Toxicity of GO and rGO suspension against *P. acnes*: physical puncture and oxidative stress

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Abstract

Acne vulgaris associated with Propionibacterium acnes (*P. acnes*) remains one of the most common skin diseases, while lacking of effective and non-resistant treatments. Graphene including graphene oxide (GO) and reduced graphene oxide (rGO) have been triggering abundant attentions due to their astonishing performances in multi research areas. Here, the GO and rGO suspensions with different concentrations and sizes against *P. acnes* were investigated. The higher the concentration while the smaller the size distribution led to the better the antibacterial performance. And the loss of viability of *P. acnes* can surprisingly achieve 72% under a 100 µg 10 000 mesh rGO existed which was induced by physical puncture and oxidative stress. Furthermore, the physiological activities of *P. acnes* will reduce the GO to rGO which further accelerate its death. This study will provide a rapid, effective and non-resistant method for the treatment of acne.

1. Introduction

Acne is currently the most common skin disease, affecting approximately 80%–90% of adolescents and young adults, while moderate and severe acne accounts for 15%–20% of cases [1, 2]. At present, it is believed that the pathogenesis of acne is mainly related to the following four factors: excessive sebum secretion, hyperkeratosis at the opening of the hair follicle sebaceous gland duct, propagation of *propionibacterium acnes* (*P. acnes*), and inflammation [3]. Among them, the skin symbiotic *P. acnes* are thought to trigger inflammation such as endocar-ditis, sarcoidosis, allergic alveolitis, hyperostosis and osteitis (SAPHO) syndrome [4–8]. Currently, antibacterial drugs are mainly divided into antibiotics and non-antibiotics. The most commonly used antibiotics are tetracycline, macrolide, clindamycin, metronidazole. While the main non-antibiotics are benzoyl peroxide, taurine bromamine, azelaic acid and topical retinoids [9–11]. In recent years, due to the extensive use of systemic or local broad-spectrum antibiotics in the treatment of acne, the phenomenon of drug resistance of this bacteria has increased significantly, and the existence of cross-resistance reduces the efficacy of drugs.

Graphene is a single-atom-thick sheet of sp²-hybridized carbon atoms in a closely packed honeycomb two-dimensional lattice, meanwhile graphene oxide (GO) is graphene attached with carboxylc, phenol hydroxyl and epoxide groups [12]. In recent years, due to the huge specific surface area, good mechanical properties, stable physicochemical properties, excellent thermal and electrical conductivity [13–15], reduced graphene oxide (rGO) and GO have already been used in many fields, such as batteries [16], sensors [17], electromagnetic shielding [18] and thermal management [19]. Furthermore, serveral GO and rGO applications in biological aspects have also been reported. In 2010, Huang Qing’s team discovered the antibacterial effect of graphene derivatives, that is, GO can destroy the cell membrane of bacteria, causing intracellular material to flow out and...
killing bacteria [20]. Because this is a potentially non-resistant physical 'antibiotic', immediately attracting widespread interests in the scientific and technological community. Subsequently, the reports of antibacterial properties of graphene composites with different forms and different dopings emerged. There are many factors can influence the antibacterial activity of carbon nanomaterials, such as electronic structure [21], wrinkles [22], and size [23], as well as the interacting conditions between carbon nanomaterials and bacterial cells, such as concentration [24], incubation time [25], medium [26], and illumination [27]. At the same time, graphene and its composites behave well in biocompatibility [28, 29], which can promote cell proliferation [30] and epidermal factor expression [31].

In this study, we use \textit{P. acnes} to evaluate the antibacterial activity of GO and rGO with different lateral size and concentration. GO and rGO can enrich \textit{P. acnes} on its sheets so that it had a more direct interaction with it. The most interesting phenomenon was that the physiological activity of \textit{P. acnes} will cause the reduction of GO resulting in accelerated death. At last, we have a conclusion that GO and rGO have an inhibition to \textit{P. acnes} through two aspects: physical puncture and oxidative stress. As far as we know, this is a report to fully clarify the antibacterial properties of GO and rGO against \textit{P. acnes} by different factors.

