicity such that it affects the peptides secondary or tertiary structures.

### 3.1. Epoxide functional groups

Epoxide (–O–) is the most dominant functional group in GO based materials (Fig. 1a). Our analysis demonstrates that epoxides are highly reactive in the presence of water molecules. Indeed, even at physiological conditions, oxygen atoms are able to detach from graphene surface and form hydroxyl anions in association with water. Some of these hydroxyl groups stay attached to the graphene layer, while others are solvated by the surrounding water, casing a high local pH, and may react with the peptide as follows:

$$\text{R-CHO} + \text{O} \xrightarrow{\text{GO(aq)}} \text{R-COOH}$$

or

$$H_2O + H^2 \rightarrow 2OH^-$$

Therefore, our simulations directly confirm the presence of ROS at high epoxide concentrations. However, the amount of free atomic

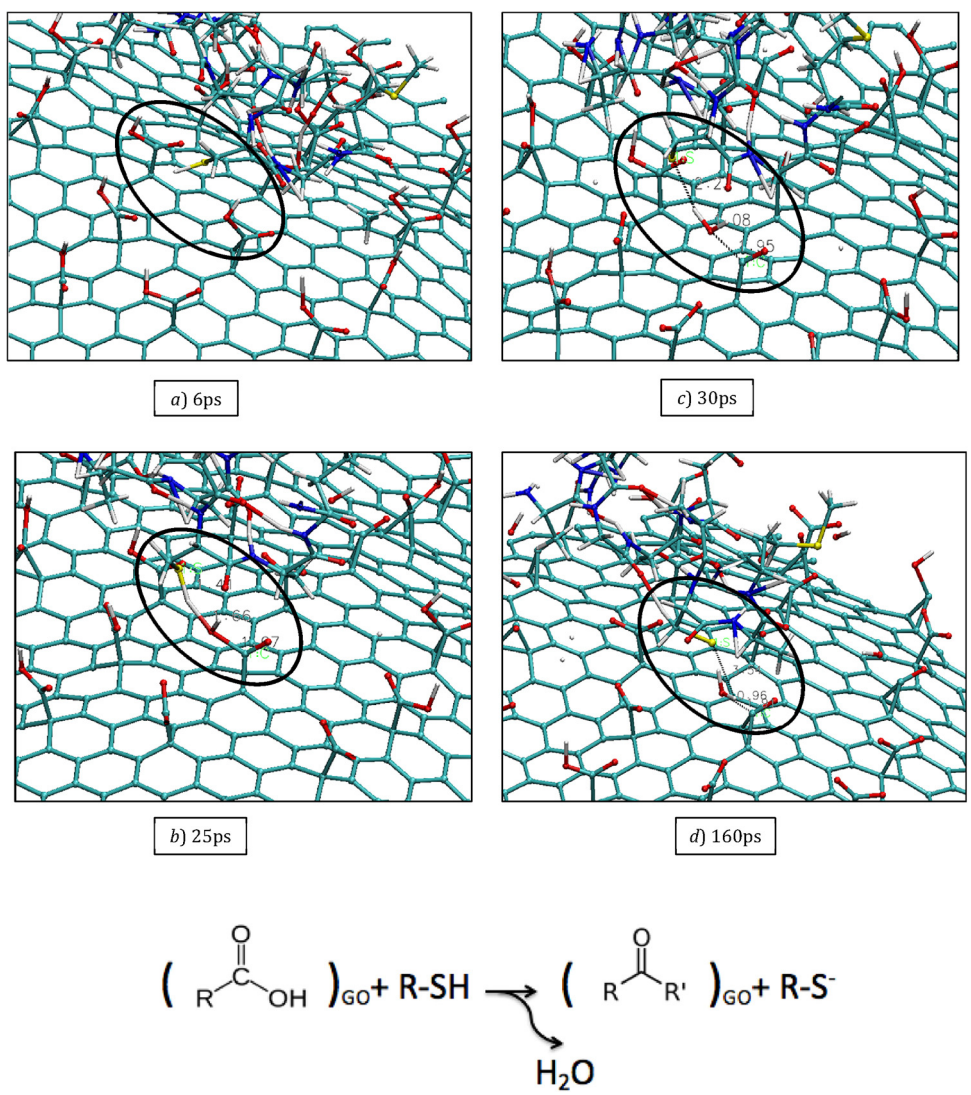



**Fig. 3.** Influence of carboxyl group on the peptide and the thiol bond deprotonation. (a) Thiol bond and the carboxyl make an H-bond; (b and c) one hydroxyl dissociates from carboxyl while thiol bond becomes deprotonated. This finally results in a water molecule, which later dissolve into the solution and one carbonyl group on the graphene sheet.

oxygen depends strongly on the density of not only the epoxides but also of the other functional groups, which will be discussed further in the upcoming sections. It is worth noting that our simulation results explain why hydroxyl groups are so likely to be observed in GO as they can be formed from reactions between water and epoxides.

