Nano-graphene oxide improved the antibacterial property of antisense yycG RNA on *Staphylococcus aureus*

Shizhou Wu 1,2, Yunjie Liu 3, Hui Zhang 1 and Lei Lei 2*

Abstract

**Background:** *Staphylococcus aureus* (*S. aureus*) has the potential to opportunistically cause infectious diseases, including osteomyelitis, skin infections, pneumonia, and diarrhea. We previously reported that ASyycG RNA reduced the transcripts of virulent genes, and biofilm formation of *S. aureus*. Currently, graphene oxide (GO) nanosheets are used to efficiently deliver nucleic acids with favorable biocompatibility.

**Methods:** In the current study, a GO-based recombinant pDL278 ASyycG vector transformation strategy was developed. The particle size distributions and zeta-potential of the GO-PEI-based ASyycG were evaluated. The ASyycG plasmids were labeled with gene-encoding enhanced green fluorescent protein (ASyycG-eGFP). Quantitative real-time PCR assays were performed to investigate the expression of yycF/G/H and icaA/B genes. Biofilm biomass and bacterial viability of *S. aureus* were evaluated by scanning electron microscopy and confocal laser scanning microscopy. We found that the expression of the yycG gene was inversely correlated with levels of the ASyycG transcripts and that the GO-PEI-ASyycG strain had the lowest expression of biofilm organization-associated genes.

**Results:** The results showed that the GO-based strategy significantly increased ASyycG transformation as a delivery system compared to the conventional competence-stimulating peptide strategy. Furthermore, GO-PEI-ASyycG suppressed bacterial biofilm aggregation and improved bactericidal effects on *S. aureus* after 24 h biofilm establishment.

**Conclusions:** Our findings demonstrated that nano-GO with antisense yycG RNA is a more effective and relatively stable strategy for the management of *S. aureus* infections.

**Keywords:** *Staphylococcus aureus*, Antisense RNA, YycG, Graphene oxide, Biofilm

Introduction

*Staphylococcus aureus* (*S. aureus*), a gram-positive coccus, is carried by about 20–30% of healthy individuals and mostly colonizes the nasopharynx [1]. *S. aureus* has the potential to cause a wide range of diseases, including osteomyelitis, skin infections, pneumonia, and even life-threatening infective endocarditis associated with considerable global human morbidity and mortality [2]. However, in some cases, *S. aureus* is resistant to multiple types of antibiotics, which has been attributed to the abuse of antibiotics, resulting in the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) [3]. Presently, more than 50% of *S. aureus* in clinical isolates in hospitals worldwide are methicillin-resistant [4]. Therefore, the identification of novel antibacterial strategies is of the utmost importance.

Graphene oxide (GO) is a graphene sheet containing functional organic groups, such as carboxyl, hydroxyl, carbonyl, and epoxy, on its basal plane [5]. The sharp edges on the GO sheet structures physically disrupt cell membranes and cause oxidative stress reactions. Therefore, it is expected to act as a novel type of bactericidal agent with a low risk of developing resistance from pathogenic bacteria [6]. In addition, the large surface area of GO sheets makes them ideal candidates for gene delivery [7]. Although GO sheets can be used to effectively deliver single-stranded nucleic acids, the ability of GO to carry double-stranded DNA (dsDNA) is limited. Polyethyleneimine (PEI) is a well-studied cationic polymer that has
been used as a common non-viral gene delivery vector when combined with GO. Compared with the PEI polymer, GO–PEI has been reported to have lower cytotoxicity and higher transfection efficiency, and thus, has high potential as a gene vector [8].

Two-component signal transduction systems (TCSs) are essential pathways for bacterial responses to environmental stimuli. Typical TCS components consist of a transmembrane histidine kinase sensor and a corresponding cytoplasmic response regulator, which can bind to specific regions to regulate the expression of targeted genes [9]. YycFG is the only essential TCS in S. aureus, contributing to its physiology, and biofilm metabolism [10]. Biofilms are microbial communities embedded within self-produced extracellular substances and are closely related to the development of infections in humans [11]. In S. aureus, polysaccharide intercellular adhesion (PIA) encoded by icaADBC is a functional factor involved in biofilm organization [12].