2. Materials and methods

2.1. Preparation of GO and RGO
First, 50 ml 98% H\textsubscript{2}SO\textsubscript{4} were added into a 500 ml beaker and stirred. Then 4 g graphite and 0.8 g NaNO\textsubscript{3} were mixed in the beaker while the temperature was kept below 5 °C. After 30 min, the beaker was transferred to 40 °C water bath. After that, 4 g KMnO\textsubscript{4} was added and the reaction maintained for 3 h. Then the deionized water (250 ml) was added slowly to keep the temperature at 100 °C. Then 30% H\textsubscript{2}O\textsubscript{2} was added into the solution generating bubbles where the color changed from brown to yellow. After that, the solution was washed with 3% hydrochloric acid and deionized water to neutral.

2.2. Preparation of rGO
1 mg ml\textsuperscript{-1} GO suspension was sonicated for 4 h and was transferred to a water bath at 80 °C and added 40 ml hydrazine (v/v = 1:20). This reaction was lasted for 8 h, and then filtered with deionized water to wash the suspension to neutral and dried.

2.3. Preparation of medium
Every 1 L medium containing tryptone 10 g, glucose 5 g, yeast extract 3 g, soluble starch 1 g, beef extract 10 g, sodium chloride 5 g, sodium acetate 3 g, L-cysteine hydrochloride 0.5 g. After mixing uniformly, resazurin (oxygen indicator) was added to the solution, causing the color of the solution changes from yellow to purple. After heating, the solution turned back to yellow, indicating that the oxygen in the solution was removed. Stopped heat and cooled to the room temperature with nitrogen. The medium was transferred to 10 ml tube with nitrogen, each tube 5 ml. Sterilized with the autoclave at 121 °C for 30 min.

2.4. Cell preparation
Thaw the frozen \textit{P. acnes} (OD = 0.6). After shaking evenly, inoculate \textit{P. acnes} into test tubes containing culture medium, 200 μl per test tube. After 20 h incubation, the OD value was measured at 600 nm and chose those achieved 0.4 which the most stable period of bacterial growth, and the influence of physiological factors can be avoided. Then added 1 ml GO or rGO suspension.

2.5. MTT method
After adding GO (or rGO) 1 h, the culture was well stirred and 1 ml of the culture was added to the polyethylene (PE) tube. After centrifuging (7000 rpm 5 min), the supernatant was taken away only \textit{P. acnes} and GO (rGO) left. 500 μl of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was added to the PE tube and cultivated 1.5 h, then centrifuged (12 000 rpm, 3 min). The supernatant was took away and replaced the MTT with the same amount of dimethyl sulfoxide (DMSO). After 20 min cultivation, 200 μl of the supernatant was took into a 96-well plate and ELISA (enzyme-linked immunosorbent assay) was used to measure the loss of viability of \textit{P. acnes} (at 490 nm). \textit{P. acnes} without GO or rGO as a control group. In order to prevent the color of GO (rGO) from affecting the test results, the pure culture medium containing 1 ml GO (rGO) with the same conditions were also prepared. The loss of viability of \textit{P. acnes} was calculated by the following formula:

\[
\text{The loss of viability of } P.\text{acnes}\% = \frac{OD_0 - (OD_b - OD_c)}{OD_0}
\]
In this paper, we prepared 1000 mesh and 10,000 mesh GO by Hummer's method. The related characterization demonstrated the outstanding effects in a short time in view of its antibacterial properties.