The consequence of ROS in biological environment can be investigated by looking at GO-peptide interactions. Fig. 2 depicts the process in which oxidative species can detach from the GO surface and react with the peptides. This process can be described as follows:

(a) The peptide approaches GO surface (Fig. 2a).
(b) Water interacts with epoxide and generates two hydroxyl groups, one of which attacks to the aldehyde (–HCO) capping end (Fig. 2b and Fig. 2c).
(c) A proton from aldehyde transfers to the second hydroxyl and form a water molecule together with a carboxyl capping end instead (Fig. 2d, Fig. 2e and Fig. 2f).

We mention that in our simulation the two ends of the peptides are capped in order to hinder the helix from unraveling quickly, as our randomly sequenced peptide is not a good helix former.

Furthermore, we claim that ROS production is accentuated by the presence of graphene sheet. To assess this hypothesis, the same system is tested again but in the absence of the graphene sheet with a constant concentration of hydroxyls. In this case, after 200 ps simulation, the aforementioned reaction did not occur which indicates the catalytic behavior of graphene oxide besides its capacity to form ROS.

### 3.2. Carboxyl functional groups

The same approach as discussed in the previous section was used to analyze carboxyl groups on GO (Fig. 1b). For the sake of comparison, the same functional group distribution is used for the carboxyl simulations as previously used for the epoxides. As expected, a system consisted of graphene sheet with a high number of carboxyl groups providing an extremely acidic solution for the peptide, resulting in protonation of the Asp residue of the peptide.

In addition to the capability to provide an acidic environment, carboxyl groups on GO may cause thiol bonds (–CSH) deprotonation in the Cys residue of the peptide (Fig. 3). When the Cys residue of the peptide is confronted with a carboxyl group on GO (Fig. 3a), it forms an H-bond with carboxyl (Fig. 3b) followed by breaking C—O bond in carboxyl (Fig. 3c). As a consequence, the carboxyl transforms to an epoxide and the Cys residue is deprotonated. In summary:

$$\text{R-COOH} + \text{R}'\text{-SH} \xrightarrow{\text{GO(aq)}} \text{R-CO} + \text{R-S} + \text{H}_2\text{O}$$

The deprotonation of Cys residue is more prominent in the presence of an additional Cys residue in another peptide. The deprotonated Cys residue is capable of forming disulfide bonds, which is important, since it can lead to significant tertiary structure change in the peptides. Disulfide bonds play critical roles in peptide folding and can lead to amyloid formation by misfolding peptides. This can cause different disease such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), prion-related disorders (PrDs), and amyotrophic lateral sclerosis (ALS) [44].

Our simulation results also indicate carboxyl catalytic behavior. To test this hypothesis, we repeated the simulation in the absence of the graphene sheet; in this case no Cys deprotonation was observed

