The Pennsylvania State University

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Department of Mechanical Engineering

# APPLICATION OF REACTIVE MOLECULAR DYNAMICS IN BIOMATERIAL SCIENCE AND COMPUTATIONAL BIOLOGY

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by

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## ABSTRACT

In this thesis, the potential applications of reactive molecular dynamics in computational biology have been evaluated. Within the last three decades a considerable amount of simulations have been performed employing non-reactive molecular dynamics, which have had significant impacts on medicine and biomaterial sciences. However, we believe reactive molecular dynamics has the potentials to predict several vital events in human body that cannot be grasped either in experiment or other computational techniques. In this thesis, we briefly introduce the essence of reactive molecular techniques and show how this method can assist computational biologist to further analyze events that can ultimately lead to biochemical disorder by modeling two different systems (I) pH-drive helical coil transition to random coil and (II) graphene oxide interactions with polypeptide helices.

We have studied alpha helix to random coil transition using ReaxFF reactive molecular dynamics as a function of pH. In addition, we show proton transfer between the solution and the peptide can break the alpha helix hydrogen bonds and consequently, at extreme pHs significant amount of helix will unravel. We also compare the effects of temperature and alpha helix length in denaturation mechanism. The ReaxFF findings are in significant better agreement with *ab initio* calculations then previous non-reactive force field results – indicating the relevance of the reactive component on helical loss.

Furthermore, we report the first study on graphene oxide (GO) toxicity at the atomic scale. This study reveals the likely destructive mechanisms of GO during its interactions with living organisms. Reactive molecular dynamics study is utilized to illuminate the toxicity pathways and assess the available hypotheses about GO biocompatibility.

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## Chapter 1

## 1 Introduction

#### 1.1 Computational Biology: when computer power merges with biology lab

In the nineteenth century, one could divide science into four major fields: physics, chemistry, mathematics and medicine. This division existed because science was in its initial stage of development and it was not much often that, for example, a physicist got involved in a problem that demanded a considerable knowledge of other territories. However, as human knowledge extended and more scientific mysteries were solved, humanity was confronted with complicated problems that could not be addressed unless a combined knowledge of all sciences was utilized. The need for interdisciplinary researches gets accentuated as, for instance, complex problems in medicine cannot be solved without the use of a significant knowledge in mathematics. Today, scientists and engineers are using the available knowledge from different fields of study to enhance our life style, boost the available technologies and invent devices that years ago could only be imagined in Jules Verne books. One of these intersecting points of different sciences is the application of computer science, physics and mathematics together with biochemistry in medicine.

Medicine is a field of study, which addresses diagnosis, prevention, and treatment of disease. Contemporary medicine uses engineering techniques to process breast images to probe the possibility of cancer in patience. It uses new material to build artificial organs to be replaced with human heart, vein, skin, lung, etc. Chemical engineers employ different techniques to facilitate protein production. Recruiting the knowledge of mechanics, engineers are able to invent artificial cartilage and bones that can have better mechanical properties even compared to natural members. As such, it is clear that contemporary medicine owes a lot to engineering disciplines. A vital connection exists between computer science and biology, leading to the important field of study known as computational biology. Computational biology is the study of biological systems using mathematical modeling with the aid of computer simulations.

In the twentieth centuries few biologist could predict how helpful computer simulations would become. During years of biological researches when enormous amount of biological data were generated, scientists were missing vital relations between these data. Therefore, they started to build some mathematical models in order to obtain a cause-and-effect form of function between such data. After a while, the efficiency of such models made computational biology an important distinct field of study. Due to a significant increase in computational power, the benefits of computational biology became even more substantial such that today huge computer resources in different labs around the world are involved in this field of research. To name some examples, IBM Blue Gene system scored a Linpack performance benchmark of 280.6 teraflops (280.6 trillions of calculation per second), a top-ranked set up, is fully dedicated for life science analysis and computational biology [1]. The Sandia National Laboratories Intel XP/S 140 Paragon supercomputer, which claimed the No. 1 position on the June 1994 list with 3,680 processors; the system ran the Linpack benchmark at 143.40 giGflop/s [2]. A lot of other big names with huge computational resources are also involved in medicine, namely Microsoft ©, Intel, Oak Ridge National Lab (ORNL), etc. All of these facilities provide the required condition for computational biology to compete beside experimental medicine.

Now that we conceptualized computational medicine, several questions would be posed; "What is the advantage of computational medicine compared to experimental medicine? Mathematical models are based on significant simplifications and numerous assumptions that are hard to be trusted on. How efficient these models are? Are they really being used in real life? How predictive are these models? What is the place for computational medicine and drug discovery in pharmaceutics? What is the area of concentration of computational medicine and where is its power bounded?"

A complete answer to these questions requires knowing computational methodologies and underlying principles. Today, scientists use different ways to employ computational resources in medicine. Each of them separately assists researchers to do vital studies that experimental medicine is incapable of. Here are some examples that describes computational medicine:

- Advanced mathematical models allows scientists to find out the underlying network of molecules that are involved in cancer and use these models to predict the risk of developing disease [3].
- *Computational physiological medicine* uses time dependent computational models to study how healthy organs disorganize to unhealthy state, which focuses on developing treatments for cancer, diabetes and heart disease [4].
- Computational anatomy uses medical images to detect changes, for example, to study brain structural changes. Researchers have found shape changes that appear to be associated with Alzheimer's disease and neuropsychiatric disorders, such as schizophrenia [5].

*Computational models of electrical activity* in the heart are on their way to being used to guide doctors in preventing sudden cardiac death and in diagnosing and treating those at risk for it [6].

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Our body is a highly organized but complex system. Experimental biologists are able to extract a large amount of useful data, however, most of these data are not representative. In another words, although they are able to implement significant tests to study different biological systems, often understanding the underlying functions that relate such data are not quite obvious. To shed light to this malfunctioning of experiment biology, we ought to discuss how a lot of pathways work inside the body. Cancer system biologists describe the signaling pathways in body as a network, which the correlation functions are usual more than just a simple cause and effect relation [7]. It means that in contrast to usual experimental methods, changing one parameter while keeping the rest constant will not yield to the same results, as it should after super positioning the results. This *"non-linearity"* makes a comprehensive conclusion hard to define, and it is where modeling approaches can be helpful. Computational biologists use simple equations to describe different complex biological systems by employing experimental data such as reaction rates. Such models are able to predict the system behavior in a meaningful manner with a reasonable precision. The main advantage of such models is that the scientists are able to change different factors simultaneously and see the effect on the whole system [7].

Another important subfield of study of computational medicine is atomistic level simulations. As we will discuss more later on, most of the biological functions are organized in atomic resolution. Therefore, if one can find the proper interactions between distinct systems in atomic scale, he



(variant)

Figure 1.1. General structure of amino acids. Roughly 20 amino acids exist which take part in forming peptides. Structure of amino acids conserves (except Proline) among 20 amino acids and they only differ in R-group.

might be able to predict human body responses to different stimuli. To further evaluate this method, we first briefly describe how human body functions in atomic scale.

If you magnify a human tissue  $10^9$  times, you will enter a new world known as "atomic spectra" in which atomic masses (~ $10^{-27}$ Kg) are significant, nano seconds are vital and so called "*small energies*" ( $10^{-21}$ J) are huge energies. Our body consists of oxygen (O, 65%), carbon (C, 18%), hydrogen (H, 10%), nitrogen (N, 3%) and other elements (4%) based on molecular masses [5?]. These four elements bind to each other to form the most important building block of living species known as "*amino acids*". Figure 1.1 shows atomic structure of amino acids. *R* denotes to various side chains and is the main distinguishing factor between amino acids. There are roughly 20 amino acids in human body, which can bind to each other and form more complicated structures known as peptides. The usual way to form a peptide structure is based on a hydrolysis reaction as follows (Figure 1.2):



Figure 1.2. Hydrolysis reaction of amino acids. This reaction plays the principle role in peptide formation.

Peptide structures can continue such hydrolysis reactions to form polypeptides. As it can be seen in Figure 1.2, there are different active sites that facilitate intra-molecular and inter-molecular interactions. For instance, oxygen (=O) in one amino acid (peptide) can form a H-bond with hydrogen (N-H) in another peptide. As a consequence, peptides show distinct conformations that have various functions and are called secondary structures. The most well known tertiary structures are known as alpha helix, beta sheets and U turns. Figure 1.3 represents alpha helix and beta sheet conformations.