Antisense RNAs (asRNAs) are a type of single-strand RNA that recognize mRNA by base-pairing and inhibiting the transcription and transduction of target mRNA [13]. Antisense RNA strategy is a promising approach for novel gene-specific antisense antibiotics to cure bacterial infections [14]. However, the efficiency of transforming antisense RNA into bacterial cells is limited without a suitable carrier system [15]. Since GO-PEI complexes are highly positively charged, effective loading with DNA plasmids can be achieved. In this study, a GO-based plasmid transformation system was developed using GO-PEI complexes that were loaded with antisense yycG plasmid (GO-PEI-ASyycG). We hypothesized that the antibacterial properties of GO to S. aureus could be enhanced by loading the gene vector with antisense ASyycG plasmids. A potential role for the clinical application GO-PEI-ASyycG as a novel antibiotic agent was proposed for the management of the S. aureus infections.

Methods and materials
Preparation of GO-PEI-ASyycG and cytotoxicity evaluation
The antisense yycG sequences (ASyycG) were synthesized by Sangon Biotech (Shanghai, China). To generate a recombinant pDL278 ASyycG plasmid, the ASyycG sequences were inserted into the BamHI and EcoRI restriction sites of a pDL278 vector [16]. To synthesize the GO-PEI complexes, GO powder (XFNANO Materials Tech, Nanjing, China) was added to ddH2O to a final concentration of 0.1 mg/mL. Next, the solution was slowly mixed with branched polyethyleneimine (BPEI, 10 kDa; Sigma-Aldrich, St. Louis, MO, USA). Then, the solution was processed with 10 cycles of ultrasonication for 60 s, with 60 s rest on ice between each sonication. The obtained solution was mixed on a shaking table overnight at room temperature. To remove redundant PEI compounds, the mixtures were washed three times with ddH2O by centrifugation (12000×g, 1 min) and resuspended with ddH2O to a final concentration of 0.1 mg/mL. pDL278 ASyycG plasmid (100 ng/μL) was added to the GO-PEI complexes at a volume ratio of 1:125 and the mixtures were incubated for 1 h at room temperature.

The working concentration of GO-PEI-ASyycG was determined by cytotoxicity assays. Briefly, the mouse embryonic fibroblast NIH/3T3 cell line (Sigma-Aldrich) at a density of 1000 cells/well were seeded into 96-well plates in 100 μL of Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and mixed with the GO-PEI-ASyycG solutions at dilutions ranging from 100 μg/mL to 0 μg/mL. After incubation for 48 or 72 h (37 ºC, 5% CO2), the plates were removed and each well was washed with phosphate buffer solution (PBS, pH = 7.4) twice. The CCK-8 cell counting kit (Dojindo Laboratories, Kumamoto, Japan) was used to test the cell viability and each well was incubated with 10 μL of CCK-8 reagent. After 2 h of culture, the OD values of each well were measured using a microplate reader (ELX800, Gene, Hong Kong, China) at 540 nm.

Particle size distribution, zeta potential, and atomic force microscopy measurements
The particle size distribution of the GO, GO-PEI, and GO-PEI-ASyycG solutions (0.1 mg/mL) was measured by dynamic light scattering (DLS) and the zeta-potential was examined by a Malvern instrument (Zetasizer Malvern Nano ZS, Instruments, Worcestershire, UK). A total of 50 μL of GO, GO-PEI, or GO-PEI-ASyycG solution was dropped onto sterile coverslips and films were prepared and air-dried in room temperature. The roughness of the films was assessed using an atomic force microscope (AFM) (SPM-9500J2, Shimadzu, Tokyo, Japan) in the contact mode. Micrographs of all films were evaluated by scanning electron microscopy (SEM; Inspect F50, FEI, Hillsboro, OR, USA) as previously described [17].