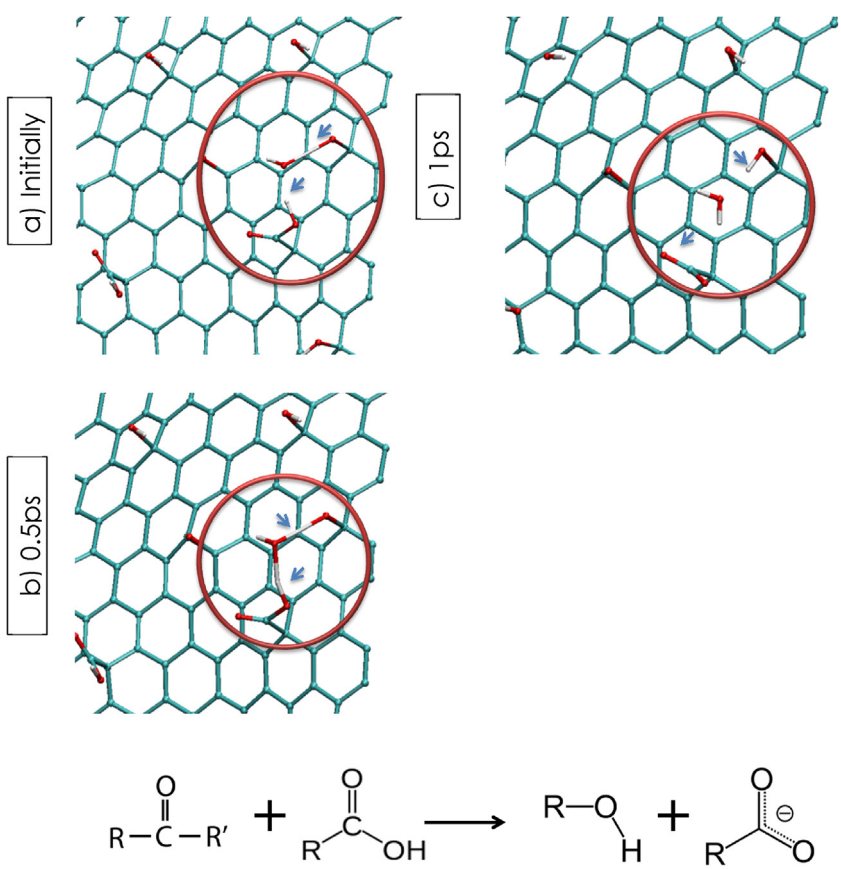



**Fig. 4.** Opposite effect of carboxyl and carbonyl on the peptide and the solution. (a) Water molecule forms a bridge between carbonyl and carboxyl groups. (b) Carboxyl, carbonyl and water are bound together by strong H-bonds and complete the bridge (transition state); (c) carbonyl harnesses the released proton from carboxyl.

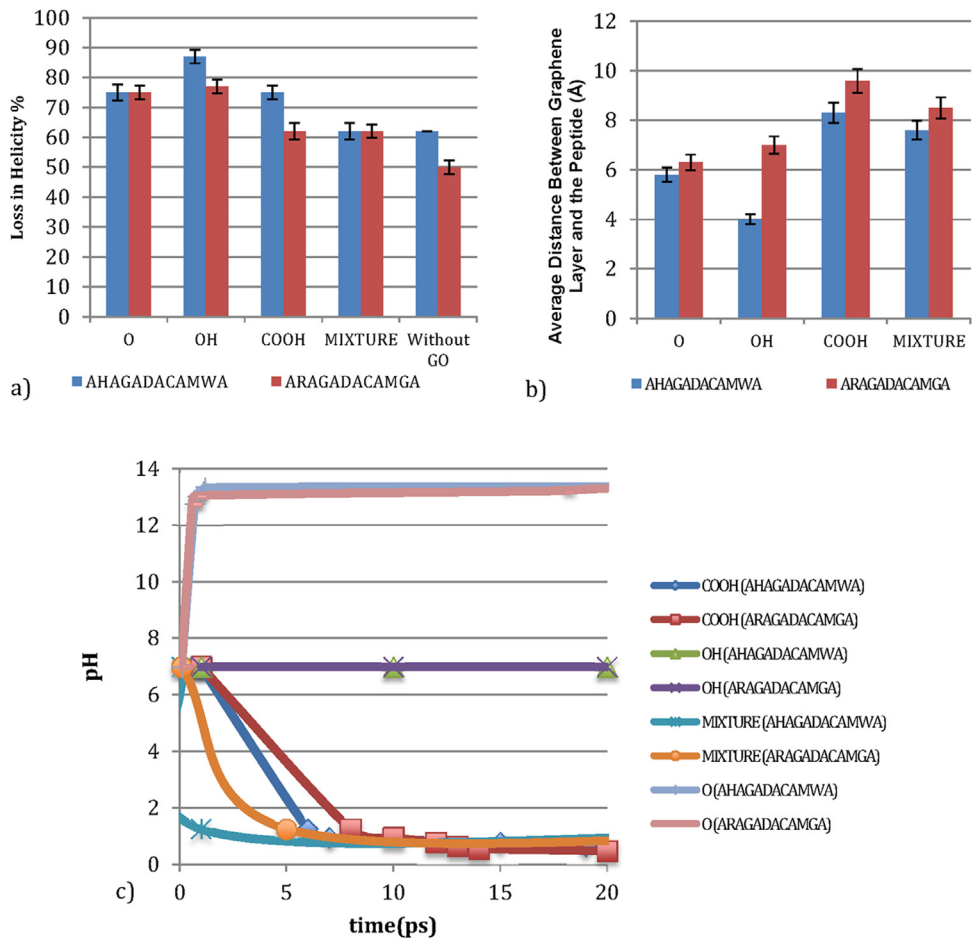



**Fig. 5.** Comparison between influences of different functional groups on the peptide: (a) secondary structure loss analysis, (b) adhesion analysis and (c) pH change over time. Error bars show one standard deviation in five series of simulations on similar configurations for each data set.

which implies that GO populated with carboxyl groups can accelerate thiol bond deprotonation.

