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Enhanced antimicrobial activities of silver-nanoparticle-decorated reduced graphene nanocomposites against oral pathogens

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ABSTRACT

As a means of capitalizing on the synergistic properties between reduced graphene nanosheets (R-GNs) and silver nanoparticles (AgNPs), an efficient and convenient chemical reduction method was used to prepare silver-nanoparticle-decorated reduced graphene nanocomposites (R-GNs/Ag). The products were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), and Raman spectroscopy, which confirmed the loading of well-dispersed silver nanoparticles on reduced graphene sheets. Their antimicrobial activities against oral pathogens such as *Candida albicans*, *Lactobacillus acidophilus*, *Streptococcus mutans*, and *Aggregatibacter actinomycetemcomitans* were investigated by MIC determination, the counting of colony-forming units (CFU), agar diffusion tests, and growth curve observation. Compared with pure R-GNs and AgNPs, R-GNs/Ag composites exhibited enhanced antimicrobial properties owing to highly dispersed AgNPs on R-GNs.

1. Introduction

Graphene, a one-atom-thick sheet of sp^2-bonded carbon atoms in a dense honeycomb two-dimensional crystal, is popular in the field of nanomaterials science due to its unique structure and extraordinary physical, electrical, and chemical properties [1,2]. In 2010, Qing Huang and his group members first reported the strong antibacterial effect as well as the minor cytotoxicity of graphene-based nanomaterials [3]. The potential applications of graphene and graphene-based nanoparticles as antimicrobials have drawn extensive research attention. In addition, the large surface area and unparalleled performance of graphene make it a good substrate for inorganic nanoparticles. In this regard, graphene-based composites such as TiO_2, ZnO, Fe_3O_4, and Ag nanoparticle-decorated graphene have been successfully synthesized, exhibiting synergistic properties and promising applications as antibacterial agents [4–6].

Silver nanoparticles (AgNPs) are widely known as broad-spectrum antimicrobial agents due to their large surface area and slow release of Ag^+ cations [7]. They kill bacteria through multiple processes, including breaking cell walls, destroying the structure of proteins, and interrupting the process of DNA synthesis [8,9]. Because of their effective antibacterial activity and biocompatibility, AgNPs have been used not only for infection treatment, wound healing, and food preservation [10–13], but also in the field of dental materials [14,15]. However, the applications of AgNPs for antibacterial purposes are often hampered by their strong tendency to self-aggregate, leading to the obvious decrease or even the total loss of antibacterial potency [16]. Their biological activities also have strong associations with the sizes and shapes of nanoparticles [17,18]. Therefore, graphene-based nanosheets have been developed as substrates to stabilize and control the aggregation of AgNPs [19].

Oral microbes have been identified as the causative factors of dental caries and periodontal disease. *Streptococcus mutans* and *Lactobacillus acidophilus*, which can produce acid by the fermentation of carbohydrates, are facultative anaerobic, Gram-positive, and the important cariogenic bacteria in oral plaque [20,21]. *Aggregatibacter actinomycetemcomitans*, facultative Gram-negative, is well-known to cause aggressive periodontitis [22]. *Candida albicans*, Gram-positive fungus, is frequently detected in the oral cavities of children and elderly persons. It has been associated with mucosal infection, root caries, and the progression of periodontal disease [23]. In numerous studies, graphene-based nanosheets decorated with AgNPs on the surface as novel composite materials showed enhanced bactericidal effects against Gram-negative *Escherichia coli* and Gram-positive bacteria *Staphylococcus aureus* [24,25]. However, the

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effects of graphene-based/silver nanoparticle composites against oral pathogens have not been explored.

In this study, we aimed to obtain novel reduced-graphene/silver nanoparticle (R-GNs/Ag) composites by covering reduced graphene sheets (R-GNs), one type of graphene derivative, with AgNPs. Four typical cariogenic and periodontopathogenic bacteria – *S. mutans*, *L. acidophilus*, *A. actinomycetemcomitans*, and *C. albicans* – were selected to evaluate the antimicrobial activity of nanoparticles. Compared with pure R-GNs and AgNPs, R-GNs/Ag presented enhanced antimicrobial effects toward oral pathogens, owing to highly dispersed AgNPs on R-GNs.

2. Materials and methods

2.1. Preparation of R-GNs/Ag composites

The power of R-GNs was synthesized by a re-expansion and exfoliation method as described in our previous work [26]. AgNPs were obtained by the efficient and convenient chemical reduction of $AgNO_3$ with hydrazine hydrate as the reductant [27]. For the preparation of R-GNs/Ag composites, $AgNO_3$ was reduced in the same way in the presence of an R-GNs suspension. In brief, 100 mg R-GNs and 200 mg poly(N-vinyl-2-pyrrolidone) (PVP) were added to 200 mL of deionized water. After being uniformly mixed, a 100-mg quantity of $AgNO_3$ powder was added to the R-GNs solution. The temperature was adjusted to 80 °C, after which a 2-mL quantity of hydrazine hydrate was gradually added, with continuous stirring for 2.5 h. The final specimen was centrifuged (10,000 rpm for 15 min), washed twice in deionized water, and then vacuum-dried at 60 °C for 24 h. All products were stored at 4 °C for further use.