Figure 1.3. Secondary structure of peptides in the form of alpha helix and beta sheet.

Secondary structures can also bind and form large structures of peptides know as tertiary structures. Such large structures are known as proteins, which have vital functions in body. Often protein functions come either from their conformational changes or their compositional changes. Proteins can interact with each other and thereby, organize several activities inside living species. Conformational changes among proteins are usually consequences of H-bond formation or breaking, although ionic interactions, electric dipole moment, side chain-side chain interaction, disulfide bonding and some other interactions are important [8].

Studying the function of proteins based on their conformations opens a new field of research known as *structural biology* in which researchers try to identify different functional segments from other parts. Many pathological pathways are based on conformation of such functional segments. For instance, several reactions inside the living cells are enzymatic, which means that

they require enzymes for reaction to take place. Enzyme substrate interaction are often conformational base, which means if enzyme and substrate shapes fit with each other properly, they will react. Proteins often use the same properties in that if, namely, in the presence of a signaling molecule the protein will experience a conformational change that suit fitting into another protein vacancy.

As a consequence, computational biology has made researchers able to predict such interactions in atomic scale. Structural biology has achieved tremendous breakthroughs over the past halfcenturies thanks to atomic-resolution models. Although static structures of proteins can be obtained through crystallography and other techniques, in reality they show highly dynamic atomic structures. Computational medicine recruits different methods to assess atomic interactions. Molecular dynamics (MD) simulation is useful approach that has been frequently employed in recent decades. Although molecular dynamics initially was introduced to study biological systems, today other fields of research such as material science, mechanics and physics are utilizing MD for their simulations.

#### **1.2** Molecular dynamics as a tool for molecular biology

Here we show how MD simulations can be considered as a useful biological tool through a number of recent studies involving conformational changes in proteins, transport across membrane, protein folding, ligand receptor binding and drug design.

#### **1.2.1** Conformational Changes

Under physiological condition, proteins and other bio macromolecules can move from different conformations and their regulatory functions depends on these conformations. One important conformation dependent super family of regulatory proteins is known as globular proteins (G-proteins) that their tendency to bind to specific G-protein-coupled receptors (GCPRs) and kinases determines remarkable number of pathways. GPCRs are the largest class of drug targets and their active vs. inactive states depend on their conformation. Several studies have been performed using MD simulations to find out their active and inactive conformations [9, 10, 11]. Dror *et al.* observed a novel conformation of  $\beta_2$ -adrenergic, which was not observed in experiment due to side effects of biochemical agents used in experiments [12]. Anton [13] used MD simulations to discover spontaneous transition of active to inactive state of  $\beta_2$ -adrenergic and answered to the question that had been raised up due to discrepancies between two different crystallography reports.

Kinases are known as enzymes that are referred to as regulatory systems that control cancer autoimmune system diseases. Mutations in kinase can cause abnormal configurations that can impair subsequent signaling and can lead to cancer. Faraldo-Gomes and Roux [14] used MD simulations on Src family tyrosine kinases and justified swift switch between active and inactive states in spite of robust binding between SH3 and SH3 auxiliary domains to its catalytic domain. They used an MD method known as "*umbrella sampling*" to probe distinct energy profiles between these two states and realized a circumventive pathway that is in favor of such transition.

## 1.2.2 Membrane Transport

A big obstacle for delivering drug into cells is cellular membrane, a defense mechanism, which primarily consists of lipid bilayer, and mostly is impervious to protein transport. MD simulations have elucidated the rate of substrate permeation, transport selectivity and membrane channel gates responses to different stimuli in a spatial and temporal manner. For instance, while crystallography results show that potassium channels are not capable of passing potassium through, Noskov et al. [15] and Bostick and Brooks [16] using MD simulations showed that selectivity of such channels depends on thermal fluctuation of the filter and thereby, justified potassium transport into cells. Therefore, these thermal fluctuations provide enough space for potassium ions to pass through these channels.

Moreover, there exist particular types of channels, which switch between two open and close states based on external voltage known as "*voltage gated ion channels*". While identifying the related voltages is considered to be a significant obstacle in experiment, Jensen et al. [17] used unbiased microsecond time scale MD simulations to assign the corresponding voltages to various voltage gated ion channels.

## 1.2.3 Protein Folding

Protein folding raises two questions that can be tackled by MD simulations. First, amongst various conformations that one protein can take (which are energetically localized minimum), which one is the most favorable conformation (global minimum)? The second question is to identify the pathway in which the protein transition between the native and deformed states occurs. Neither of these questions can be solved through experiments partly because some of conformations are so unlikely to occur or are so short-lived that they cannot be captured easily.



Figure 1.4. MD simulation results [50] show a good agreement with experimental results in finding the stable configuration of various protein folds.

Several studied have been performed to determine different protein folds. For example, Lindorff-Larsen et al. [18] used MD to verify the native states of 12 proteins, which fit reasonably well with experimental reports. Figure 1.4 shows 12 important conformations that are obtained using MD by Lindorff-Larsen et al. [18].

#### 1.2.4 Ligand Binding and Drug Design

Two important factors that define the quality of one specific drug might be called as ligandreceptor binding and affinity. As we discussed earlier, three-dimensional structure of proteins can strongly influence their binding. Today computational medicine scientists use MD methods to identify protein binding by calculating their accessible surface area in a process known as *"protein docking calculations"*. Such calculations can efficiently demonstrate binding rate of different proteins, e.g. anti-body anti-gen binding. Likewise, using MD simulations facilitate assessing the binding energy of ligand and receptors to evaluate affinity of enzyme and substrate. Such evaluations can assist researchers to enhance the quality of drugs by increasing the binding energies.

In summary, computational biology has enabled us to probe biological systems in a different way, which is rather fast, cheap and precise. Knowing such tools can help us circumvent obstacles that we might face while utilizing experimental methods. In the up coming sections we further discuss the details of different computational techniques.

## 2 Methods

In the previous section, we discussed how computational methods such as MD simulations could provide us a unique possibility to investigate several problems that tackling them experimentally is either infeasible or expensive. Here we describe the basics of MD methods and illustrate the essence of reactive MD together with its benefits over traditional MD methods.

## 2.1 Hierarchy of Computational Techniques

You would not use microscope to measure a screw diameter nor would you use a ruler to measure the atom diameter. This statement also can be applied to territory of application of each computational method. Indeed, a researcher first is responsible to determine the length and time scale of his calculations in order to select the appropriate method. As it is shown in Figure 2.1, although some computational methods have very high precision, they are incapable of simulating long-time processes over large length scales.

Figure 2.1 depicts various computational methods that might be recruited while addressing a biological problem ranging from centimeters and month (e.g. simulation of ruptured abdominal aortic aneurysms using finite element method (FEM)) up to nano-scale problems (e.g. protein-protein or protein-DNA interaction using MD or Density Functional Theory (DFT)).



Figure 2.1. Hierarchy of computational techniques. A researcher should use the proper method to analyze different systems ranging from nm to Km length scale.

## 2.2 Objectives of the Upcoming Work

In this work, we are going to study protein-environment reactions at atomic level. This study is aimed to evaluate the potential of reactive molecular dynamics simulations in predicting properties of biomaterials and its applications in computational biology. Although simulating large segments of biomaterials is infeasible, given the fact that most of the properties that describes biomaterial arise from its surface composition rather bulk properties, we will probe protein interaction with surrounding environment whose thickness is around a few number of atoms. The main goals of this work can be summarized as follows:

- Analyzing pH dependent alpha helix to random coil transition
- Revealing Graphene Oxide Toxicity Mechanisms

## 2.3 Introduction to Atomistic Simulations

When your subject matter requires high level of precision, all-atom molecular dynamics would be a reasonable candidate despite its high computational cost. Various methods for atomistic simulations are introduced each of which addresses systems with special properties. Here we briefly mention some of the most famous and popular all-atom simulation methods and discuss advantages and disadvantages of each of them.