Bacterial culture and transformation
A single colony of S. aureus was selected from a tryptic soy agar (TSA) plate and cultured in tryptic soy broth (TSB) medium to the mid-exponential phase, which was determined by an OD600 value of 0.5. For the S. aureus GO group, 250 μL of mid-exponential S. aureus was incubated with 2 μL GO solution (final concentration determined by cell viability assay). In the ASyycG group, 2 μL of recombinant pDL278 ASyycG plasmid was mixed with 250 μL of mid-exponential S. aureus as in our previous studies [16]. For the GO-PEI-ASyycG strains, 250 μL of mid-exponential S. aureus was co-cultured with prepared GO-PEI-ASyycG. All S. aureus strains were...
cultured at 37 °C in 5% CO₂ for 1 hour, then diluted into 5 mL of fresh TSB medium.

**Transformation efficiency of GO-PEI-ASyycG in vitro**

The ASyycG plasmids were labeled with gene encoding enhanced green fluorescent protein (ASyycG-eGFP). The sequences of ASyycG and eGFP were synthesized by Sangon Biotech (Shanghai, China) and are listed in the Additional file 1. The ASyycG-eGFP and GO-PEI-ASyycG-eGFP strains were constructed based on the transformation procedures described above. Both strains were grown in TSB medium until an OD₆₀₀ value of 0.5 was reached. A total of 50 μL of bacterial suspensions was dropped onto coverslips and air-dried at room temperature for 30 min. Confocal laser scanning microscopy (CLSM) was applied to determine the expression level of eGFP. The transfection efficiency was determined by comparing the green fluorescence intensities.

Real-time polymerase chain reaction (RT-PCR) assays were conducted to assess the expression of ASyycG in all *S. aureus* strains. Briefly, total RNA was extracted from *S. aureus* suspensions from the mid-logarithmic growth phase in TSB medium using an RNA purification Kit (MasterPure, Epicentre, Madison, WI, USA) according to the manufacturer’s instructions. Total RNA was reverse transcribed using an RT Reagent Kit (PrimeScript, Takara, Kyoto, Japan). Quantitative RT-PCR assays were carried out using the primers listed in Table 1 using a LightCycler 480 system (Roche, Basel, Switzerland). The 16S rRNA gene was used as an internal control [17].

**Growth conditions of *S. aureus* strains**

After transformation, all strains were diluted in TSB at a ratio 1:20 and incubated in 96-well plates. The bacterial growth curves were monitored by measuring the OD₆₀₀nm with a microplate reader (ELX800, Gene, Hong Kong, China) every 60 min for 24 h. The proportions of live bacteria cells were estimated by confocal laser scanning microscopy (CLSM, FV1000; Olympus Corporation, Tokyo, Japan) at ×40 magnification. Live cells were stained with SYTO9 dye (LIVE/DEAD Bacterial Viability Kit reagent; BacLight, Invitrogen, Grand Island, NY, USA) and dead cells were labeled with propidium iodide (PI). Three-dimensional reconstruction was conducted and analyzed using Imaris 7.0.0 software (Imaris 7.0.0, Bitplane, Zurich, Switzerland) as previously described [18].

**Evaluation of *S. aureus* biofilms**

Crystal violet (CV) assays were applied to compare the biomass of *S. aureus* biofilms cultured in 24-well polystyrene plates for 24 h. As previously described, the biofilms were stained with 0.1% (w/v) crystal violet for 15 min at room temperature [17]. The dye bound on the biofilms was collected using 1 mL of de-staining solution (8:2 ethanol:acetone). Then, the solution was transferred to a new plate and the OD₆₀₀nm was read by a microplate reader (ELX800, Gene, Hong Kong, China).

Sterile coverslips were immersed in 24-well plates with different *S. aureus* strain suspensions (OD₆₀₀nm = 0.5). After 24 h of co-culturing, the planktonic suspensions were removed and the biofilms grown on the coverslips were washed three times with PBS (pH7.2). Then, the biofilms were fixed in 2.5% glutaraldehyde for 4 h at room temperature and dehydrated with serially concentrated ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100%). The prepared biofilms were dried to critical-point at room temperature and coated with gold powder. Scanning electron microscopy (SEM; Inspet F50, FEI, Hillsboro, OR, USA) was used to estimate the morphologies of all *S. aureus* biofilms by selecting three random areas from each sample [19].

