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ROS generation by reduced graphene oxide (rGO) induced by visible light showing antibacterial activity: comparison with graphene oxide (GO)

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Reduced graphene oxide (rGO) generates reactive oxygen species (ROS) under visible light in air via singlet oxygen – superoxide anion radical pathway which readily kills *Enterobacter sp.* The rGO+ intermediate reacts with hydroxyl ion to produce graphene oxide (GO) as a coat on the surface of rGO resulting enhanced fluorescence and slowing down the photo induced ROS formation. GO is not toxic but on ageing gets surface coating of rGO to show its toxicity.

The synthesis of graphene oxide from graphite is dominated by the classic method introduced by Hummers et al.[1] This GO became soluble in water as it contains several functional groups like carboxylic, hydroxyl groups.[2] This solubility feature attracted several studies to explore its utility in diversified fields. GO is used in catalytic oxidation[3-5], biotechnology[6-9] and as a surfactant.[10] However, a straight forward ready conversion of GO to graphene is not observed as it is difficult to get rid of all those attached hydrophilic oxo groups in GO. Graphene oxide under reduction converts into a reduced GO form improving the electrical conductivity.[11, 12] The reactivity of GO under varied chemical environments have been investigated. One important aspect of such a reaction is the reduction of GO. It was observed that GO is changed to reduced graphene oxide (rGO) under exposure with reducing chemicals or bio- molecules including bacteria or even under bright light.[13] This reduction of GO to rGO diminished its dispersibility in water and fluorescent properties as well [14].The physicochemical changes of GO on reduction to rGO led to the development of newer optoelectronic properties which are being used in physical and biochemical applications.[13] It is now known that graphene and GO are by large non-toxic to humans [15]. However, rGO, the intermediate species, perhaps, with no fixed stoichiometry with respect to C : O (representing the attached oxo functional groups) in between GO and graphene, has not been checked for its benign role towards human health. Therefore, we undertook the work and herein show that rGO generates ROS from aerial oxygen even under visible light and such reaction though harmful to human but may be used to combat hospital pathogens.

The GO for this work was prepared by the well-known Hummers method[1] and was characterized. This GO was reduced by using four conventional reducing agents like hydrazine hydrate, sodium borohydride, hypophosphorous acid and sodium dithionite to get rGOs of different shade (see S1). All these rGOs are now subjected to sunlight (or indoor tungsten lamp of 60W light irradiation) using water filter and glass tube to cut off thermal and UV irradiation. In a typical experiment rGO, (hydrazine hydrate reduced) (0.5mgmL^{-1}) was just dispersed mechanically in water containing 0.6 µmol nitro blue tetrazolium chloride (NBT). The pale yellow solution of the mixture slowly changed to blue under light exposure within 30 minutes (figure 1).

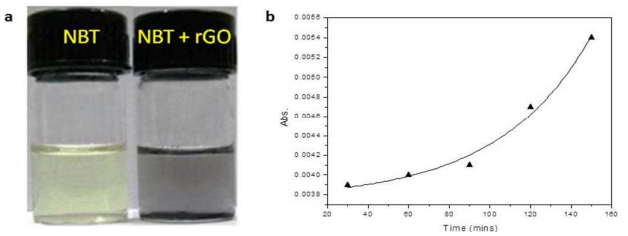

Figure 1 a, Blue diformazan formation on adding rGO (5 mg) in sunlight into (0.6 µmol NBTsolution) ; b, the increase in diformazan formation with the increase in the light exposure time.

The change in yellow to blue colour of the NBT solution is characteristic to the formation of diformazan dye[16]. On extending the time of light exposure the intensity of the blue colour steadily increased (a part of diformazan is precipitated out when formed in excess) to a point and on further irradiation ceased to develop further. Similar reactions in the

