Nano-graphene oxide with antisense vicR RNA reduced exopolysaccharide synthesis and biofilm aggregation for Streptococcus mutans

Shizhou WU¹,², Yunjie LIU³, Hui ZHANG¹ and Lei LEI²

¹ Department of Orthopedics, West China Hospital, Sichuan University, Chengdu, 610041, China
² State Key Laboratory of Oral Diseases, Department of Preventive Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu, 610041, China
³ West China School of Public Health, Sichuan University, Chengdu, 610041, China

Corresponding author, Lei LEI; E-mail: leilei@scu.edu.cn

Streptococcus mutans (S. mutans) has been proved to be crucial cariogenic pathogens. Antisense vicR RNA reduced the transcription of virulence genes and lead to a reduction in biofilm formation. In the current study, a graphene-oxide plasmid transformation system was developed using interacted GO-polyethylenimine (PEI) complexes loaded with antisense vicR-expressing plasmid (GO-PEI-ASvicR). The particle size distribution and zeta potential of the GO-PEI-based ASvicR were evaluated. Quantitative real-time PCR assays were used to investigate the expression of S. mutans virulence genes. The exopolysaccharide (EPS) production in biofilm were evaluated by confocal laser scanning microscopy and anthrone method. We showed that GO-PEI could efficiently deliver the ASvicR-expressing plasmid into S. mutans cells and support excellent transcripts of ASvicR. Furthermore, GO-PEI-ASvicR significantly reduced virulent-associated gene expressions, suppressed biofilm aggregation and inhibited EPS accumulation. Our reports demonstrated that preserving nano-graphene oxide with antisense vicR RNA will be a more effective strategy for dental caries management.

Keywords: Streptococcus mutans, Antisense vicR RNA, Graphene oxide, Exopolysaccharide

INTRODUCTION

Dental caries refers to regional destruction of dental hard tissues by acidic products of bacterial metabolism of dietary carbohydrates and the pathobiology of dental caries is widely recognized that the formation of dental plaque biofilm¹. The polysaccharides, proteins, and eDNA are secreted by bacterial biofilm colonized on the teeth surfaces which provides protection from desiccation, host defenses and enhanced resistance to antimicrobial agents, resulting in the development of caries cavities². Streptococcus mutans (S. mutans), a gram-positive coccus, has been proved to be crucial cariogenic pathogens. The exopolysaccharide (EPS) of S. mutans, mainly produced by glycosyltransferases (Gtfs), attributes to the formation of the stable glucan biofilm scaffolds³,⁴. Oral microbial cells acquired the ability to sense local conditions and respond readily to environmental stimulus such as pH, osmotic pressure, temperature, redox potential, nutrient availability, and toxic chemical exposures⁵. Two-component signal transduction systems (TCSs) are essential in the regulation of bacterial responses to environmental changes. The typical TCS consists of a transmembrane sensor protein (a histidine kinase) with a sensor domain, and a conserved cytoplasmic response regulator which can bind to the regulatory regions of targeted genes to promote a biological process. Fourteen TCSs are identified in the genomes of S. mutans⁶. The VicRK is the essential TCSs for viability of S. mutans which was initially identified from Bacillus subtilis⁷. The VicRK induces the genes expression, including gtfB, gtfC and gbpB, for synthesis of extracellular insoluble glucan which is the major extracellular matrix component of S. mutans biofilms.

Antisense RNA is a single-strand RNA which can bind to the base-paired mRNA and result in inhibition of downstream transcription and translation⁸. Our previous results indicated that a novel ASvicR RNA associated with a role on posttranscriptional dysregulation of VicR protein production and decreased biofilm biomass of S. mutans⁹. However, the application of those therapeutic antisense oligonucleotides in bacterial cells is limited by their poor transformation efficiencies without a suitable and effective carrier system. A cell-penetrating peptide is the conventional agent for transformation facilitating to antisense RNA access process⁵. Nevertheless, the inherent toxicity and poor stability of these peptides restricts the further applications⁶. Hence, there is an urgent need to a novel and effective vector with favorable biocompatibility for antisense RNAs transformation.

