LEVERAGING ELECTROCHEMISTRY TO UNCOVER THE ROLE OF NITROGEN IN THE BIOLOGICAL REACTIVITY OF NITROGEN-DOPED GRAPHENE

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Introduction

While there is substantial research on the role of nitrogen (N) doping in broadening graphene applications (energy conversion and storage, electronics, sensors),[1-3] significantly less is known about the influence of N doping on the bioactivity of graphene. Resolving the material properties that govern impacts of N-doped graphene (NG) at bio interfaces is critical to: (i) developing rational design guidelines to meet desired functional performance outcomes while minimizing the potential for unintended consequences, and (ii) enabling their biomedical and bioanalytical development.

Our previous study on reduced graphene oxide (GO) demonstrates the positive correlation between electrochemical activity and the propensity to induce biological oxidative stress.[4] On the other hand, in our efforts to probe underlying mechanisms of electrochemical and biological reactions of oxygen functionalized carbon nanomaterials,[4-6] we identified electron transfer properties common to both activities that are similarly tuned by different surface functional groups. In this work, we investigate the potential for N doping to (i) decouple function and biological reactivity of graphene, and (ii) establish the foundation for a new paradigm linking inherent electronic and biological activities of carbon nanomaterials. Two important electrochemical reactions, oxygen reduction reaction (ORR) and oxygen evolution reaction (OER), are used as representative functional performance metrics and to characterize inherent electronic behaviour of NG as the potential mechanism underlying bioactivity.

Materials and Methods

The NG samples were synthesized by a hydrothermal method followed up with thermal annealing. Nitrogen precursors are mixed with GO in a hydrothermal autoclave reactor, and nitrogen doping occurs concomitantly with the elimination of oxygen groups on the GO. Two different precursors were used herein, urea and uric acid. The samples after the one-pot hydrothermal reaction with urea and uric acid are labelled as NG-U and NG-UA, respectively.

There are four nitrogen configurations that are commonly observed in NG, namely, pyridinic-N, pyrrolic-N, graphitic-N, and N-oxide (Figure 1).[2, 7-9] The type of N introduced into the carbon lattice of our samples was tailored using thermal annealing at different temperatures (650, 800, 950 ℃) after the hydrothermal reaction, leveraging different thermal stabilities of the N-types: graphitic-N > pyridinic-N > pyrrolic-N.[3, 10] The samples after annealing NG-U at 650 ℃ and 950 ℃ are referred to as NG-U-650 and NG-U-950, respectively. The samples after annealing NG-UA

at 650 ℃ and 800 ℃ are denoted by NG-UA-650 and NG-UA-800, respectively.

X-ray photoelectron spectroscopy (XPS) was used to quantify the amount and distribution of N-types on our different samples. Raman spectroscopy was used to characterize the changes in defect density by comparing the intensity ratio of the D and G peak (I_D/I_G) to differentiate the doping types (n- or p-type) based on the shift of G peak position.[11-13] Transmission electron microscopy (TEM) was used to observe the changes in the graphene morphology.

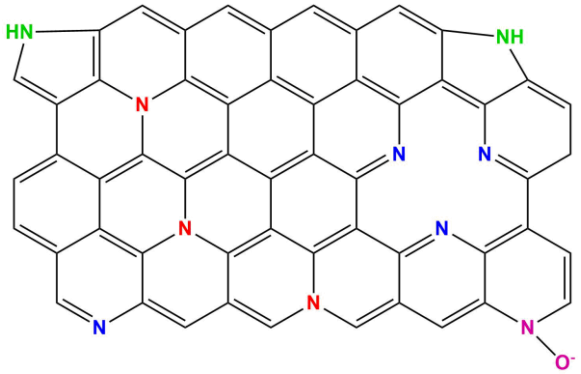

Figure 1. Schematic N-doping configurations in N-doped graphene, including pyridinic-N (blue), pyrrolic-N (green), graphitic-N (red), and N-oxide (purple).

The bioactivity of the prepared materials is evaluated as the inactivation of a bacterial model organism, *Escherichia coli* (*E. coli*), and the propensity to oxidize the intracellular antioxidant, glutathione (GSH). The GSH oxidation was evaluated using Ellman's assay while the bacterial inactivation was determined by colony counting method.