### 3.3. Hydroxyl functional groups

Analogous to our previous analyses for epoxide and carboxyl, the functional groups on the GO were replaced by hydroxyls (Fig. 1c). Interestingly, our simulation results indicate that hydroxyls are chemically inactive groups that tend to stay on graphene sheet without any direct reaction. However, there seems to be only a very small amount of electron sharing between carbon in graphene sheet and oxygen of hydroxyl, which causes the graphene–OH bond to be remarkably polar. As a consequence, we observe an unusually strong H-bond between the hydroxyl and the peptide side chains. This argument is supported by DFT calculation results, which suggest that the H-bond energy between a water molecule and the hydroxyl group of GO (R-OH$\cdots$OH$_2$) ($\sim$10.8 kcal/mol) is more than twice as the H-bond energy between water and methanol (CH$_3$OH$\cdots$OH$_2$) ($\sim$4.3 kcal/mol) [45,46]. This strong H-bond is able to essentially glue the peptide to GO surface in the presence of hydrophilic side chains. Our simulation results show significant decrease in the graphene-peptide distance and H-bonding between GO and the peptide. Moreover, the aforementioned H-bonding is able to unravel the helical structure, if enough polar side chains are present, resulting in secondary structure denaturation, which is attributed to many abnormalities [44].

It is worth to re-iterate that our randomly sequenced peptide is not a good helix former. Thus, to remove the self-induced portion of unraveling of the helix, the same simulation has been repeated in the absence of the graphene layer and in the presence of the graphene layer but without any functional groups on it. For this system, the amount of denaturation of secondary structure is calculated correspondingly and is used as a control system. We reached approximately the same amount of denaturation for no graphene and with de-functionalized graphene cases. Moreover, the graphene sheet alone showed no chemical reactivity in our simulations.

### 3.4. Lerf-Klinowski model [36]

After evaluating the individual functional groups separately, we incorporated the Lerf-Klinowski (LK) model (Fig. 1d) in our analysis to see the combined effects of the functional groups. For the sake of comparison, we keep the concentrations and the distributions of all groups on GO to be similar even though LK model suggests that carboxyl groups should be placed at the edges. We will also consider the exact LK system to study the effect of peripheral carboxyl groups later.

Our LK simulation results obtain the same conclusion for hydroxyl regarding its inactivity. However, it illustrates that carboxyl and carbonyl have opposite roles in that carboxyl groups are capable of attenuating the carbonyl destructive function. This can justify the endocytosis of carboxylized graphene versus apoptosis in the presence of pristine graphene [37]. Fig. 4 clearly depicts how ROS arising from carbonyl can be harnessed by the presence of carboxyl groups. As it is shown in Fig. 4a, the water molecule acts as