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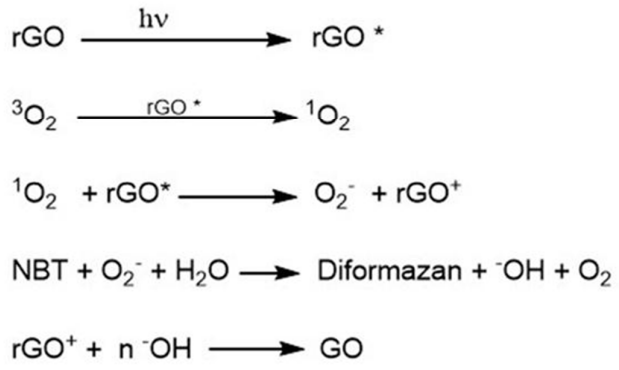
presence of either sodium azide or dimethylsulfoxide of fresh rGO with NBT does not produce any blue diformazan .As azide ion and DMSO are well known quenchers of singlet oxygen ($^1O_2$) and hydroxyl radical respectively [17] so it is apparent that the light induced reaction of rGO with oxygen proceeded via the formation of such species.

Thus the reaction proves that light induced excited rGO* transfers its energy to triplet oxygen ($^3O_2$) to form singlet ($^1O_2$) at the initial stage and finally have other reactive oxygen species including hydroxyl radical. The next step reaction could be the excited rGO* reduces $^1O_2$ to superoxide ion, $O_2^{\cdot-}$ which is known to react with NBT involving abstraction of protons from water (scheme 1).



Scheme 1. Reaction scheme for photo-induced superoxide generation by rGO, The self generation of other ROS including OH radical from superoxide radical anion has not been shown.

Therefore the diformazan formation increases the pH of the reaction medium with the progress in time (figure 2a) and this generated OH^- can react with rGO^+ to produce GO (scheme 1). The alternate direct hydroxylations by hydroxyl radical of rGO to produce GO is possible. The formation of hydroxyl radical is anticipated as observed by the quenching ability of DMSO in this reaction [17].

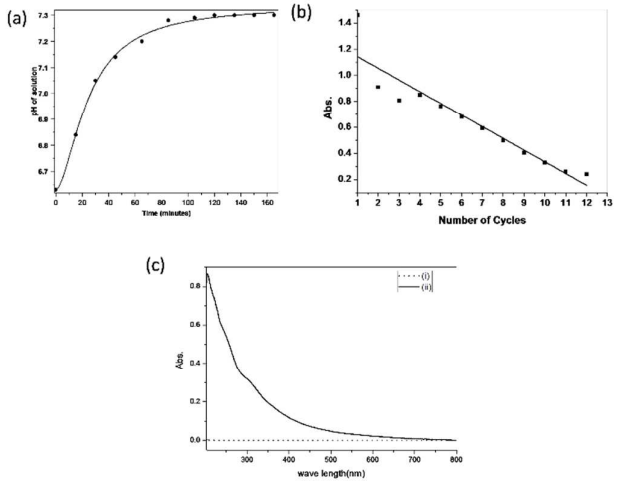


Figure 2 a) The increase in pH of NBT-rGO solution on exposure to sunlight λ≥300 nm with time intervals of 15 mins. b) The slowdown of diformazan formation (O_2.- generation by rGO on its reuse in several cycles (photo-exposure time per cycle was an hr), c) electronic spectrum of formed GO in ethanol(i) where rGO is virtually insoluble(ii) (broken line)

Whether rGO is catalytic or not has now been tested by re - using the used rGO with fresh NBT solution in several subsequent cycles. We observe that the production of diformazan decreases gradually in subsequent cycles (figure 2b). This shows that after every cycle the ability of rGO to generate superoxide radical reduces. Interestingly, washing the used rGO with 10% sodium hydroxide followed by its acid wash workup with 6N HCl and drying under anaerobic condition in the dark showed a weight loss of around 30% but the residual alkali-acid treated rGO regains its ability to produce superoxide anion (diformazan production) from air under exposure with light. The NaOH leached part on neutralization with dilute HCl acid and after evaporation has been checked. This residue on extraction with ethanol when subjected to electronic absorption displayed spectrum of GO in Figure 2c. Interestingly rGO is not soluble in ethanol (Figure 2c). Therefore such apparent solubility difference in between GO and rGO can readily be made to identify both the species.