Electrochemical ORR and OER are commonly carried out in a hydrodynamic condition to enhance mass transfer by the induced convection so that the limiting current can be achieved for slow kinetics of reaction. Rotating ring-disk electrode (RRDE) and rotating disk electrode (RDE) measurements were performed for ORR and OER, respectively.[14]

Results and Discussion

*Differentially treated NG samples exhibit different propensities for GSH oxidation and E. coli inactivation, with the oxidative stress identified to be the dominant mechanism (**Figure 2**).*

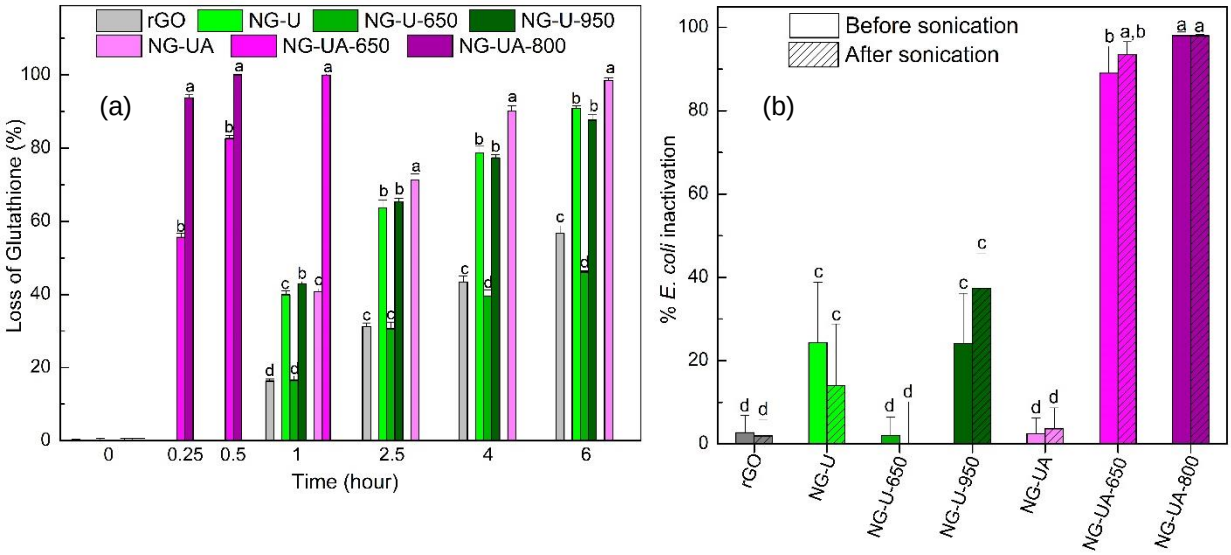

Figure 2. (a) GSH oxidation by rGO and NG samples for a total 6 h of incubation compared to the control (no rGO/NG). The mass loading of samples is 0.05 mg mL^{-1} and the initial concentration of GSH is 0.4 mM. (b)

Cytotoxicity of rGO and NG samples to *E. coli* after 4 h of reaction to 0.2 mg mL^{-1} sample before and after 10 min bath sonication to release viable bacteria wrapped in aggerated graphene sheets. The data were normalized to the control (saline solution without rGO/NG), the cell concentration of the control remained constant after the 4 h incubation and after the 10 min sonication. Means suffixed with different letters (a-d) for each time point are significantly different from each other at $P < 0.05$. Error bars denote the standard deviations of sample replicates.

GSH oxidation serves as an indicator of oxidative stress and correlates with the cytotoxicity resulting from the chemical pathway.[4, 15-17] Significant differences in GSH oxidation is observed across the NG samples. TEM images (not shown) indicate similar morphology, suggesting any contribution of the physical mechanism will be similar for all samples. The physical wrapping mechanism is typically investigated by releasing trapped cells through a mild bath sonication after exposure and prior to plating.[17-19] No statistical difference is observed before and after sonication, thus, cell entrapment is not observed. The similar trend in GSH oxidation and *E. coli* inactivation suggests a dominant chemical mechanism of cytotoxicity.