**Data analyses**

All data were processed using SPSS software (SPSS version 20, IBM, Armonk, NY, USA). The values are expressed as mean ± standard deviation (SD) for the indicated number of samples. The quantification cycles for describing gene expression were relatively quantified by real-time PCR using 16S as an internal control and calculated based on the ATCC29213 expression, which was set to 1.0. Bartlett’s test was employed to assess the homogeneity of data variance and the Shapiro-Wilk test was conducted to determine the normal distribution of the data. One-way analysis of variance was used to compare the data, followed by pairwise multiple comparisons. The differences were considered significant if the p value was < 0.05.

**Results**

**Cytotoxicity and characteristics of GO-PEI-ASyycG films**

The viability of the 3T3 fibroblasts cells was significantly decreased after 48 or 72 h treatment GO-PEI-ASyycG concentrations higher than 50 μg/mL (Fig. 1a). Our

### Table 1 Sequences of primers used for qRT-PCR analysis

| Primers | Sequence 5′-3′ (forward/reverse) | Reference |
|---------|---------------------------------|-----------|
| RT-qPCR |                                  |           |
| icaA    | 5′-GATTATGTAAAGCCTTGG-3′ / 5′-ACTGTCGGCACCATT-3′ | This study |
| icaD    | 5′-ATGGCTAAGCACCAGACAG-3′ / 5′-CTGTCTTCCAAATTGCAA-3′ | This study |
| icaB    | 5′-CACATACCAAGCTTTCGTTA-3′ / 5′-TGGAGTGCAGCTTTC-3′ | This study |
| yycF    | 5′-TGCGGAAAGGAAAGCACTA-3′ / 5′-AACCGTCAAATATCTG-3′ | This study |
| yycG    | 5′-CGGGCGCGTACCAAGACTTT-3′ / 5′-CTGAGACCTTTGACACACGT-3′ | This study |
| 16S rRNA| 5′-CTGCTGCGCCATGGAATCC-3′ / 5′-CGCAGCTGCTGAAATACA-3′ | This study |
results indicated that the GO-PEI-ASyycG complexes were not toxic at concentrations lower than 50 μg/mL (Fig. 1a). Dynamic light scattering measurements were applied to determine the hydrodynamic sizes of GO, GO-PEI, and GO-PEI-ASyycG in deionized water. Z-average sizes of 420 nm and 280 nm were obtained for GO and GO-PEI, which were much smaller than the average size of GO-PEI-ASyycG (490 nm) (Fig. 1b). The surface charge (zeta potential) value of GO was approximately at −23.1 mV. After mixing with the cationic PEI polymer, the GO-PEI and GO-PEI-ASyycG complexes demonstrated positive surface charges of 15.7 mV and 38.4 mV, respectively (Fig. 1c). Using AFM, the roughness analysis of the membranes revealed that the GO-PEI-ASyycG nanosheet roughness averaged 8.9 nm, significantly higher than the GO and GO-PEI films, which averaged 3.4 nm and 4.5 nm, respectively (n = 10, *p < 0.05; Fig. 1d). Using SEM, the results showed rougher and denser surface morphologies on the GO-PEI-ASyycG films compared to GO and GO-PEI (Fig. 1e).

**GO-PEI-ASyycG increased ASyycG transformation and significantly reduced biofilm formation-associated gene expression**

Using confocal laser scanning microscopy, higher levels of GFP-expression were observed in samples induced with GO-PEI-ASyycG compared to pure ASyycG (Fig. 2a). A 200% increase in GFP-expression transcripts in GO-PEI-ASyycG transformed strains was found, a statistically significant increase compared to the expression in ASyycG
cells \((n = 10, p < 0.05; \text{Fig. 2b})\). Quantitative RT-PCR assays demonstrated that expression levels of AS\textit{yycG} RNA in the AS\textit{yycG} and GO-PEI-AS\textit{yycG} strains were significantly increased 2.8-fold and 6.5-fold, respectively, compared to \textit{S. aureus} ATCC29213 strains \((n = 10, p < 0.05; \text{Fig. 2c})\). Accordingly, the gene expression levels of \textit{yycG} were significantly downregulated in the AS\textit{yycG} and GO-PEI-AS\textit{yycG} strains \((n = 10, p < 0.05)\). Consequently, the expression levels of PIA synthesis-associated genes \textit{icaA/D/B/C} were the lowest in the GO-PEI-AS\textit{yycG} strain among all groups \((n = 10, p < 0.05)\).