3. Results and discussion

3.1. Antibacterial Activity of GO and rGO dispersions

In this paper, we prepared 1000 mesh and 10,000 mesh GO by Hummer's method and obtained the corresponding rGO through chemical reduction. The related characterization demonstrated the outstanding characteristics of our GO and rGO (figures S1–S3 available online at stacks.iop.org/MRX/8/045402/mmedia) supporting information). P. acnes is a typical facultative anaerobe and hoped to have more obvious therapeutic effects in a short time in view of its actual treatment. Considering this, we added GO (rGO) in the most stable period of bacterial growth (OD = 0.4), after that we measured the OD value and the loss of viability after 1 h of drug-bacterial interaction to evaluate their antibacterial ability. It should be noted that all experiments in this article were in triplicate at least. As shown in the figure 1(a), the OD value of GO (rGO) significantly decreased proving the inhibitory effect, while the controled group was increased to 0.48. As the concentration of GO (rGO) solution increased, the OD value gradually decreased with the trend slowed down. Moreover, the antibacterial properties of GO (rGO) were size dependent. GO with smaller size distribution possessed stronger antibacterial effect. And the MTT method was carried out to measure the loss of viability of P. acnes (as described in supporting information). Within a certain cell number range, the amount of MTT crystallization is proportional to the number of living cells. Figure 1(b) illustrated the loss of P. acnes viability steadily increased with the extending concentration of GO and rGO. For 1000 mesh and 10,000 mesh GO solutions, the loss of P. acnes viability reached 5%, 12%, 31% and 7%, 15%, 38%, respectively. While for 1000 mesh and 10,000 mesh rGO solutions, the loss of viability could achieve at 15%, 31%, 65% and 20%, 39%, 72% respectively. Owing to more
edges of the GO (rGO) with smaller sizes, it had more opportunities to pierce the cell membrane of \textit{P. acnes} resulting in higher loss of \textit{P. acnes} viability. The above results indicated that rGO solutions had much higher antibacterial activities than GO solutions at the same condition. Moreover, the higher the concentration and the smaller the layer, the more death of \textit{P. acnes}. The antibacterial mechanism will be described in detail in subsequent chapters.

3.2. Aggregation of \textit{P. acnes} and oxidation of glutathione

Surprisingly, a black flocculent substance appeared in the tube after adding 1 ml 100 \(\mu\)g ml\(^{-1}\) GO solution (figure 2(a) inset) and 100 \(\mu\)g ml\(^{-1}\) rGO solution 1 h (figure S4 supporting information). The SEM images showed that the black flocculent substance was formed by a large mass of \textit{P. acnes} and GO sheets (figure 2(a)). It seemed that GO had an affinity for \textit{P. acnes} that caused them to accumulate on GO sheets. GO (rGO) can adsorb proteins through non-covalent bond self-assembly [33], which may cause \textit{P. acnes} gathered on its sheets due to the enrichment of nutrients. There was an absorption peak at 194 nm standing for peptide bond which widely existed in proteins (figure 2(b)). The intensity of medium contained GO (rGO) was lower compared with pure medium, and between them, GO possess a stronger adsorption than rGO. The sizes of the sheets had no effect on the adsorption (figure S5 supporting information). Moreover, due to the excellent electrical conductivity of rGO, the close contact between \textit{P. acnes} and rGO sheets will cause oxidative stress based on the electronic structures. Their resistances were measured by pressing GO or rGO of the same quality into a shape of 4 cm in diameter and 1 mm in thickness as shown in (figure S6 supporting information). GO was electrically insulating while rGO had high conductivity as shown in figure 2(c). GO contained many oxygen-containing functional groups on the sheets, which can grab the movable \(\pi\) electrons in the sheet layer, resulting in a decrease in conductivity. After reduction, the \(\pi-\pi\) conjugated structure of the sheets was restored leading a higher conductivity.
Glutathione (G-SH) which contains sulfhydryl groups is a tripeptide consisting of glutamic acid, cysteine and glycine with detoxification and antioxidant effects. G-SH can help maintain the redox environment of cells and is believed to regulate the biological state of eukaryotic cells (such as proliferation, differentiation, and apoptosis). Intracellularly, G-SH is predominantly found in its reduced form, while carbon-based materials in close contact with the cell membrane can act as a conductive bridge exposing the electrons of G-SH to the external environment and leading to an increase in oxidized glutathione (G-S-S-H) [34, 35]. In vitro experiments were carried out to verify the oxidation of glutathione, and it was found that the loss of G-SH was consistent with the loss of P. acnes viability (figure 2(d)). For rGO dispersions, the loss of G-SH was respectively 10%, 28% and 60% with the concentration increased while GO was 6%, 15% and 32% respectively due to their different electronic properties. Stronger electrical conductivity can oxidize more G-SH leading to more death of P. acnes.