2.2. Characterization of nanoparticles

The compositions and morphology of the as-prepared products were characterized by scanning electron microscopy (SEM, FEI, Quanta 400F) and transmission electron microscopy (TEM, FEI, FEI Tecnai G2 Spirit) at an acceleration voltage of 120 kV. The X-ray diffraction (XRD) patterns were obtained by means of powder X-ray diffractometry (XRD, PANalytical, Empyrean) with Cu Kα radiation. The Raman spectra were acquired on a Laser Micro-Raman Spectrometer (Renishaw inVia) with 532-nm laser excitation.

2.3. Microorganisms and culture conditions

Four oral pathogenic strains were used in this study. *S. mutans* (UA159), *L. acidophilus* (ATCC4356), and *A. actinomycetemcomitans* (ATCC29523) were grown in brain heart infusion broth (BHI) in an anaerobic chamber (N_2 80%; H_2 10%; CO_2 10%). *C. albicans* (SC5314) was grown aerobically in Sabouraud glucose broth (SGB) under 150 rpm shaking speed. Culture temperature was kept at 37 °C for all strains. Cells were harvested by centrifugation (8000 rpm for 2 min) and washed by phosphate-buffered saline (PBS) at the exponential growth phase. The cellular densities were set by a spectrophotometer.

2.4. Antimicrobial effects of R-GNs, AgNPs, and R-GNs/Ag on oral pathogens

The minimum inhibitory concentrations (MICs) of R-GNs/Ag nanocomposites against the four strains were determined by the micro-dilution method. Cells were dispensed in double-concentration culture medium with concentration of 10^5–10^6 CFU/mL. Then 100 μL of bacterial cell suspensions were inoculated into 96-well microtiter plates containing 100 μL of various concentrations of serially diluted R-GNs/Ag in each well, starting at an initial concentration of 5.12 mg/mL. After 24 h of incubation, the MIC was determined as the lowest concentration at which visible bacterial growth was totally inhibited.

For comparison of the antimicrobial efficiency of R-GNs, AgNPs, and R-GNs/Ag, a colony-forming units (CFU) counting method was also conducted. Strains were incubated with R-GNs, AgNPs, or R-GNs/Ag as above. Fresh nutrient medium was inoculated with microorganisms, but no NPs were set as control groups. Strains were incubated at 37 °C for 24 h. Then 10-fold series dilutions of each culture were spread on agar plates and incubated at 37 °C for 24–48 h. Tests were performed two times in triplicate. The viability of bacteria and fungus was evaluated by the counting of colony-forming units.

2.5. Agar diffusion method

The antibacterial activity of R-GNs/Ag was also examined by the agar diffusion method modified by the previous study [28]. Sterile solid agar medium was prepared on plates in advance. Strains were cultured in broth at 37 °C for 24 h. Then, an 80-μL quantity of inoculum was added to test tubes containing 8 mL melted BHI or SGB agar at 50 °C and poured onto an agar plate prepared in advance. After solidification of agar, wells were made and a 40-μL quantity (about 100 μg, 200 μg, 400 μg) of R-GNs/Ag (sample concentration 2.56 mg/mL, 5.12 mg/mL and 10.24 mg/mL) was loaded. Plates were dried and incubated at 37 °C for 24 h.

2.6. Effects of R-GNs/Ag on growth curves

Next, we further assessed the effects on the bacteria and yeast growth of R-GNs/Ag at concentrations that showed effective antimicrobial properties. *S. mutans*, *L. acidophilus, A. actinomycetemcomitans,* and *C. albicans* were added in double-concentration culture medium to obtain cell suspensions of ~10^5 CFU/mL. Then we mixed the medium with equal volumes of material suspensions or deionized water (control group) and incubated them at 37 °C for 32 h. After reacting for 2, 4, 6, 12, and 32 h, 100-μL aliquots of mixture were removed and counted by the CFU method described as before.

2.7. Statistical analysis

The experiments were performed in triplicate, and data were expressed as mean ± standard deviation. The statistical analysis was done by SPSS software (IBM SPSS Statistics V21.0). Level of significance was determined by one-way ANOVA combined with a Student-Newman-Keuls (SNK) post hoc test. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Preparation and characterization of nanoparticles

R-GNs/Ag composites were formed by the loading of AgNPs on the surfaces of reduced graphene nanosheets. First, we synthesized the pristine product, multi-layer reduced graphene, by a high-quantity production method using graphite as the starting material. To produce AgNP-decorated reduced graphene, PVP had been added to modify the reduced graphene surface. The presence of PVP facilitated the loading of Ag^+ onto reduced graphene surfaces. Ag^+ was further reduced to AgNPs with hydrazine hydrate as the reducing agent. After being centrifuged and purified, R-GNs/Ag composites were obtained and appeared to be well-dispersed.

The structure and surface morphology of materials were investigated by SEM (Fig. 1) and TEM (Fig. 2). Fig. 1A presents the image of R-GNs in a thin-layer structure with crumpled sheets on the edge. Fig. 1B displays the AgNPs in different shapes of spherical micelles, deformed spherical micelles, and short rod-like micelles. Particles of ~100-nm size are stacked tightly in clumps, while spherical AgNPs are uniformly dispersed on reduced graphene sheets with little obvious aggregation. The SEM of R-GNs/Ag results confirmed the attachment of AgNPs to the reduced graphene sheets (Figs. 1C, D). To further characterize the structure clearly, we also took TEM images of as-prepared materials and composites. Fig. 2A shows the thin, crumpled, and wrinkled