**GO-PEI-AS\textit{yycG} decreased cells viability and suppressed biofilm formation**

When growth was monitored in the different strains, the results showed that the time before entry into the log phase in the GO-PEI-AS\textit{yycG} strains was obviously prolonged compared to that of the AS\textit{yycG} strains (Fig. 3a). The most impaired formation of biofilms was observed in GO-PEI-AS\textit{yycG} strains compared to GO, AS\textit{yycG}, and ATCC29213 (Fig. 3b). Quantitatively, these results were confirmed by the OD values of the biofilm biomasses, which were the lowest in the GO-PEI-AS\textit{yycG} strains \((n = 10, p < 0.05, \text{Fig. 3b})\).

SEM observation demonstrated reduced levels of extracellular matrix components in the biofilms of the GO, AS\textit{yycG}, and GO-PEI-AS\textit{yycG} cells that were separated by blank areas (Fig. 3c). Exopolysaccharide-enmeshed cell clusters were greatly decreased in the GO-PEI-AS\textit{yycG} strain biofilms (Fig. 3c).

**Discussion**

Our previous work indicated that antisense \textit{yycG} RNA (AS\textit{yycG}) could inhibit the target gene \textit{yycG} in the MRSA strain. We found that AS\textit{yycG} strains inhibited biofilm organization and increased antibiotic sensitivity [20]. However, one of the major obstacles to the use of antisense oligonucleotides is that, without a suitable and effective vector, the uptake by bacterial cells is limited [21]. In the current study, a GO-based recombinant pDL278 AS\textit{yycG} vector transformation strategy was used to electrostatically combine the vector with cationic GO-PEI complexes. We showed that GO-PEI could efficiently deliver AS\textit{yycG} plasmid into \textit{S. aureus} cells with efficient transcripts of AS\textit{yycG}. GO has been reported to ionically bind to cationic PEI polymers [8]. These positive surface charges could interact with the negatively charged cellular surface and promote bacterial transformation [22]. A
previous study reported that 50 μg/mL of GO-PEI (or lower) did not have toxic effects on the cellular apoptosis rate [23]. In the present study, we demonstrated that the synthesized GO-PEI-AS\_yyc\_G was not toxic at concentrations less than 50 μg/mL (Fig. 1a). Therefore, 50 μg/mL of GO-PEI-AS\_yyc\_G was adopted as the working concentration.

The results of AFM observation indicated that the surface roughness of the GO-PEI-AS\_yyc\_G nanosheets was increased compared to GO and GO-PEI material films (Fig. 1d). The surface characteristics of the GO nanosheets films were assessed using SEM, which showed a rougher and denser surface morphology in the GO-PEI-AS\_yyc\_G films compared to those of GO and GO-PEI (Fig. 1e). Because the surface roughness of membrane films could influence bacterial colonization and adhesion, the increased surface characteristics of the GO-PEI-AS\_yyc\_G material films probably indicated enhanced adhesive force.

To evaluate the vector transformation efficiencies, AS\_yyc\_G recombinant plasmids were labeled with gene encoding enhanced green fluorescent protein. The levels of GFP-expression indicated the presence of AS\_yyc\_G transcripts and revealed higher transformation efficiencies in S. aureus cells induced by GO-PEI-AS\_yyc\_G compared to pure AS\_yyc\_G plasmids. In particular, the quantitative RT-PCR assays showed that the fold change in AS\_yyc\_G expression in the GO-PEI-AS\_yyc\_G strain was roughly 3-fold higher in the AS\_yyc\_G strain transformed with competence-stimulating peptide. We speculated that the GO-based strategy significantly increased AS\_yyc\_G transformation as a delivery system and reduced transcripts of the yyc\_G gene.