## 2.3.1 Quantum Chemistry

When in late 1926, Ervin Schrodinger attributed wave properties to matters and formulated wave behavior of matters by his famous equation as:

$$\widehat{\mathcal{H}}\Psi(\vec{r},t) = i\hbar\frac{\partial}{\partial t}\Psi(\vec{r},t)$$
(2.1)

where  $\hat{\mathcal{H}}$  is system Hamiltonian and is defined as:

$$\widehat{\mathcal{H}} = \frac{-\hbar^2}{2m} \nabla^2 + V(\vec{r}, t)$$
(2.2)

Although solution to (2.1) can describe almost any behavior of materials at electronic level, analytical solution only exists for hydrogen atoms. Therefore, one may solve (2.1) numerically which also has a tremendous computational cost such that even the strongest super computers are not able to simulate more than 100 atoms interacting with each other. Hence, inevitably, one should consider some simplifications on (2.1) so that it can be used in practice.

## 2.3.2 Density Functional Theory

The idea of density functional theory (DFT) originates from Thomas and Fermi by one to one correspondence of electron density and electron wave function. In this method, by defining specific density functional, and considering it as a linear function of basis sets, computational cost reduces significantly compared to calculation of wave function. The coefficients of such linear functional could be found easily by different methods.

1) Local Density Approximation (LDA) methods, (2) methods with Gradient-correction factor and (3) hybrid methods which incorporate a combination of Hartree-Fock and DFT approximation to electron-exchange energy are three principle DFT methods. Although DFT calculations have high level of accuracy and can be utilized to study many atomic level phenomena rather precisely, its computational cost is still high and even strongest resources can not simulate a system more than 200 atoms in a reasonable time.

## 2.3.3 Molecular Dynamics Simulation

Molecular dynamic simulation (MD) can be viewed as a bridge between quantum mechanics and classical Newtonian mechanics. In this method, atoms are considered as spherical masses that can interact with each other and their kinetics obey Newtonian mechanics i.e.:

$$\vec{F} = m\vec{a} \tag{2.3}$$

It means that given the intra-atomic interaction, particle motion can be found by Newton second law. Whereas the kinetic of particles can be found by classical mechanics, their interaction can be found either empirically or using quantum calculations.

We know in general that the aforementioned interaction can be found by

$$F = -\nabla V(\vec{r}) \tag{2.4}$$

where  $V(\vec{r})$  is the potential energy and depends on the system properties and will be discussed in detail in the upcoming sections.

## 2.3.4 Ensemble Theory

An ensemble is a collection of micro systems that statistical average of those micro systems can represent the time average of the macro system. In other words, this theory finds the average of different system (but energetically equal) instead of calculating the time average of one system. To shed light on this concept, consider the experiment of tossing a coin *N* times. Statistically speaking, the outcome should be equal as tossing *N* coins simultaneously.

#### **2.3.4.1** Micro-Canonical Ensemble (NVE)

This ensemble composes of distinct isolated systems each of which has similar number of particles (N), volume (V) and energy (E). Note that in this ensemble there is no energy transfer between subsystems *i.e.* internal energy is inherently conserved.

## 2.3.4.2 Canonical Ensemble (NVT)

Akin to NVE ensemble, in this ensemble also number of particles and volume remains constant; however, energy transfer between ensembles is allowed. Instead, ensemble temperature will remain constant. This ensemble is the most popular ensemble, which is used in molecular dynamics simulation. To implement NVT ensemble in molecular dynamics, one should keep temperature constant. This goal can be achieved by means of system temperature controls known as thermostat. Various algorithms to render such thermostats have been proposed which Berendsen thermostat [19] and Nose-Hoover thermostat [20] are the most famous ones. Briefly, Berendsen thermostat scales the temperature by comparing the temperature with an external bath using

$$\frac{dT}{dt} = \frac{1}{\tau} \left( T_{bath} - T(t) \right) \tag{2.4}$$

## 2.3.4.3 Grand Canonical Ensemble (µVT)

Analogous to previous ensembles, this ensemble again considers constant volume beside constant temperature; however, the number of particles can change to make chemical potential ( $\mu$ ) to remain constant.



Figure 2.2. A description of periodic boundary condition. In this special boundary condition, identical duplicates of one system will be positioned side by side.

## 2.3.5 Periodic Boundary Condition

When you deal with a system at atomic scale, surfaces become important. In other words, surface energy of materials at atomic scale overcomes bulk energies. However, you are not going to study surface energy of different systems frequently. To avoid surfaces in our simulations, you can recruit periodic boundary condition concept.

In this type of boundary condition, series of system duplicates are generated and exposed to the system. The essence of this concept can be realized from Figure 2.2. Consider the bolded atom in Figure 2.2, which is moving through the right edge of the simulation box. Upon the exit of that atom from the box, an identical atom will be inserted to the system from the left edge of the simulation boxes; so thereby, system properties remain the same.

#### 2.3.6 Choice of Force Fields

Coming back to Section 2.3 a vital step in performing an MD simulation is obtaining intra-atomic forces (force field). The choice of force field is problem wise and a compromise between precision and computational cost. Generally the force fields can be divided into two groups: *non-reactive* force fields vs. *reactive* force fields.

## 2.3.6.1 Non-reactive Molecular Dynamics

Non-reactive molecular dynamics encompasses the most of available force fields and would be used where any chemical reaction *i.e.* chemical bond formation or breaking is not much of interest. Although this method is incapable of simulating many processes, which certain reactions are essentially involved, it has lower computational cost than reactive molecular dynamics and can be widely used for various simulations consisting of remarkable number of atoms e.g. up to several millions of atoms. Wide range of non-reactive simulations have been performed such that most of non-reactive force fields are combined in commercial packages such as AMBER [21] and CHARMM [22]. Although these force fields have been improved significantly within the last three decades to seem more and more precise, still their liability in modeling a lot of reaction mediated processes such as enzymatic reactions, proton transfer, disulfide bond formation, DNA hydrolysis and a lot of other biological phenomena requires other methods to be employed.

## 2.3.6.2 Reactive Molecular Dynamics

In contrast to non-reactive molecular dynamics, reactive molecular dynamics is able to take into account bond formation and bond breaking during simulation. Although a few reactive molecular

dynamics force field exist, here we focus on ReaxFF force field. ReaxFF is an intra-atomic potential that is introduced and developed by Dr. Adri Van Duin and William Goddard at Caltech, which uses the idea of bond order to simulate chemical reactions [23]. Here we briefly describe the essence of ReaxFF and one may find the details elsewhere [24].

Among reactive molecular dynamics methods, QM/MM is also of great importance. This method combines the strengths of the QM (accuracy) and MM (speed) approaches, thus allowing for the study of chemical processes in solution and in proteins. The QM/MM approach was introduced in the 1976 paper of Warshel and Levitt [25]. They, along with Martin Karplus, won the 2013 Nobel Prize in Chemistry. This method can also be used for our purpose because of its ability to address relatively significant number of atoms in addition to considering bond formation and bond breaking.

#### 2.3.6.2.1 Bond Order Concept

The bond order concept breaks the putative notion of chemical bonds and contributes an energetic perception to it. The traditional way of approaching bond orders is to ascribe a bond order of one to  $H_2$  or two to  $O_2$ . However, ReaxFF revisits this concept and assigns a smooth function to bond order and relates it to bond energy. Thereby, zero bond order pertains to bond breaking point whereas bond order of three is the strongest bond order on hand. ReaxFF, therefore, has the capability to change bond order between two atoms to mimic bond formation and bond breaking. By contrast, traditional non-reactive force fields, assign infinite energy to the bonds when two atoms separate from each other, which is unrealistic and is solved in reactive MD. To calculate bond order, ReaxFF uses the following formulation as:

$$BO_{ij} = BO_{ij}^{\sigma} + BO_{ij}^{\pi} + BO_{ij}^{\pi\pi}$$
$$= \exp\left[p_{bo1}\left(\frac{r_{ij}}{r_o^{\sigma}}\right)^{p_{bo2}}\right] + \exp\left[p_{bo3}\left(\frac{r_{ij}}{r_o^{\pi}}\right)^{p_{bo4}}\right] + \exp\left[p_{bo5}\left(\frac{r_{ij}}{r_o^{\pi\pi}}\right)^{p_{bo6}}\right]$$
(2.5)

where  $p_{bo}$ s are ReaxFF parameters. Bond order is a continuous function that makes sure that it reaches to zero at long bond length (Figure 2.3).



Figure 2.3. A comparison between bond order functions for various bond natures.