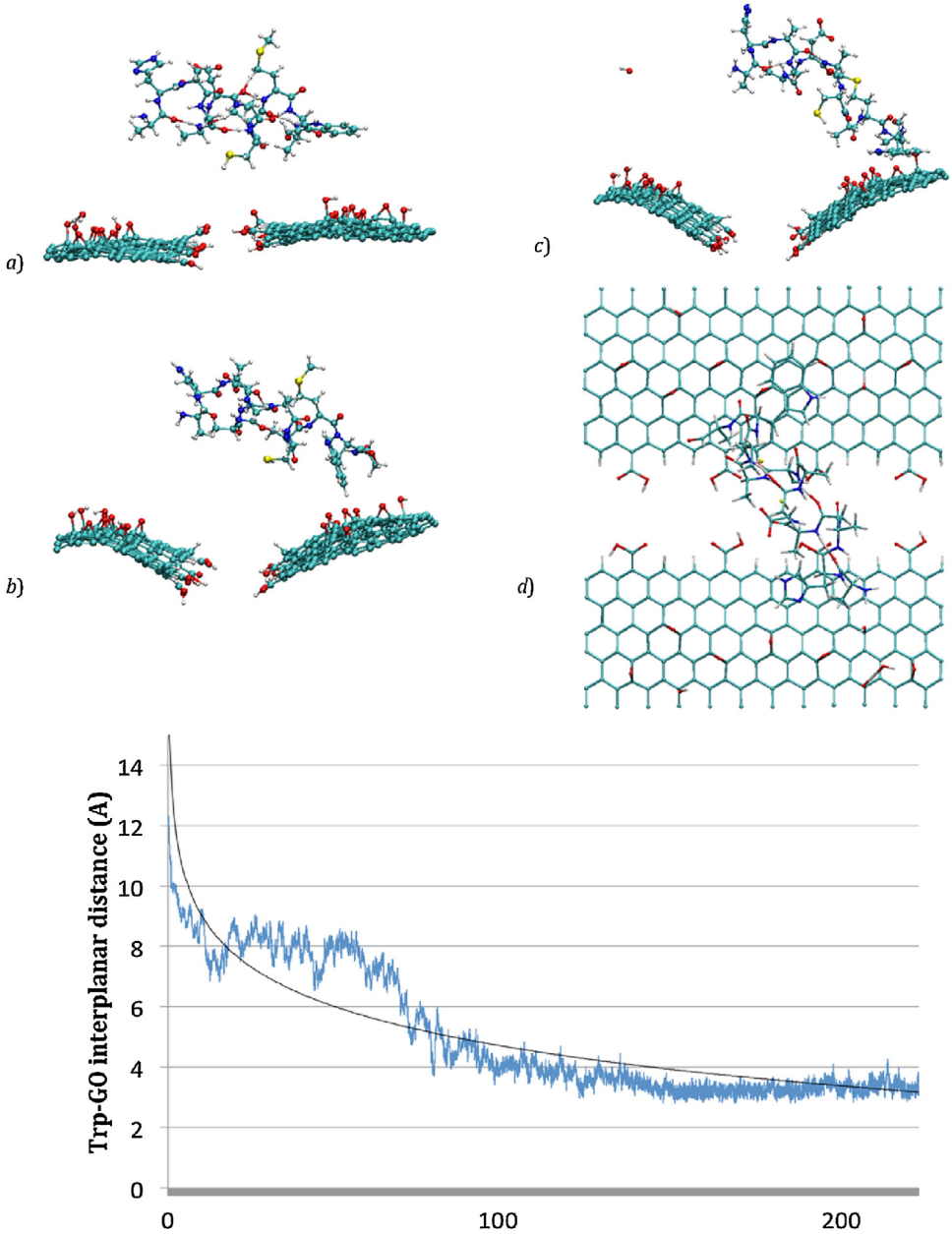



**Fig. 6.** Lerf-Klinowski model for graphene oxide. Snap shots show π–π stacking interaction between the graphene layer and Trp. (a) Initially the peptide stays far away from the graphene sheet, (b) then comes to the vicinity of the sheet and Trp reorients to be parallel to the sheet and (c) stays there at equilibrium distance of 3.3 Å. (d) top view of the peptide and the graphene sheet orientation. (e) diffusion of the peptide to adhere to the graphene layer.

a bridge between the carbonyl and the carboxyl. When the water molecule is inserted between the two functional groups, it forms strong H-bonds, which facilitates proton transfer between the carbonyl and the carboxyl (Fig. 4b). The neighboring carboxyl supplied the hydroxyl anion and the required proton (Fig. 4c). Hence, interestingly, this event can show that in the presence of all functional groups, carboxyl cannot acidify the solution significantly. Furthermore, the ROS coming from the existence of the epoxides is no longer straightforward to be captured in our simulations.

At this stage, we can begin to compare the toxicity of each group. Our criteria to describe toxicity of each group are based upon:

(1) Secondary structure denaturation or any factor that impairs the helical configuration.
(2) *Adhesion*: The average distance between the peptide atoms and the GO surface is characterized as adhesion criterion. In other words, the closer the helix to the graphene sheet, the more cohesive the layer.
(3) *pH change as a function of the composition*: Although not essentially toxic, extreme pHs can impair different physiological systems. Therefore, pH evolution of each system during the time is calculated to show how each functional group can influence the acidity of the solution. We calculated pH by counting the free protons available in the solution at each moment.

Fig. 5a compares the secondary structure loss as a function of different groups on GO. As it is shown, the maximum loss pertains to hydroxyl followed by epoxide. Although not significant, comparing to the control case (no graphene), GO seems to be able to accelerate the helical unraveling. This can be justified by considering the strong H-bond between the peptide side chains and the functional groups on GO as we discussed earlier.