Furthermore, GO-PEI-AS\_yyc\_G significantly suppressed bacterial growth and biofilm aggregation (Fig. 3). Using SEM observation, few randomly distributed microcolonies were identified and exopolysaccharide-enmeshed cell clusters were greatly decreased in the GO-PEI-AS\_yyc\_G strain biofilms compared to the GO and AS\_yyc\_G strains. After 24 h of biofilm establishment, CLSM findings revealed that GO-PEI-AS\_yyc\_G greatly reduced the cellular viability (Fig. 4). These results suggested that S. aureus was markedly inhibited by GO-PEI-AS\_yyc\_G, which improved the bactericidal effects of AS\_yyc\_G on the S. aureus biofilms.

Biofilm-forming capacity is an essential factor in the development of S. aureus-induced infections and results in significant increases in morbidity and mortality [24]. In S. aureus, PIA is a crucial component for biofilm organization [25], which is mostly synthesized by
glycosyltransferase enzymes encoded by the ica operon [12, 26]. In the current study, the GO-PEI-ASyyG strain had the lowest expression of yycF/G/H and icaA/D genes and biofilm formation, indicating that the pathogenesis of S. aureus was further decreased by the GO-PEI complexes, improving ASyyG transformation. Injectable GO-PEI-ASyyG could be useful in orthopedic applications to manage osteomyelitis lesions and reduce the use of antibiotics. Future directions will need to extend the applications of GO-PEI-ASyyG strategy as a potential way of managing the antibiotic resistance of S. aureus infections. At an appropriate concentration, GO-PEI-ASyyG could potentially improve the antibacterial properties of irrigation fluid. However, a limitation of the current study was the lack of in vivo experiments which are needed to confirm the effective concentration of this novel antibacterial agent before clinical application.

In the current study, a GO-based recombinant pDL278 ASyyG vector transformation strategy was developed. We found that the expression of the yycG gene was inversely correlated with the levels of ASyyG transcripts and that the GO-PEI-ASyyG strain had the lowest expression of biofilm organization-associated genes. The GO-based strategy significantly increased ASyyG transformation as a delivery system compared to the conventional competence-stimulating peptide strategy. Furthermore, GO-PEI-ASyyG suppressed aggregation of bacterial biofilms and improved the bactericidal effects on S. aureus after 24 h of biofilm establishment. Thus, our data demonstrated that nano-GO with antisense yycG RNA may be an effective and relatively stable strategy for the management of S. aureus infections.

**Fig. 4** GO-PEI-ASyyG suppressed the vital cells in S. aureus biofilms. a Double labeling of the biofilms in the untreated S. aureus and ASyyG-, GO-, and GO-PEI-ASyyG-treated strains. Green, vital cells (SYTO 9); red, dead cells (PI); scale bars, 100 μm. The three-dimensional reconstruction of the biofilms was performed using Imaris 7.0.0. b Volume ratio of the vital bacterial biomass in the biofilms (n = 10, *p < 0.05)

### Additional files

**Additional file 1:** The sequences of ASyyG and eGFP were synthesized by Sangon Biotech (Shanghai, China) and were inserted into BamHI and EcoRI restriction sites of a pDL278 vector. The initial sites of eGFP were underlined and bold in red. (DOCX 14 kb)

### Abbreviations

- AFM: Atomic force microscopy
- CLSM: Confocal laser scanning microscope
- CV: Crystal violet
- EPS: Extracellular polymeric substances
- GO: Graphene oxide
- PIA: Polysaccharide intercellular adhesion
- S. aureus: Staphylococcus aureus
- SEM: Scanning electron microscopy
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Authors’ contributions
Setting up the research was done by LL and ZH. Experiment section was major done by WSZ and Y.JL. Statistical analysis was done by WSZ and LL. Manuscript preparation was done by WSZ. Supervising was done by ZH and LL. All authors read and approved the final manuscript.

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Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests

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