After performing bond order calculation, one can evaluate bond energy by using the obtained bond orders as:

$$U_{bond} = -D_e^{\sigma} B O_{ij}^{\sigma} \exp\left[p_{be1} \left(1 - \left(B O_{ij}^{\sigma}\right)^{p_{be2}}\right)\right] - D_e^{\pi} B O_{ij}^{\pi} - D_e^{\pi\pi} B O_{ij}^{\pi\pi}$$
(2.6)

where  $D_e^{\sigma}$ ,  $BO_{ij}^{\sigma}$  represent dissociation energy and bond-order for sigma bond between atom i and j and correspondingly  $D_e^{\pi}$ ,  $BO_{ij}^{\pi}$  for a double bond and  $D_e^{\pi\pi}$ ,  $BO_{ij}^{\pi\pi}$  for a triple bond.  $p_{be1}$  and  $p_{be2}$  are parameters of the potential function. Similar bond-order dependent potential functions have been defined for  $U_{angle}$  &  $U_{torsion}$  functions.

Knowing the energy function, one can easily reach intra-atomic interactions and thereby, one can specify further atomic coordinates.

In this chapter we briefly introduced our methods that is going to be used. Our approach for MD simulations can be used to model several biological events. Here we consider two systems: a) effects of pH in protein denaturation, b) toxicity of graphene oxide. The properties of reactive MD provide us a unique opportunity to tackle these two systems because it is able to study reactions. For instance, it is feasible to analyze protein denaturation in different pHs using traditional MD methods; however, they are not able to show protonation of peptides that as we will show plays critical role in denaturation process. Moreover, we address the interaction between graphene oxide and the peptides using reactive MD. Thereby, any reaction that can lead to compositional change in the proteins can be elucidated.

## **Chapter 3**

#### 3 Case Study 1 - pH dependent analysis of alpha helix to random coil transition

We have studied alpha helix to random coil transition using ReaxFF reactive molecular dynamics as a function of pH. Urea binding to peptides and associated interference with back bone H-bonds and charged



side chains interactions, which can both denature the helices have been studied previously using non-reactive force fields [26]. This study reveals new proton-transfer mechanisms of denaturation of alpha helical structures, which cannot be captured by non-reactive molecular dynamics. In addition, we show proton transfer between the solution and the peptide can break the alpha helix hydrogen bonds and consequently, at extreme pHs significant amount of helix will unravel. We also compare the effects of temperature and alpha helix length in denaturation mechanism. The ReaxFF findings are in significant better agreement with ab initio calculations then previous non-reactive force field results – indicating the relevance of the reactive component on helical loss.

#### 3.1 Preface

After more than two centuries biochemical studies on proteins, we have learned a significant amount about how three-dimensional structures of peptides can influence various signaling pathways in human bodies. Regenerative disease such as Alzheimer's, Parkinson's or Diabetes all are caused by protein misfolding [27]. A protein's function can be lost if it is denatured. Denaturation can occur as a result of chemical treatments or elevated temperatures. Often, if the protein remains in solution, it can reform if its normal environment returns. Denaturation can lead to loss of protein-DNA and protein-enzyme interactions which themselves can cause diseases, such as Alzheimer's and Creutzfeldt-Jacob disease or Charcot-Marie-Tooth disease [28]. There is no doubt about the significance of maintaining the appropriate three dimensional structures to avoid serious disease. However, how different factors in the solution can alter such structures are still obscure. Proteins are mainly composed of alpha helices and beta sheets and some other intermediate structures. Changes in temperature, solution ionic strength and pH can affect their conformation. Several experiments have been performed to investigate each effect ranging from short sequence peptides to macro molecular structures [29-31]. However, none of them has shown the mechanisms of denaturation and the possible reactions that can occur in extreme pHs.

Zimm-Bragg [32] and Lifson-Roig [33] models are considered as the most classic way of studying this transformation. It has been tried to improve these statistical mechanics based approaches to capture more experimental observations [34]. Despite their valuable analytical formulations, they cannot take into account chemical reactions using such models.

Another reasonable method to address this problem is utilizing the molecular dynamics techniques. A significant number of simulations have been performed to show time dependent structural changes in different environments [34-37]. Concerning molecular dynamics, one can use various methods each of which differs mainly in the force field of which is being used. The well-known classical non-reactive force fields such as AMBER [38], CHARMM [39] and others [40-43], can properly simulate a wide array of macromolecules and can show different environmental effects on the proteins [44-46]. Early on, H. Kovacs and coworkers used the GROMOS force field to study the effect of chloroform, methanol and water on the extend of helicity [47]. Furthermore, using the same force field, P. Doruker and I. Bahar showed the sequence dependent helix denaturation of short peptides in water and vacuum [48]. Nevertheless, while these force fields are capable of studying a large number of molecules, none of them can show the possible intervening chemical reactions that are likely to happen in ionic solutions and the upcoming denaturation. By contrast, ab initio methods such as DFT can capture such reactions. I. A. Topol [1], for example, solved the debate between controversial results obtained by different non-reactive molecular dynamics techniques and experiments. However, *ab initio* techniques are very computationally demanding are not able to show time-resolved mechanisms of denaturation and cannot be utilized for large, complex systems.

ReaxFF, on the other hand, is not only able to take into account chemical reactions including bond breaking and bond formation, but also can present a time dependent evolution of the micro structure. Here, we use our previously reported force field for biomolecules [49, 50] to study pH dependent stability of peptides. We calculate helix percentage of an Ala based peptide in a wide range of pHs in the presence of H+ and OH– ions. Results show the backbone torsional angles change in extreme pH, which no longer can be described as a helix. Moreover, we show how proton transfer between the solution and the peptide in different pHs occur, which can clarify the denaturation mechanisms.

Temperature dependency and length effect are also studied. We also studied  $Ala_{13}$  in vacuum and observed that  $3_{10}$  helix is the stable configuration and alpha helix to  $3_{10}$  helix transition is likely. Our results show a good agreement with *ab initio* results and non-reactive molecular dynamics [51-54].

#### 3.2 Simulation Methods

As shown in [48], Ala has the most propensities to form helical structures among amino acids. To achieve the maximum possible helical structures, we employ pure Ala oligomers in our alpha helix design. However, one can repeat our simulations to study the sequence dependency of our conclusions.

Two different polypeptides are generated for the purpose of our simulations to capture the size dependency: Ala<sub>13</sub> and Ala<sub>78</sub>. Both of the structures are in zwitterionic form *i.e.* they are neutral, with NH3<sup>+</sup> and COO<sup>-</sup> ends and are stable at pH 7. Ala<sub>13</sub> is placed in a 30Å cubic box while for Ala<sub>78</sub> a rectangular 30 by 30 by 150Å box is used. The box is then filled with water to have a density equal to 1 g/cm<sup>3</sup>. To mimic acidic and basic pH, HCl and NaOH are added to solutions, respectively. Both Ala<sub>13</sub> and Ala<sub>78</sub> would show helicity around 100% in solution in the beginning. Both peptide and water are minimized at 1K and then 400ps NVT simulations at 310K (unless otherwise mentioned) are performed using ReaxFF force field. Verlet-Berendsen thermostat [45] with 100.0fs damping constant is recruited for NVT simulations and the time step is considered to be 0.1fs.

In contrast to previous studies on helix to coil transition, which have employed non-reactive force fields such as AMBER, CHARMM and GROMOS, ReaxFF is able to capture bond formation and bond breaking during simulation. In this force field the total energy of the system is described as:

$$E_{sys} = E_{bond} + E_{over} + E_{under} + E_{lp} + E_{val} + E_{coulomb} + E_{vdw} + E_{H-bond}$$
(3.1)

The parameters of this force field are obtained by means of quantum mechanical calculations. This procedure is described in our previous work [25]. The partial contributions in Eq. (1) include bond energies ( $E_{bond}$ ), energy contributions to penalize over-coordination and (optionally) stabilize under-coordination of atoms ( $E_{over}$  and  $E_{under}$ ), lone-pair energies ( $E_{lp}$ ), valence angle energies ( $E_{val}$ ) and terms to handle non-bonded Coulomb ( $E_{coulomb}$ ), van der Waals ( $E_{vdw}$ ) interaction energies and hydrogen bond energies ( $E_{H-bond}$ ).