Further the alkali washes were found to be fluorescent (not shown) and this part contains GO as shown in Figure 2c. The leaching of GO in alkaline medium showing enhanced fluorescence has been reported [18]. These results led to conclude that the excited rGO donates an electron to singlet oxygen to create superoxide ion. The NBT-superoxide-water reaction generates diformazan dye liberating HO^- ions. This hydroxyl ion reacts with rGO^+ to produce GO which may coat the surface of the insoluble bulk rGO to passivate it for the subsequent reaction to occur. The superoxide anion generates other reactive oxygen species (ROS) like hydroxyl radical that may directly react with rGO to produce GO. This establishes that the activity of rGO to produce superoxide is hindered by the deposition of GO on its surface produced by light induced ROS. The general belief as GO generates superoxide radical from aerial oxygen and thus toxic to produce ROS is wrong as freshly prepared GO has been checked and found that it does not have the capability to generate superoxide radical in air under light exposure (see S2). These experiments when carried out in phosphate buffer saline (PBS) (0.01M) in the pH range between 6.8 and 7.4 under tungsten light (60 W) indoor showed similar result indicating that rGO is capable to produce ROS to damage cells under physiological pH. The starting rGO and light exposed rGO at pH 6.8 have been subjected to fluorescence microscopy to show that rGO does not show any observable fluorescence but after tungsten light irradiation fluorescence in all the three visible lights are observed (figure 3) which is characteristic to GO.

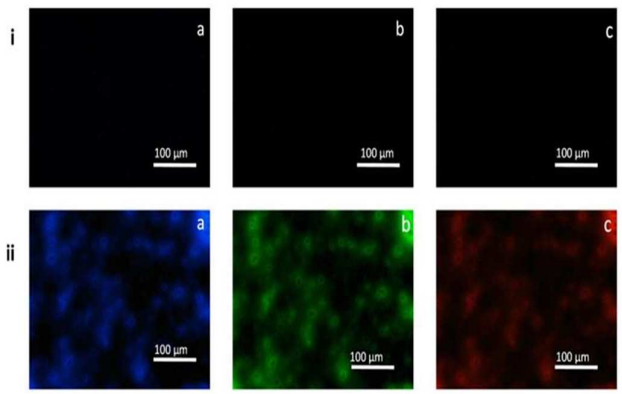


Figure 3 i) Fluorescence of fresh rGO, ii) fluorescence of rGO after light exposure in air for two hr in PBS buffer, pH,6.8. The excitation wavelengths are in the range of a) 385, b) 488, c) 561 nm.

This ability of rGO to generate ROS under indoor light in air made us to search its antibacterial property. This is more important to combat strain like New Delhi metallo-beta-lactamase1 (NDM1 producing Enterobacteriaceae[19]. *Enterobacter sp.* was chosen to find the effect of rGO under light and air. 20 µl of *Enterobacter sp.* suspension was inoculated in two sets of nutrient broth (Hi-Media Laboratories, India) separately. To one of these sets 0.5 mg rGO was added in the culture media inoculated with *Enterobacter sp.* and another set of *Enterobacter sp.* culture was used as control .Both the sets were allowed to stand under 60 W glowing tungsten bulb light for 2 hours at 37°C. Then these bacterial cultures were incubated for 24 hours at 37°C. After 24 hours, 10 µl of cell suspension from both rGO-treated and control were taken and smeared on two clean glass slides separately. The smears were observed under Nikon inverted microscope (figure 4). Similar operation when carried out under argon (in the presence of the necessary amount of CO_2 in all the cases) treated incubation of the bacterial culture showed distinct difference in the growth rate. The lesser growth of the bacterial cells in the presence of rGO compared with the control clearly established the influence of rGO in generating superoxide. The traces of air present under argon medium showed residual proliferation.