We believe that the antibacterial mechanism is as follows: firstly, GO (rGO) could induce P. acne to accumulate on their sheets by adsorbing proteins. And then, due to the close contact between P. acnes and the sheets, GO (rGO) can act as a conductive bridge to mediate the transfer of conductive electrons from inside of the P. acnes to external environment leading to its death.

3.3. Physical puncture effect
As we all know, graphene as a two-dimensional material has atomic sharp edges and it will puncture the cell membrane, resulting in bacterial death. And in this study, GO and rGO can adsorb protein making P. acnes aggregated on them. During the aggregation, the sharp edges would puncture the cell membrane and kill P. acnes. As shown in figures 3(a) and (b), after incubating with GO or rGO suspension, the shape of P. acnes changed from rod structure to withered and irregular. In order to verify the physical sterilization of rGO and GO, rotating and fixed modes were carried out as shown in figure 3(c). After 1 h cultivation with 1 ml 1000 mesh GO, the OD value of the rotation medium was lower, as the edges of the sheets leading more opportunity to contact with P. acnes. rGO and GO can act as knives to cut P. acnes leading to its death.

![Figure 3. Physical puncture effect. (a) and (b) Were the SEM images of normal P. acnes and P. acnes after 1 h incubation with rGO. It showed a significant change in the morphology of the P. acnes after the addition of rGO. (c) The OD value of rotating and fixed bacterial culture medium. The OD value of the rotated was significantly lower than that of the fixed culture, indicating a decrease in bacteria.](image-url)
3.4. Reduction of GO by the activities of P. acnes

There were black particles in bacterial culture medium with GO solution after 1 h incubation and even appeared black precipitate in 100 μg ml⁻¹ GO solution (figure S4). Generally, the color of GO solution is brown while rGO is black, so we suspected that GO may be reduced by substances in solution or P. acnes. We compared the FTIR of the GO solution, rGO solution and black precipitate. There were many oxygen-containing functional groups on the GO sheets such as hydroxy (–OH) at about 3400 cm⁻¹, keto (–C=O) at around 1700 cm⁻¹, carboxyl (–COOH) at about 1400 cm⁻¹. Because the difficulty of a large number of functional groups to be completely removed by the reducing agent, the rGO sheets still contained oxygen-containing functional groups. The peak shape of the black precipitate was basically the same as that of rGO indicating the reduction of GO. The movement of the XRD characteristic peak position can also explain the reduction of the black precipitate (figure S7 supporting information). To verify that it was the physiological activity of P. acnes caused the reduction, a control medium that did not contain P. acnes was set up. After cultivating 1 h, the FTIR of the controlled group was slightly reduced the GO but far from the black precipitate (figure 4(c)). It proved that the physiological activity of bacteria restored GO consistent with previous studies [36]. After reduction, the loss of viability of P. acnes enhanced proving that rGO was more toxic (figure 4(d)).

4. Conclusion

In conclusion, we first discovered the antibacterial effect of GO and rGO solutions against P. acnes and verified it by testing the OD value and the loss of viability of P. acnes. GO (rGO) with smaller size distribution and higher concentration possessed stronger antibacterial effect. In our study, 100 μg ml⁻¹ 10 000 mesh rGO solutions behaved the best performance with 72% P. acnes inactivation. GO (rGO) solution can induce P. acnes to accumulate on their sheets by adsorbing nutrients. What’s more, the physiological activities of P. acnes will reduce GO to rGO speeding up its death. The toxicity of GO (rGO) can be attributed to damage of bacterial...
membranes and oxidative stress. By direct contact, the sharp edges of GO and rGO can pierce bacterial membranes and conductive rGO mediated stronger oxidative stress than almost insulating GO. Our study can provide new ideas for the treatment of acne without drug resistance.

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Supporting information

The Supporting Information is available free of charge on the website.

Notes

The authors declare no competing financial interest.

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