The advantage of using reaxFF rather than non-reactive force fields is the capability of reaxFF to see the direct interactions between ionic residues ( $H^+$ ,  $OH^-$ ,  $Na^+$  and  $CI^-$ ) with the helix such as likely proton transfer from solution to the peptide in acidic environment or from peptide to solution in basic environment.

In order to study helix to coil transition as a function of pH change, we perform 6 separate simulations in pHs equal to 0, 1, 7, 13 and 14 plus one simulation in vacuum. Then we calculate the amount of helicity and interpolate the missing pHs. It is noteworthy that acidic and basic environments are provided by ionic species *i.e.* by direct adding of 16 HCl at pH 0 (2 HCl at pH 1) or 16 NaOH at pH 14 (2 NaOH at pH 13). Furthermore, the same simulations are done for Ala<sub>78</sub> to question the size dependency of helix to coil transition.
In addition, temperature change can have significant effect on denaturation results. Thus, a set of simulations is performed to take temperature effect into account. Ala<sub>13</sub> in pH 7 is exposed to temperature variations from 310K to 500K.

To illustrate the amount of helicity, two different representations are offered: traditional Ramachandran plots and a modified hydrogen bond length based criteria. Previously Doruker and Bahar [48] have used the hydrogen bond length as a criterion for helicity. However, one can question such measurements from two perspectives. First, in their measurements polypeptide would be considered alpha helix only if hydrogen bond length is less than 2.3Å. This assumption might seem unfair for the sequences with hydrogen bonds close to this value but with a little difference. Thus, in the present work to avoid such problems helix to random coil transition is described by a linear line between the full helix configuration at 2.1Å (or less) and full coil at 2.5Å (or more).  $\pm 0.2$ Å tolerance is considered to take into account the maximum amount of fluctuations. Second, the backbone torsional angles are ignored in their evaluations. Therefore, here the peptide is considered as alpha helix if it follows the following conditions:

$$Helix \% = \frac{1}{n} < \sum_{i=1}^{n} Res_i >_{[t,t+100]} \times 100$$
(3.2)

where the summation is over the amino acid residues,  $\langle * \rangle_{[t,t+100]}$  is averaging of results over time on 100 steps intervals to reduce the simulation fluctuations, *n* is the number of residues and



Figure 3.1. Alpha helix definition based on hydrogen bonds and back bone torsional angles

 $Res_i = \frac{1}{3}(f_1 + f_2 + f_3)$ .  $f_i$  and back bone torsional angle constrains are described in Figure 3.1. It should be noted that the same formulation could be used for  $3_{10}$  helical structures by counting *i*, *i*+3 hydrogen bonds.

# 3.3 Results and Discussion

#### 3.3.1 Helix to Random Coil Transition Measurements

Helix to coil transition is studied as a function of pH change of Ala<sub>13</sub> solution. 400ps reactive MD simulations show different amounts of helix to coil transition for different pHs. Initially all the peptides have their full helical structure. At neutral pH helical structure remains almost intact although some fluctuations can be observed at the final stages (Figure 3.2), but they seem to be temporal unraveling because of the end effects. By increasing or decreasing pH values, the denaturation accelerates. Results show ~65% ionization of HCl and NaOH within the first 10ps at pH0 and pH14. A qualitative depiction of helices at different pHs is presented in Figure 3.2.



Figure 3.2. Qualitative pH dependent alpha helix to random coil transition

In order to have a quantitative evaluation for the amounts of helix to coil transition, helicity is measured by the two discussed methods. The modified Doruker's method shows helix unraveling during time as is presented in Figure 3.3. After an initial stabilization, all the environments show helix to coil transition but with different rates.

Figure 3.4 shows a direct comparison between the effects of each pH on helicity. To be fair, the helix percentage is calculated using data collected for the last 20ps of the simulation. A fourth order polynomial is used for interpolation. As it is shown in Figure 3.4, extreme pHs lead to a significant amount of unraveling whereas for neutral pH, little unraveling is observed. It should be noted that helix to coil transition at neutral pH primarily occurs from the termini while at extreme pHs, both the internal H-bonds between N-H and C=O and the termini were destroyed.



Figure 3.3. pH dependent alpha helix denaturation using out modified criteria



Figure 3.4. Helicity as a function of pH. Neutral pH has the most helicty remained.

Ramachandran plots are also provided in Figure 3.5 to give insight about torsional dihedral angle changes. As it can be seen in Figure 3.5, extreme pHs distort  $\phi$  and  $\psi$  angles the most.

### 3.3.2 Denaturation Mechanisms

The advantage of reactive MD simulations over traditional, non-reactive empirical simulations [36] is that one can investigate reaction pathways that cause helix to coil transition. As mentioned earlier, all the peptides are in their zwiterionic form initially. However, after a while proton transfer between the solution and the peptide would change the protonation state of the peptides. Table 3.1 shows that proton transfer results in positively charged peptides in acidic environment and proton abstraction results in negatively charged peptides in basic environment. Also, proton transfer in pH 0 and 14 is about twice as fast as it occurs at pH 1 and13.

Our results show (Figur 3.2) that although often denaturation initiates at the C terminus, sometimes the denaturation begins from the central residues. Reactive molecular dynamics

simulation shows that this kind of denaturation primarily is because of the activity of ionic species in the vicinity of hydrogen bonds. Snapshots of 500fs simulation (Figure 3.6) at pH 0 shows a novel reaction mechanism between H+, Cl- and the oxygen of the peptide bond The mechanism of proton transfer to the peptide can be described as follows (Figure 3.6): a) HCl attacks to the hydrogen bond and stays in the vicinity by making a hydrogen bond with = O site. b) In return the regular hydrogen bond length between = O and – NH increases. c) HCl ionizes and the resulted proton binds strongly to = O (bond length about 1.1Å.) d) Finally, the Cl- ion moves away and –OH opens the helix groove.

The same mechanism affects other Ala<sub>13</sub> systems at different pHs but to different extend. Specifically in low proton concentrations, the proton is not able to make a strong bond with the carbonyl oxygen and stays in longer distances to keep its hydrogen bond. This also can open the helical grooves but to lesser amount. Some other mechanisms (Figure 3.7) that can lead to helix to random coil transition have been discussed in literature [26]. O. D. Monera *et al.* have shown how urea can bind to peptide bonds and interfere with back bone H-bonds [52]. Moreover, M. Bycroft and A. R. Fersht have described how intervening with charged side chains can denature the helices [53]. However, reactive molecular dynamics can also show how a proton might attack the helix and breaks the hydrogen bond, a phenomenon that is impossible to capture with common non-reactive force fields. Simulations on other peptides consisting of more active side chains (*i.e.* with charged or polar side chains) may show similar proton transfer reactions, which could also play some roles in helical denaturations.

Our simulation results also show a temperature dependency in helical structures. Three different simulations at neutral pH have been performed in various temperatures, 310K, 350K and 500K to

study temperature effects. As it seems, although in physiological temperature the helix remains, higher temperature can lead to a significant helix to coil transition even at neutral pH such that almost no helical structure can be remained in temperatures more than 350K. (Figure 3.8)



Figure 3.5. Ramachandran plots. Ala13 shows dihedral angle change mostly at extreme pHs.

pН	Helix%	Proton Transfer	Proton Transfer	Denatured
	after 400ps		Time (ps)	terminus
0	65.0	Receive H+ from C-terminus	117	C-terminus
1	69.3	Receive H+ from C-terminus	221	C-terminus
7		N/A	N/A	C and N-
	79.2			terminus
13	74.7	Release H+ from N-terminus	398	C-terminus
14	62.3	Release H+ from N-terminus	126	C-terminus

Table 3.1. Summary of chemical reactions observed by reactive MD simulations.



Figure 3.6. Helical structure begins to unravel by HCl attack to a central hydrogen bond. a) Minimized structure; b) HCl creates a hydrogen bond with oxygen of the peptide bond; c,d) HCl ionizes and proton forms a bond with oxygen which breaks the regular hydrogen bond between amide and oxygen; e) Ribbon representation of denaturation of helical structure.



Figure 3.7. Water molecules get inserted into hydrogen bonds and break them down primarily from two termini (yellow arrows). In pH0 proton transfer from H-Cl to C=O suddenly breaks the center of the helix (purple arrow). Other protons also facilitate hydrogen bond breaking (blue arrows).