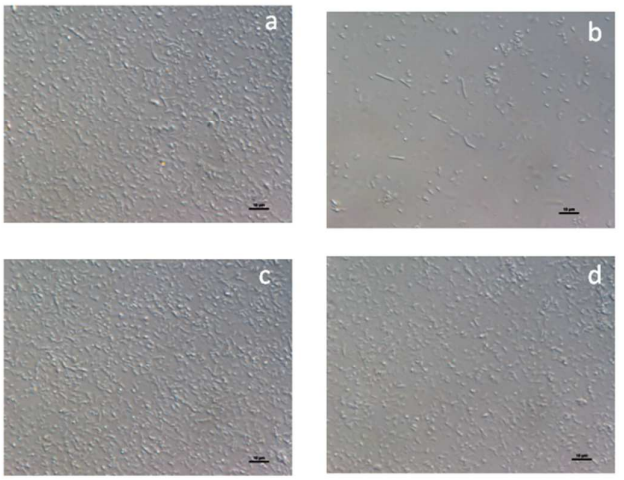


Figure 4. Activity of rGO on the growth of *Enterobacter sp.* treated under visible light : a) without rGO, b) with rGO. , a and b in air, c) without rGO and d) with rGO , c and d in argon .

This clearly suggests that microbial contamination can be readily avoided by using rGO to combat the growth of hospital pathogens. The gradual loss of the activity of rGO may be replenished by the reduction of the formed GO coat using hydrazine vapour or by washing off the GO coat with alkali.

Conclusions

In summary this work demonstrates that handling of rGO under ambient condition in air and under visible light to be carried out with caution as it readily generates harmful ROS. To combat hospital pathogens the careful use of light and air should be made and precaution to its safe use lies to keep it in the dark when not needed. This work sends a caveat that one should use GO with utmost care as it gets slowly reduced even in the open environment on ageing with the formation of a surface coating of non-stoichiometric formation of rGO . Such toxicity is related to ROS generation rests on the rGO contamination that is not due to GO.

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Notes and references

1. W. S. Hummers and R. E. Offeman, *J Am Chem Soc*, 1958, **80**, 1339-1339.
2. H. He, J. Klinowski, M. Forster and A. Lerf, *Chem Phys Lett*, 1998, **287**, 53-56.
3. D. R. Dreyer, H.-P. Jia and C. W. Bielawski, *Angew Chem Int Ed*, 2010, **49**, 6813-6816.

4. T.-F. Yeh, J.-M. Syu, C. Cheng, T.-H. Chang and H. Teng, *Adv Funct Mater*, 2010, **20**, 2255-2262.
5. J. Pyun, *Angew Chem Int Ed*, 2011, **50**, 46-48.
6. V. C. Sanchez, A. Jachak, R. H. Hurt and A. B. Kane, *Chem ResToxicol*, 2012, **25**, 15-34.
7. Y. Wang, Z. Li, J. Wang, J. Li and Y. Lin, *Trends in biotechnology*, 2011, **29**, 205-212.
8. Y. Guo, L. Deng, J. Li, S. Guo, E. Wang and S. Dong, *ACS Nano*, 2011, **5**, 1282-1290.
9. Y. Song, Y. Chen, L. Feng, J. Ren and X. Qu, *Chem Commun*, 2011, **47**, 4436-4438.
10. J. Kim, L. J. Cote, F. Kim, W. Yuan, K. R. Shull and J. Huang, *J Am Chem Soc*, 2010, **132**, 8180-8186.
11. S. Gilje, S. Han, M. Wang, K. L. Wang and R. B. Kaner, *Nano Lett*, 2007, **7**, 3394-3398.
12. D. R. Dreyer, S. Park, C. W. Bielawski and R. S. Ruoff, *Chem Soc Rev*, 2010, **39**, 228-240.
13. K. P. Loh, Q. Bao, G. Eda and M. Chhowalla, *Nat Chem*, 2010, **2**, 1015-1024.
14. G. Eda, Y.-Y. Lin, C. Mattevi, H. Yamaguchi, H.-A. Chen, I. S. Chen, C.-W. Chen and M. Chhowalla, *Adv Mater*, 2010, **22**, 505-509.
15. M. Saxena and S. Sarkar, *RSC Adv*, 2014, **4**, 30162-30167.
16. M. A. Pathak and P. C. Joshi, *Biochim Biophys Acta,* 1984, **798**, 115-126.
17. R. Franco, M. I. Panayiotidis and J. A. Cidlowski, *J Biol Chem*, 2007, **282**, 30452-30465.
18. D. Pan, J. Zhang, Z. Li and M. Wu, *Adv Mater*, 2010, **22**, 734-738.
19. D. Raghunath, *Indian J Med Microbiol*, 2010, **28**, 189.

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