*Evaluation of functional electrochemical activities (ORR and OER) of NG samples validate different electron transfer properties as a function of nitrogen type (**Figure 3**).*

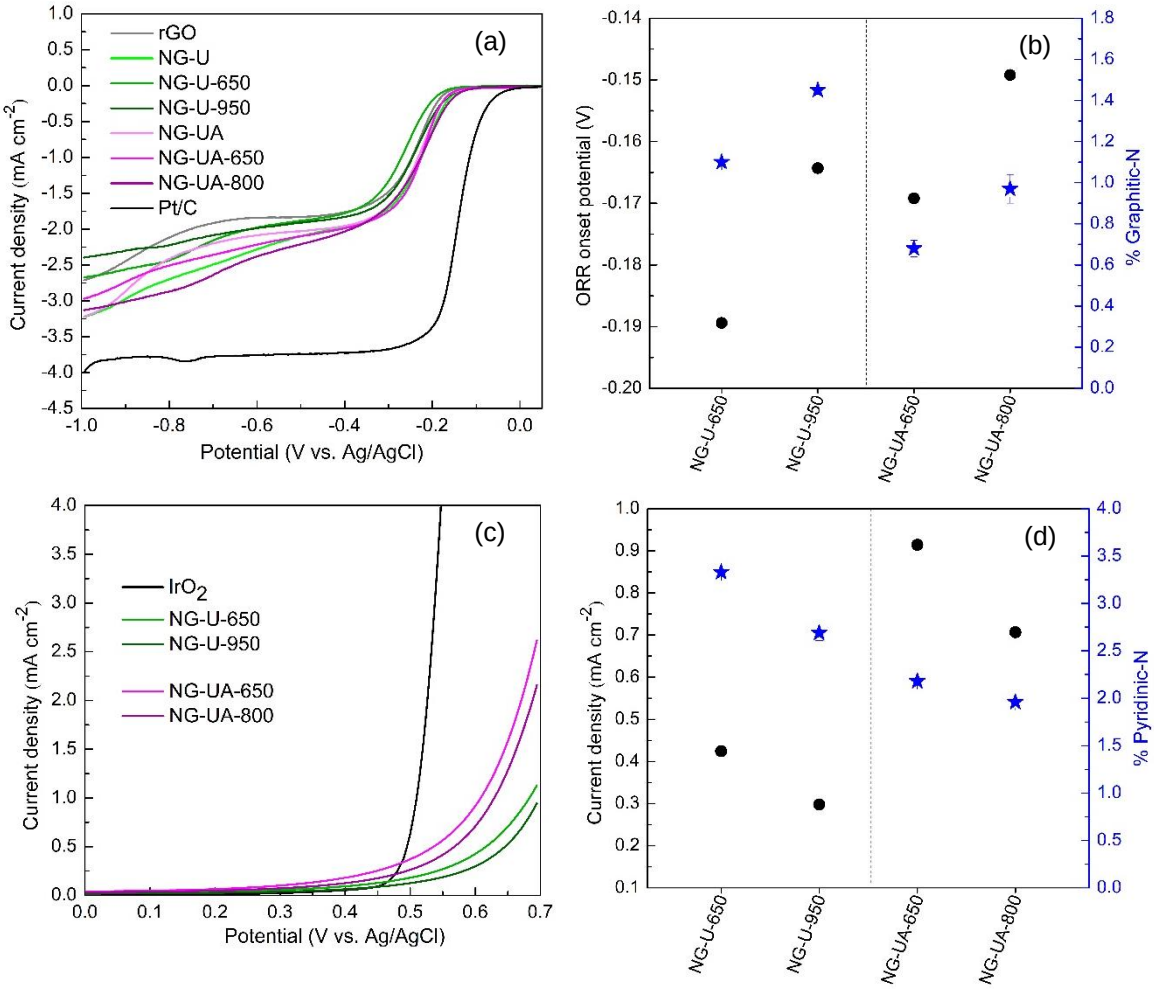

Figure 3. (a) ORR polarization curves of rGO, NG samples, and Pt/C on RRDE in O_2 saturated 1 M KOH with a rotation rate of 1600 rpm and a scan rate of 5 mV s^{-1}. (b) The correlation between the ORR performance of

NG (represented by the onset potential) and the percent of graphitic-N (determined by XPS). (c) OER polarization curves of annealed NG samples and IrO_2 on RDE in O_2 saturated 1 M KOH with a rotation rate of 1600 rpm and a scan rate of 5 mV s^{-1}. (d) The correlation between the OER performance of annealed NG samples (represented by the current density at a potential of 0.6 V) and the percent of pyridinic-N (determined by XPS).