Figure 3.8. Temperature dependent denaturation of alpha helices

#### 3.3.3 Alpha helix to 3-10 helix transition

Quantum chemical calculations [26] have shown that alpha helix is not the stable structure in vacuum; rather, alpha helix to  $3_{10}$  helix transition would occur. However, there are controversial results regarding helix stability from non-reactive force fields simulations [48]. To test the validity of force field, 300ps simulation on Ala<sub>13</sub> has been performed in vacuum at 350K (Figure 3.9). Helicity of Ala<sub>13</sub> is calculated based on our modified hydrogen bond criteria except that ii+3 H-bonds are enumerated for  $3_{10}$  helix. As it can be seen in Figure 3.9, alpha helix is not stable in vacuum; rather, it tends to transition to a higher density structure and form  $3_{10}$  helical structures as predicted by DFT and *ab initio* calculations [26]. However, this transition process is quite slow and therefore, to further illustrate the validity of such transition, we studied a helix, which initially is in its  $3_{10}$  form under similar conditions. Interestingly, the simulation results illustrates that in this condition  $3_{10}$  helix in vacuum can be justified by noting that  $3_{10}$  structure has more hydrogen bonds compared to alpha helix and energetically more favorable as well. Note that helicity percentage is found by the same formula used by Druker [26], i.e. hydrogen bond will break if and only if its length is more than 2.3Å so that results can be compared.



Figure 3.9. Comparison between non-reactive and reactive molecular dynamics simulation results for Ala13 in vacuum and in pH7 at 350K. At pH7 reactive molecular dynamics and non-reactive molecular dynamics are in good agreement; however, they differ in vacuum condition.

### 3.3.4 Length Dependency



Figure 3.10. Alpha helix to 310 helix transition at vacuum.

To see the length effect on helix to coil transition, five series of simulations are done on longer peptide *i.e.* Ala<sub>78</sub> in various pHs (pH 0,1,7,13,14). In these longer peptides we do not observe any helix to coil transition within the first 400ps simulation in 310K. It implies a size dependency for helix denaturation. Size dependency of alpha helix to random coil transition at neutral pHs can be described by Lifson Roig theory 33]. Length dependency of helix de naturation is primarily because of the end effects. In other words, for short poly Ala alpha helices (shorter than 14 residues), end effect plays an important role; however, for poly Ala alpha helices with more than 14 residues, end effects are negligible. In addition, we predict that long chains of peptides can also influence the total stability of the polypeptide in that central helix unraveling is also unlikely to occur for longer peptides. D. J. Jacobs and G. G. Wood have reported length dependency on helical content of alpha helix structures using Distance Constraint Model [54]. They showed up to

some rigidity correlation length, long strings of overlapping H-bonds incorporate together which can generate an additional entopic force which can save the alpha helical content more than what the shorter helices show. This explanation indicates that the mechanisms that break H-bonds at extreme pHs in shorter peptides cannot easily compete with the helix-retaining forces present in long helices.

### 3.4 Conclusion

ReaxFF reactive molecular dynamics is used to study pH dependent helix to coil transition. In contrast to non-reactive molecular dynamics, ReaxFF is able to capture proton transfer between helix and solution. Therefore, we are able to report a new mechanism for helix protonation, which leads to helix to random coil transition. In the presence of ionic structures, protons and hydroxyl groups in solution attack the hydrogen bonds and strongly bind to = O or N – H. It causes unraveling of helical structure and significant change in torsional dihedral angles. From the simulation results, the stability of Ala<sub>13</sub> alpha helix was found to strongly depend on pH. The presented results using reactive force field in pH 7 are in agreement with that of non-reactive simulations at 310K, 350K and 500K which demonstrates that in the high temperature conditions, helix will be distorted rapidly. Alpha helix to  $3_{10}$  helix transition is also observed in our gas phase simulations, which are in agreement with *ab initio* calculations.

pH variations are also studied on Ala<sub>78</sub> to capture the length effect on helix to coil transition and it is found out that for long poly peptides, neither end effect nor proton transfer can denature the helix. This observation is compatible with Distance Constraint Model predictions on length dependency.

# **Chapter 4**

### 4 Case Study 2 - Revealing Graphene Oxide Toxicity Mechanisms

Here we report the first study on graphene oxide (GO) toxicity in atomic scale to reveal the likely destructive mechanisms of GO during its interactions with living organisms. Reactive molecular dynamics study is utilized to illuminate the toxicity pathways and assess the available



hypothesis about GO biocompatibility. In this study, for the first time, we clarify the role of each GO functional group during its interactions with biological milieu. Our study suggests the possible chemical reactions between the GO sheet and different proteins that lead to oxidative stress, acidic or basic pHs and cell surface adhesions. It can be inferred from our analysis that the hypothetical oxidative stress arises primarily from epoxy groups (=O) while hydroxyl groups (-OH) are responsible for protein secondary structure denaturation and cell-surface adhesion as a result of strong H-bonding. Carboxylic groups (-COOH) which are easier to be noticed at the edges can play a soothing effect since they have opposite effects of epoxy to some extend. Moreover, a novel catalytic effect of GO is observed which can be ascribed to epoxy and carboxylic groups and can accelerate denaturation of tertiary structures. Finally, we show how the peptides containing aromatic rings (e.g. Tryptophan) adhere to the edges of the graphene sheet as a result of a stable  $\pi$ - $\pi$  stacking interaction with an equilibrium distance of 3.2-3.5Å

## 4.1 Preface

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 [51]. Amongst such unique applications, medical applications of graphene have recently been in the center of attentions [52-56]. Fullerene, nano-diamond, carbon nanotubes (CNTs), and graphene oxide (GO) all are accounted as a family of carbon driven materials and are used widely in places where living tissues are involved [57]. Given its considerable applications in medicine and biotechnology such as biosensors, gene delivery and drug delivery [58-60] a deep investigation on the possible hazards that it can pose on living organisms seems vital [57, 61, 62]. Despite various studies on toxicity of different carbon deriving materials, there are still controversial reports regarding the amounts of toxicity of the underlying mechanisms that give hazardous properties to them [63-65].

Among different graphene based materials, graphene oxide has its own particular applications in medicine because of its hydrophilicity. Graphene oxide is similar to graphene; however, some other functional groups are added to it to enhance its 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 epoxy (=O), hydroxyl (-OH) and carboxyl (-COOH) as the main underlying functional groups that form GO family. Density and range of these functional groups varies based on different manufacturing methodologies [66]. While Hofmann and Holst's suggest a model, which only consists of epoxy groups [67], Ruess considers a variation of this model by adding hydroxyl groups to it [68]. Nakajima and Matsuo, on the other hand, changed the

hypothetical sheet geometry whereas keeping the functional groups and thereby, suggested a lattice structure analogous to  $(C_2F)_n$  [69, 70]. Other functionalizations are also reported by adding amines [71], polyethylene glycol [72] and polyethylenimine [73].

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 [74]. To explore such possibilities different studies have been performed at cellular level [74, 75-82]. Not only are they controversial, but they also do not represent the precise mechanisms that bring about the described cytotoxicity. For instance, whereas several studies show that graphene based materials that are functionalized with chitosan, peroxide or PEG have excellent biocompatibility, others demonstrate toxicity that arises from oxidative stress, metal toxicity and sharp edges of graphene sheets and can lead to membrane rupture [83]. However, because of the complex interactions between GO and cellular membrane, final conclusion has often been postponed to further investigations ahead. 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 influence of different functional groups on GO-cell interactions at atomic level. Series of reactive molecular dynamics simulations have been performed 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. In addition, to see the validity of recent reports on the importance of edge effects and the geometry of GO on its cytotoxicity, the interactions between the peptides and GO strips are studied as well. Our results show epoxy and carboxyl groups have catalytic effects and can bring about tertiary structure denaturation. Such effects can be ascribed to accelerated thiol bond (-SH) breaking and aldehyde to carboxyl transition in the presence of oxidative stress. On the other hand, hydroxyl is primarily responsible for secondary structure denaturation. Hydroxyl groups on graphene sheets can form strong hydrogen bonds with hydrophilic side chains that not only plays an important role in secondary structure denaturation, but also can augment cell-surface adhesion as reported by Lee *et. al.* [84]. Moreover, our model describes how  $\pi$ - $\pi$  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 [85]. Our procedure to question GO toxicity can also be employed for further studies on other functional groups on graphene.

