Bioactive Materials 3 (2018) 341–346

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: http://www.keaipublishing.com/en/journals/bioactive-materials/

Combination types between graphene oxide and substrate affect the antibacterial activity

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ARTICLE INFO

Article history:
Received 26 February 2018
Received in revised form
25 April 2018
Accepted 1 May 2018

Keywords:
Graphene oxide
Antibacterial
Titanium
Combination type
S. aureus

ABSTRACT

Duo to their superior physicochemical properties, graphene and its derivatives (GDs), such as graphene oxide (GO) and reduced graphene oxide (rGO), have attracted extensive research interests around the world. In recent years, antibacterial activities of GDs have aroused wide concern and substantial works have been done. However, the underlying antibacterial mechanisms still remain controversial. Antibacterial activities of GDs vary with various factors, such as size, number of layers, oxygen-containing groups, and experimental surroundings. We assume that combination types between graphene oxide and substrate may affect the antibacterial activity. Therefore, in this work, GO was fixed on the titanium surface with three kinds of combination types including drop with gravitational effects (GO-D), electrostatic interaction (GO-APS) and electrophoretic deposition (GO-EPD), and the antibacterial activities *in vitro* were systematically investigated. Results showed that combination types affected the ability of GO for preventing *Staphylococcus aureus* (*S. aureus*) from gathering, sharpness of wrinkles or edges and reactive oxygen spices (ROS) levels. Once *S. aureus* are in the form of separation without aggregation, GO can effectively interact with them and kill them with sharp wrinkles or edges and high ROS levels. GO-EPD could effectively prevent *S. aureus* from gathering, own sharp wrinkles or edges and could generate higher ROS levels. As a result, GO-EPD exhibited optimal antibacterial activity against *S. aureus*, followed by GO-APS and GO-D.

1. Introduction

Graphene, a sheet of two-dimensional single layer carbon atoms with sp^2 hybridized, has attracted extensive research interests around the world in recent years [1–6], duo to its superior physicochemical properties, including excellent thermal conductivity [7], high carrier mobility at room temperature [8], high Young's modulus [9], large specific surface area [10] and so on. With the exceptional properties, graphene and its derivatives (GDs), such as graphene oxide (GO) and reduced graphene oxide (rGO), are widely used in the fields of supercapacitors [11,12], fuel cells [13,14], photocatalysis [15,16] and biomedicine [17–20] and so on.

In recent years, antibacterial activities of GDs have aroused wide concern and substantial works have been done [21–23]. Currently, several predominant antibacterial mechanisms have been proposed, such as nanoknives, oxidative stress, and wrapping or trapping. However, the underlying antibacterial mechanisms still remain controversial [23]. Physicochemical properties of GDs, such as size, shape and surface functionality might affect their antibacterial activities. Lateral size of GDs can influence their adsorption ability and the amount of corners and sharp edges, which are important to the interactions between GDs and bacteria [24]. Liu et al. [25] reported that GO sheets with larger sizes exhibited stronger antibacterial activity against *E. coli* than those of smaller sizes. Number of layers of GDs can also influence the antibacterial activity. Increasing the number of layers of GDs can increase the thickness of GDs, which in turn weaken the nanoknives effect. At the same time, Increasing the number of layers of GDs can lead to agglomeration, which will reduce the chance of contact between

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Peer review under responsibility of KeAi Communications Co., Ltd.

https://doi.org/10.1016/j.bioactmat.2018.05.001

GDs and bacteria [23]. The existence of oxygen-containing groups can also alter the antibacterial activities of GDs by affecting the nanoknives effect and amphipathy. Akhavan et al. [26] found that rGO shown inhibition of cell proliferation of *E. coli* while GO was biocompatible with *E. coli*.

GDs mentioned above were almost dispersed in the solution. However, GDs, dispersed in the solution or fixed on the substrate surface, may exhibit different antibacterial activities. In our previous studies [27], we found that increasing the number of layers of GO on titanium surfaces could improve the antibacterial activity by increasing the reactive oxygen spices (ROS) levels which was opposite to the result as mentioned above. Moreover, we also found that GO on titanium surface presented stronger antibacterial activity than rGO which were reduced by vacuum heat treatment, hydrazine hydrate and sodium borohydride, respectively [28]. From above we can know that antibacterial activities of GDs vary with various factors, such as size, number of layers, oxygen-containing groups, experimental surroundings. Based on this, we wonder whether combination types between GO and substrate affect the antibacterial activity or not. To figure it out, in this work, GO fixed on the titanium surfaces with three kinds of combination types were fabricated and the antibacterial activities *in vitro* were systematically investigated.