ORR is driven by electron-donating graphitic-N while OER is driven by electron-withdrawing pyridinic-N. Graphitic-N possesses the electron-donating characteristic[11, 20, 21] and greater charge carrier transport over other N-types.[20, 22] Graphitic-N atoms can lower the O_2 adsorption barrier by decreasing the repulsive interaction between graphene π electrons and lone pair electrons of O_2,[23, 24] and can facilitate the donation of electrons to the adsorbed O_2 to form OOH species,[25, 26] both of which are key steps to enhance the reduction of O_2 in alkaline solution. Carbon atoms adjacent to electron-withdrawing pyridinic-N atoms carry a partial positive charge (δ^+), thus serving as electrophiles facilitating the adsorption of intermediates necessary for water oxidation (e.g., OH^-, OOH^-).[25, 27] In addition, the polarized pyridinic-N (δ^-) is not favourable for the reduction of O_2 due to its high density of N lone pair electrons that cause strong repulsive interaction with the O_2 approaching its adjacent carbon atoms.[24]

*The similarity between GSH oxidation and ORR activity reveal the role of graphitic-N as the active site in oxidative stress related bioactivity (**Figure 4**).*

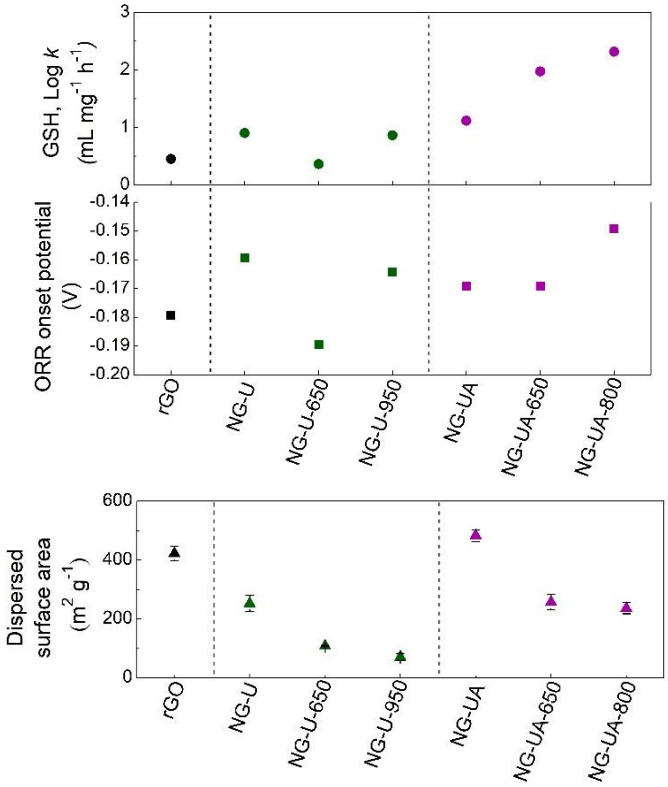

Figure 4. (a) The correlated trends between the rate constants for GSH oxidation and the onset potentials for the ORR activity. (b) The dispersed surface areas in suspension determined by the adsorption of methylene blue (MB).

ORR shares similar mechanism with GSH oxidation, both of which are dominated by O_2-mediated catalytic process to reduce O_2 that starts with O_2 adsorption on the active sites of graphene materials.[16, 28] Samples prepared using uric acid as the precursor have lower amounts of graphitic-N than samples prepared using urea yet have higher surface area, which contributes to the overall higher oxidative potential. Collectively, the data supports multiple factors synergistically influencing the NG-mediated oxidation of GSH. In addition to the defect density (data not shown) and dispersed surface area, uncovering the role of graphitic-N in both ORR and GSH oxidation elucidates the underlying mechanisms of oxidative-stress induced biological activity. The similarities in ORR and GSH trends suggests the potential to employ electrochemical tools to evaluate the relative bioactivity of graphene materials via chemical pathways.

Conclusions

Graphitic-N is responsible for ORR and O_2 mediated oxidative stress in bioactivity while pyridinic-N is responsible for enhancement in OER and does not catalyze O_2 mediated oxidative stress. Defect density (data not shown) and surface area are consequential properties after N doping and work in concert with chemical composition (N types) to modulate both electrochemical and biological activities. N types can be leveraged as a good design handle toward rational design of graphene. Identified correlation between electrochemical ORR activity and oxidative stress related bioactivity of graphene-based nanomaterials in our work[4-6] uncovers the importance of electronic nature of graphene, informing the ability to design materials for electrochemical applications while also advancing predictive toxicity capabilities.

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