### 4.2 Methods

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 routes that protein-graphene adhesion may arise from are studied.

The objective of the upcoming study is to deeply analyze chemical interactions between graphene oxide functional groups and the cellular membrane at atomic scale to clarify the amount of cytotoxicity of GO and the underlying mechanisms of such interactions. In practice, it is hardly possible to purify GO in order to obtain specific functional groups on GO and sometimes not

feasible. Hence, using simulation is the only reasonable and possible way to perform such studies, primarily because there are no such obstacles ahead. Additionally, by recruiting molecular dynamics techniques, a time dependent manner can be achieved that one nicely can see atom-by-atom interactions, which can ultimately describe the toxicity of the material with the precision of fs. 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 final conclusion about the essence of each of these groups.

Nevertheless, it is essential to mimic the biological environment in our simulations so that the obtained results are trustable. To do so, the first step would be modeling the cellular proteins like FAKs, ligand binding receptors, and nuclear pore complex. It is impossible to analyze all such organelles and therefore, a 12-residues alpha helix is replaced by other proteins. Each peptide is designed somehow that they cover most of the significant activities of amino acid side chains such as ionic interactions, H-bonds, hydrophobic interactions and  $\pi$ - $\pi$  stacking interaction. In order to show that the presented results are not sequence specific, a mutated version of the first peptide is also studied (A<u>H</u>AGADACAM<u>W</u>A -> A<u>R</u>AGADACAM<u>G</u>A).



Figure 4.1. Graphene oxide in the presence of *a*) epoxy, *b*) carboxyl, *c*) hydroxyl, *d*) combination of all functional groups. *e*) shows the polypeptide, *f*) R->H and G->W mutation.

Graphene with periodic boundary conditions (29.55882 by 34.15774 Å) is built for all studies unless otherwise mentioned. Then different functional groups are added to graphene each of which in a separate system. Chemical formula of  $C_{96}O_7$ ,  $C_{96}(OH)_7$  and  $C_{96}(COOH)_7$  are used for epoxy, hydroxyl and carboxyl, respectively. Figure 4.1 shows 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 abovementioned subsystems are then placed in the vicinity (with an initial 3Å gap) and then the simulation box is filled with water to reach a density of 1g/cm<sup>3</sup>.

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

Among different choices of simulations, reactive molecular dynamics seems the most reasonable option. For instance, non-reactive force fields are not able to capture the reactivity of GO because of the weakness in taking 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.* [86] 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 cannot describe the reaction dynamics and neither can they simulate such enormous system. ReaxFF [87] force field is recruited for our purpose. Briefly, the total energy of the system can be described as:

$$E_{sys} = E_{bond} + E_{over} + E_{under} + E_{lp} + E_{val} + E_{coulomb} + E_{vdw} + E_{H-bond}$$
(4.1)

The parameters of this force field are obtained by means of quantum mechanical calculations. This procedure is described in our previous work [87]. The partial contributions in Eq. (1) include bond energies ( $E_{bond}$ ), energy contributions to penalize over-coordination and (optionally) stabilize under-coordination of atoms ( $E_{over}$  and  $E_{under}$ ), lone-pair energies ( $E_{lp}$ ), valence angle energies ( $E_{val}$ ) and terms to handle non-bonded Coulomb ( $E_{coulomb}$ ), van der Waals ( $E_{vdw}$ ) interaction energies and hydrogen bond energies ( $E_{H-bond}$ ).

#### 4.3 **Results and Discussion**

As discussed earlier, to quantify the toxicity of graphene oxide driven materials, we assessed the direct interactions between graphene functional groups and the peptides in 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 (it is assumed that the peptides be representative of the cellular membrane proteins) is defined as toxicity such that it some how affects proteins secondary or tertiary structures.

# 4.3.1 Epoxy

The principle composition of GO based materials is coming from epoxy group (=O) (Figure 4.1.*a*). Our analysis demonstrates epoxy high reactivity in the vicinity of water. 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 stays on graphene layer, whereas, the rest will be solvated inside the water and may attack the peptide in a reaction as follows:

$$R - CHO + O \xrightarrow{GO(aq)} R - COOH$$
  
or  
$$H_2O + O^{2-} \rightarrow 2OH^{-}$$

Therefore, our simulations obviously show oxidative stress at epoxy high concentration. However, the amount of such atomic oxygen strongly depends on the density of not only epoxy group but also other groups as well. This will be discussed further in the upcoming sections. It is worthy note that our simulation results can justify why hydroxyl groups are so likely to be observed in GO as they can be arose from reactions between water and epoxies. The consequence of oxidative stress in biological environment can be investigated by looking through GO-peptide interactions. Figure 4.2 clearly depicts the process in which atomic oxygen can be detached from GO surface and attacks the peptides. This process can be described as follows:

- a) The peptide approaches GO surface (Figure 4.2.*a*).
- b) Water gets involved in interaction with epoxy group and provides two hydroxyl groups, one of which attacks to the aldehyde (-HCO) capping end (Figure. 4.2.*b* and 4.2.*c*).
- c) Proton from aldehyde transfers to the second hydroxyl and form a water molecule together with a carboxyl capping end instead (Figure 4.2.*d*, Figure 4.2.*e* and Figure 4.2.*f*)
  We stipulate that two ends of the peptides are capped in order to hinder the helix from unraveling

fast as our randomly sequenced peptide is not a good helix former.

Furthermore, we content that such oxidative stress is accentuated by the presence of graphene sheet. To check the validity of this idea, the same system is tested again but in the absence of the graphene sheet. (The concentration of hydroxyls is remained constant.) Nonetheless, after 200ps the aforementioned reaction did not occur which can itself attribute a catalytic behavior to graphene oxide beside its capacity to form oxidative stress.







Figure 4.2. Effects of epoxy functional group on the peptide, mechanisms of oxidative stress production and aldehyde to carboxyl transformation. *a*) water molecule approaches to the graphene sheet and *b*) form Hbond 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 to the solution.

# 4.3.2 Carboxyl

The same approach is recruited to analyze carboxyl groups on GO (Figure 4.1.*b*). For the sake of comparison, the same distribution as epoxy is used for carboxyl test. Clearly one might predict proton transfer from carboxyl groups on GO to solution because of acidic behavior of COOH. As it was expected, a system consisted of graphene sheet with a significant number of carboxyl groups renders an extremely acidic solution for the peptide such that Asp residue of the peptide gets protonated (Figure 4.3).



Figure 4.3. Influence of carboxyl group in protonation of Asp side chain.

In addition to the capability to provide an acidic environment, carboxyl groups on GO may cause the thiol bonds (-SH) breaking in Cys residue of the peptide (Figure 4.4). When Cys residue of the peptide is confronted with carboxyl group on GO (Figure 4.4.*a*), it forms an H-bond with carboxyl (Figure 4.4.*b*) followed by breaking C-O bond in carboxyl (Figure 4.4.*c*). The consequence would be transformation of carboxyl to epoxy and deprotonating of Cys residue. In other words:

$$R - COOH + R' - SH \xrightarrow{GO(aq)} R - CO + R - S + H_2O$$

The significance of the deprotonating of Cys residue *i.e.* thiol bond breaking, would be more clear in the presence of Cys residue in other peptides. The deprotonated Cys residue is capable of forming disulfide bonds, which is important in tertiary structure change of the proteins. Disulfide bonds plays critical role in protein folding and can bring about amyloid formation by misfolding proteins which itself 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) [88].

The simulation results also support carboxyl catalytic behavior. To test this allegation, we repeated the simulation in the absence of the graphene sheet and no Cys deprotonation was observed which implies that GO with carboxyl group can accelerate thiol bond breaking.





Figure 4.5. Influence of carboxyl group on the peptide and the thiol bond breaking. *a*) thiol bond and the carboxyl make an Hbond, *b* and *c*) one hydroxyl dissociates from carboxyl while thiol bond gets deprotonated. This finally results in a water molecule, which later dissolve into the solution and one ketone group on the graphene sheet.