**Table 1**
Summary of the comparisons between the influences of different functional groups on the secondary structure of the peptides.

| Groups | O | OH | COOH | Mixture | W/O GO |
|---|---|---|---|---|---|
| pH | 13.4 | 7 | 0.4 | 0.7 | 7 |
| Secondary interaction | × | √ | × | Not significant | – |

Accordingly, carboxyl and epoxide show potential for applying compositional change on the side chains, which might be interpreted as an effect on tertiary structure of the peptides.

Our adhesion criterion is based on the distance between the GO sheet and the peptide. We calculated these distances and the results are presented in Fig. 5b. Apparently, hydroxyl group have the strongest tendency to grab the peptide, which is not surprising given the unusually strong H-bond between the GO-hydroxyl and the peptides.

Next, pH changes as a function of different groups are calculated during the time and presented in Fig. 5c. As expected, pH changes are not sequence dependent. Moreover, as it is discussed earlier, the hydroxyl shows no chemical activity and therefore, the corresponding pH is close to 7 while carbonyl and carboxyl show basic and acidic pH, respectively. Table 1 summarizes the comparisons between the influences of different functional groups on the secondary structure.

### 3.5. *Lerf-Klinowski [36] model with peripheral carboxyl*

Finally, we used LK system with a density and distribution of functional groups closer to reality. For instance, LK model predicts carboxyl groups should be placed in the periphery of the graphene sheet especially at the edges. In addition, we reduced the concentrations of different groups to see if the toxic behavior is dose dependent [7]. Also we assessed the experimentally observed GO adhesive properties because of π–π interaction between Trp residue and the graphene sheet [35] (Fig. 6).

After 400 ps simulation at physiological temperature, this LK GO shows low activity compared to the previous simulations. It is observed that the peripheral carboxyl displays a reduced tendency for deprotonation compared to the conditions when they are placed in the center. Also, the observed ROS in the previous simulations diminished remarkably. Indeed, we report almost neutral pH ∼ 7 for GO with LK geometry. (Note that a few protons dissociated from carboxyl groups are neutralized by forming hydroxyls from epoxides.)

Results also show that neither secondary structural change nor pH evolution is sequence dependent. However, adhesive behavior strongly depends on the peptide sequences. In addition to aforementioned H-bond between the side chains and GO, strong π–π interaction is observed in our simulation.

As it is shown in Fig. 6, given a low concentration of epoxide and carboxyl groups on GO sheet, H-bonding is not significant and Trp residue obtains the required space to reorient to be parallel to graphene and form a π–π stacking interaction (Movie S1). We report an equilibrium intra-distance of 3.3 (±0.1 Å) between two aromatic rings which is in a good agreement with DFT calculation for 3.2–3.5 Å [47]. This event leads to a significant adhesion characteristic to peptides with Trp (and probably other residues with aromatic rings). Interestingly, our results verify this prediction such that the average distance of AHAGADACAMWA is 2 times smaller than ARAGADACAMGA (without Trp). Therefore, our simulation confirms the peptide adsorption on the graphene sheet as observed by Liu et al. [35].

## 4. Conclusion

Here we evaluated the toxicity of graphene oxide based material using reactive molecular dynamics. Our method is able to probe the probable reaction mechanisms between the graphene oxide sheet and the membrane peptides at an atomic level. By describing the chemical behavior of each functional group on graphene, we provide a better understanding of potential hazards of GO.

We tested epoxide, carboxyl, hydroxyl and a combination of them to see the corresponding mechanisms of denaturation of the secondary structure of peptides. Results imply that the carbonyl and the carboxyl groups have opposite chemical behavior and provide basic and acidic pHs, respectively. Besides, in a catalytic pathway, the epoxide-groups have the potential to produce ROS whereas the carboxyl can accentuate thiol bond denaturation. Graphene oxide and hydroxyls are able to form unusually strong H-bonds, which play an important role in the cell-surface adhesion.

Moreover, using the Lerf-Klinowski model for graphene oxide with mixed functional groups, we showed how π–π stacking interaction can influence cell-surface adhesion. Also, studying the system that contains all the functional groups indicates that while each functional group is active separately, their mixture is observed to be less active. For instance, the local chemical effects of the carboxyl and the carbonyl can cancel each other out. On the other hand, moving carboxyl groups from surface to the peripheral sides of the graphene sheet effectively removes the carbonyl groups, causing new epoxide groups to be formed.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.md.2015.10.001.

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