2. Materials and methods

2.1. Sample preparation

Commercial pure titanium plates with the dimensions of $10 \times 10 \times 1\ mm^3$ were polished with series abrasive papers to a mirror plane on one side of the titanium plates, which were denoted as Ti. Samples with different combination types between GO and titanium substrate were fabricated as follows. With gravitational effects, 100 μL of single layer GO aqueous solution (0.06 mg/mL, purchased from Hangzhou Gaoxi Technology Co., Ltd) was dropped on the Ti surface, dried in the air and denoted as GO-D. To adhere GO on the titanium surface with electrostatic interaction, Ti was immersed in NaOH aqueous solution (5 M) for 12 h to obtain Ti-OH on titanium surfaces, and then reacted with 5% of 3-animopropyl-trimethoxysilane (APS) for 2 h with continuous ultrasonic to get amino (NH_2) on titanium surfaces. Finally, GO was adhered to the titanium surfaces via electrostatic interaction between GO and amino with impregnation method, and the corresponding samples were denoted as GO-APS. A combination type different from GO-D and GO-APS was achieved on titanium surface with cathode electrophoresis deposition (EPD). To be more specific, GO, absorbed with metal cation (Zn^{2+}) which made GO positively charged, was deposited on titanium surface with cathode EPD and the corresponding samples were denoted as GO-EPD.

2.2. Surface characterization

Field-emission scanning electron microscope (FE-SEM, Magellan 400, FEI, USA) with an accelerating voltage of 2 KV was used to observe the surface morphologies of Ti, GO-D, GO-APS and GO-EPD. Chemical compositions of various samples were determined with X-ray photoelectron spectroscopy (XPS, PHI 5300) with an Mg K_α source (250 W, 14 KV). Raman spectra on various sample surfaces were obtained by Raman microscope system (LabRAM, Horiba Jobin Yvon, France) with an excitation wavelength at 514 nm using an Ar-ion laser. X-ray diffraction (XRD, D8 discover, Bruker) using Cu Kα as the radiation source with 1° glancing angle was applied to investigate the phase compositions on the titanium surfaces.

2.3. In vitro antibacterial experiments

2.3.1. SEM observation

Gram-positive *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) was used to assess the antibacterial activities of Ti, GO-D, GO-APS and GO-EPD. To clearly observe the morphology and integrity of cell membrane, SEM observation was performed as follows. First, Ti, GO-D, GO-APS and GO-EPD were sterilized with 75% (v/v) ethanol for 2 h and dried in the super clean bench. Subsequently, bacterial suspensions (60 μL, 10^7 CFU/mL) were seeded on various sample surfaces and cultured at 37 °C for 24 h. After culturing for 24 h, the bacteria on sample surfaces were fixed with 2.5% glutaraldehyde solution overnight, dehydrated in gradient ethanol solution (30, 50, 75, 90, 95 and 100% (v/v)) and dried in hexamethyl disilazane ethanol solution series. Finally, the images were taken using SEM (S-3400 N, Hitachi, Japan) with an accelerating voltage of 15 KV.

2.3.2. Agar culture observation

Antibacterial activities of Ti, GO-D, GO-APS and GO-EPD were further assessed with agar culture. For agar culture, after culturing for 24 h at 37 °C, the samples with bacterial suspensions were transferred into test tubes with 4 mL of 0.9% NaCl solution and shook to detach the bacteria from the samples. And then, bacterial suspensions were serially diluted with 0.9% NaCl solution in tenfold steps. At last, 100 μL of diluted bacterial suspensions were introduced to a standard agar culture plate (Tryptic Soy Broth for *S. aureus*) for further cultivation with 24 h at 37 °C. Finally, photographs of agar culture plates were taken.

2.3.3. Bacterial viability assessment

Bacterial viability assessment of Ti, GO-D, GO-APS and GO-EPD was performed with alamarblue assay kit. After culturing for 24 h at 37 °C, 500 μL of 10% alamarblue was added into each sample and cultured for another 2 h at 37 °C. Finally, 100 μL medium was transferred to a 96-well black plate and the corresponding fluorescent intensity (FI) was detected with an excitation wavelength at 560 nm and an emission wavelength at 590 nm. The antibacterial ratio was calculated as follows,

$$\text{Antibacterial ratio}\ (\%) = \frac{FI_{control} - FI_{experiment}}{FI_{control}} \times 100$$

Where $FI_{control}$ was the fluorescent intensity of control group, $FI_{experiment}$ was the fluorescent intensity of experiment group.

2.4. Agar diffusion assay

To figure out whether release of Zn ions on GO-EPD contributes to antibacterial effect, agar diffusion assay was performed on all the samples. First, 100 μL of bacterial suspensions with a density of 10^7 CFU/mL were introduced to the Trypticase Soy Broth (TSB) agar culture plates. Then, various specimens were put on TSB agar culture plates and cultured for 24 h at 37 °C. Finally, images of agar culture plates were taken.

2.5. Intracellular reactive oxide species assay

ROS levels in bacteria cells were investigated using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) which can be deacetylated with intracellular esterases into nonfluorescent 2′,7′-dichloro-dihydrofluorescein (DCFH). And then, DCFH can be oxidized with ROS into fluorescent 2′,7′-dichlorofluorescein (DCF). Therefore, the fluorescent intensity of DCF presents the ROS level to some extent. To determine the intracellular ROS levels, bacteria were cultured on Ti, GO-D, GO-APS and GO-EPD for 24 h at 37 °C, and then, 500 μL of