4.3.3 Hydroxyl

Analogous to our previous analysis for epoxy and carboxyl, functional groups are replaced by hydroxyl (Figure 4.1.c). Interestingly, simulation results illustrate hydroxyls as highly inactive groups that tend to stays on graphene sheet without any reaction. However, there seems to be a very small electron share between carbon in graphene sheet and oxygen of hydroxyl, which causes OH to be remarkably polar. As a consequence, it is expected to observe a strong H-bond between hydroxyl and the peptide side chains. This argument can be supported by DFT calculation results, which suggest that the H-bond energy between a water molecule and hydroxyl group of GO (R-OH---OH<sub>2</sub>) (~10.8 kcal/mol) is more than twice as the H-bond energy between water and methanol (CH<sub>3</sub>OH---OH<sub>2</sub>) (~4.3 kcal/mol) [89, 90]. This strong H-bond is able to adhere the peptide to GO surface in the presence of hydrophilic side chains. Our simulation results show a 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 to reach to maximum H-bonding if enough polar side chains are present. The effect would be secondary structure denaturation, which is attributed to many abnormalities [88]. It is worthy to note 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 graphene layer and in the presence of graphene layer but without any functional group on it. Then, the amount of denaturation of secondary structure is calculated correspondingly and is defined as control system. Not surprisingly, we reached approximately the same amount of denaturation for no graphene and with graphene (but no functional group on it)

case. Moreover, graphene sheet alone showed no chemical reactivity in our simulation and one might attribute graphene sheet possible toxicity to its sharp edges (a physical agent).

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Nevertheless, the essence of observed oxidative stress out of graphene sheet [91] remains ambiguous.

#### 4.3.4 Lerf-Klinowski model (42)

Then we incorporate Lerf-Klinowski (LK) model (Figure 4.1.*c*) in our analysis to see the combined effects of the functional groups. For the sake of comparison, we keep the concentration and distribution of all groups on GO to be similar even though LK model suggests that carboxyl groups should be placed at the edges. We will next consider the exact LK system to study the effect of peripheral carboxyl groups.

Our simulation results repeat the same conclusion for hydroxyl regarding its inactivity. On the other hand, it illustrates that carboxyl and epoxy have opposite role to the effect that carboxyl groups are capable to subdue epoxy groups destructive function which itself can justify the endocytosis of carboxylized graphene versus apoptosis in the presence of pristine graphene [91]. Figure 4.5 clearly depicts how oxidative stress arising from epoxy can be harnessed by the presence of carboxyl groups. As it is shown in Figure 4.5.*a*, water molecule attacks the existing epoxy on graphene and form H-bond. Afterwards, analogous to the epoxy case, it will result in two hydroxyls one of which stays on graphene while the other one gets solvated (Figure 4.5.*b*). Hydroxyl anions, however, have the tendency to get protonated and form a water molecule instead. The neighboring carboxyl renders hydroxyl anion the required proton (Figure 4.5.*c*). Hence, interestingly, this event can show in the presence of all functional groups, carboxyl cannot acidify the solution significantly. Also the oxidative stress coming from the existence of the epoxy groups is no longer easy to be captured.

Now, it is time to compare the toxicity of each group. Our criteria to describe the amount of toxicity of each groups is based upon:

- 1) Secondary structure denaturation or any factor that impairs the helical configuration.
- Tertiary structure denaturation or any factor that changes the polarity or reactivity of the side chains of the helix including thiol bond breaking, protonation state of electrically charged residues or any compositional change in the side chains.
- 3) Adhesion: The average distance between the peptide atoms and GO surface is characterized as adhesion criteria. In other words, the closer the helix to the graphene sheet, the more cohesive the layer would be considered.
- 4) 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 calculate pH simply by counting the free protons available in the solution at each moment.



Figure 4.6. Opposite effect of carboxyl and epoxy on the peptide and the solution. *a*) Water molecule interacts with epoxy and form two hydroxyls. Similar to the aforementioned scenarios, while one binds to the surface, the second one goes into the solution. *b*) The hydroxyl in interaction with carboxyl gets protonated. *c*) This results in one new water molecule, which diffuses into the solution.

Figure 4.6.*a* compares the secondary structure loss as function of different groups on GO. As it is shown, the maximum loss pertains to hydroxyl followed by epoxy. Although not significant, comparing to no graphene case (control), GO seems to be able to accentuate helical revelation. This can be justified by considering the strong H-bond between peptide side chains and functional groups on GO as we discussed earlier.

Impacts on tertiary interactions can be inferred from our recent discussion by looking through the accompanying reactions that have occurred to the side chains. Accordingly, carboxyl and epoxy

show potential for applying compositional change on the side chains, which might be interpreted as an effect on tertiary structure of the proteins.

Our adhesion criterion is based on the distance between the GO sheet and the peptide. We calculated such distances and the results are presented in Figure 4.6.*b*. Apparently, hydroxyl group have the most tendency to grab the protein, which is not strange by taking its strong H-bond into account.

Next, pH changes as a function of different groups are calculated during the time and presented in Figure 4.6.*c*.






Error bars show one standard deviation in five series of simulations on similar configurations for each data set.

Not surprisingly, pH changes are not sequence dependent. Moreover, as it is discussed earlier, hydroxyl shows no chemical activity and therefore, the corresponding pH is close to 7 while epoxy and carboxyl shows basic and acidic pH, respectively. Table 4.1 summarizes the comparisons between the influences of different functional groups on secondary and tertiary structures.

Groups	0	ОН	СООН	MIXTURE	W/0 G0
рН	13.4	7	0.4	0.7	7
Secondary	×	$\checkmark$	×	Not	-
Interaction				Significant	
Tertiary	1	×	1	Not	-
Interaction				Significant	

Table 4.1. Summary of the comparisons between the influences of different functional groups on secondary and tertiary structures.

## 4.3.5 Lerf-Klinowski [92] model with peripheral carboxyl

Last but not least, 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 concentration of groups to see if the toxicity effect is dose dependent [97]. We also added edges into the system to assess the edge effects especially the alleged adhesion to the edges of GO sheet because of  $\pi$ - $\pi$  interaction [85] (Figure 4.7).

This time the similar system is used but GO is modified. After 400ps simulation at physiological temperature, LK GO shows a surprisingly low activity compared to the previous simulations. It is observed that peripheral carboxyl depicts low tendency for deprotonation compared to the conditions when they are placed in the center. Also, the observed oxidative stress in the previous simulations diminished remarkably. Indeed, we report almost neutral pH~ 7 for GO with LK geometry. (Note that even few protons dissociated from carboxyl groups are neutralized by arisen hydroxyls from epoxy groups.)

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

As it is shown in Figure 4.7, given a low concentration of epoxy 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  $\pi$ - $\pi$  stacking interaction (Movie S1). We report an equilibrium intra-distance of 3.3 (±0.1 Å) between two aromatic rings in a good agreement with DFT

calculation for 3.2 to 3.5 Å. This event leads to a significant adhesion characteristic to proteins with Trp (and probably other residues with aromatic rings). Interestingly, our results verify this prediction such that the average distance of A<u>H</u>AGADACAM<u>W</u>A is 2 times smaller than A<u>R</u>AGADACAM<u>G</u>A (without Trp). Therefore, our simulation is able to justify the protein adsorption on the graphene sheet observed by Liu *et. al.* [85].



Figure 4.8. Lerf-Klinowski model for graphene oxide. Snap shots show  $\pi$ - $\pi$  stacking interaction between graphen 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 it and *c*) stays there at equilibrium distance of 3.3 (±0.1 Å). *d*) shows top view of peptide and the graphene sheet orientation. *e*) represents the diffusion of the peptide to adhere to the graphene layer.

## 4.4 Conclusion

Herein, we evaluate the amount of toxicity of graphene oxide driven material using reactive molecular dynamics. Our method is able to probe the probable reaction mechanisms between the graphene oxide sheet and the membrane proteins at atomic level and thereby, describe chemical behavior of each functional group on graphene, which may lead to its claimed toxicity. We tested epoxy, carboxyl, hydroxyl and a combination of them to see the corresponding secondary and tertiary denaturation mechanisms. Results imply that epoxies and carboxyl groups have opposite chemical behavior and provide basic and acidic pHs, respectively. Besides, in a catalytic pathway, epoxy has the potential to produce oxidative stress whereas carboxyl can accentuate disulfide binding. Hydroxyls are able to form strong H-bonds and plays important role in cell-surface adhesion.

Moreover, using Lerf-Klinowski model for graphene oxide, we show how  $\pi$ - $\pi$  stacking interaction can influence cell-surface adhesion. Also it justifies the fact that while each functional group is active, their mixture in practice is observed to be relatively less active.

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