Graphene oxide (GO) presents good antibacterial effects due to its sharp edges as sheet structures which can damages cell membranes physically and causes oxidative stress reactions which is supposed to reduce the possibilities of pathogenic bacteria resistance, and is expected to be a new type of bactericidal agent⁷. Also, it has potential roles on efficient nucleic acid delivery, increasing the uptake process of genes and protecting nucleic acids from the lysosomal pathway, when ionically...
bonded to cationic polymers such as polyethylenimine (PEI)\textsuperscript{12-15}. In current study, we developed a graphene-based plasmid transformation system using GO-PEI complexes loaded with antisense \textit{vicR} plasmid (GO-PEI-\textit{ASvicR}) by electrostatic interactions. As there is an association between consumption of antimicrobials and antimicrobial resistance in human beings, the complex of GO-PEI-\textit{ASvicR} will be a more effective and stable strategy in treating \textit{S. mutans} infections without antibiotics and decreasing the possibility of antimicrobial resistance emergence\textsuperscript{16}. Furthermore, the antibacterial efficacy of this nanocomplexes against \textit{S. mutans} was also investigated.

**Graphical abstract**

We showed that GO-PEI could efficiently deliver the \textit{ASvicR}-expressing plasmid into \textit{S. mutans} cells and support excellent transcripts of \textit{ASvicR}. Furthermore, GO-PEI-\textit{ASvicR} significantly suppressed biofilm aggregation and inhibited EPS accumulation (Fig. 1).

**MATERIALS AND METHODS**

**Preparation of GO-PEI-\textit{ASvicR} and cytotoxicity evaluation**

The antisense \textit{vicR} sequences (\textit{ASvicR}) were synthesized by Sangon Biotech (Shanghai, China). As previously described, the \textit{ASvicR} sequences were inserted into BamHI and EcoRI restriction sites of pDL278 vector to generate a recombinant pDL278 \textit{ASvicR} plasmid\textsuperscript{3}. The GO powder (XFNANO Materials Tech, Nanjing, China, Batch NO.100602) was characterized by the Fourier Transform Infrared spectroscopy (Fig. 2) and mixed with ddH\textsubscript{2}O to a final concentration of 0.1mg/mL. Next, the branched polyethylenimine (BPEI; Sigma-Aldrich, St...
Louis, MO, USA) was slowly added into GO solution for the synthesis of GO-PEI complexes. Then, the solution was ultrasonicated ten cycles of 60 s with one min rest on ice. The mixed solution was shaken on a shaking table for overnight. The mixture was wash three times by centrifugation (12,000×g, 1 min) in ddH₂O, and then suspended with ddH₂O. The pDL278 ASvicR plasmid were added into GO-PEI complexes and incubated for 1 h at room temperature.

The optional concentration of GO-PEI based ASvicR was determined by cytotoxicity assays. In briefly, 3T3 fibroblasts cell lines at a density of 1,000 cells/well were seeded into 96-well plates cultured with Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and mixed with GO-PEI-ASvicR solution at a range of concentrations from 1,000 μg/mL to 0 μg/mL. After 48 h to 72 h incubation PEI-AS solution at a range of concentrations from 10% of fetal bovine serum (FBS) and mixed with GO-PEI-ASvicR solution at a range of concentrations from 1,000 μg/mL to 0 μg/mL. After 48 h to 72 h incubation (37°C, 5% CO₂), the culture medium was removed. Then, the cells were washed with phosphate buffer solution (PBS, pH=7.4) twice. The cell counting kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was applied for the cellular vitality. Ten microliters of CCK-8 was added in each well and the OD values were detected at 540 nm by using a microplate reader (ELX800, Gene, Hong Kong, China) after 2 h culture.

Particle size distribution, zeta potential and atomic force microscopy measurements

The particle size distribution of prepared samples was measured by using dynamic light scattering (DLS) and the zeta-potential were evaluated by a Malvern instrument (Zetasizer Malvern Nano ZS, Malvern Instruments, Worcestershire, UK). The prepared GO-PEI-ASvicR solution (50 μL) was dropped at the coverslip, dried in room temperature and characterized by using atomic force microscope (AFM; SPM-9500J2, Shimadzu, Kyoto, Japan) using the contact mode.

Bacterial transformation and culture

The single colony of S. mutans UA159 was selected from BHI agar plates, then were cultured in BHI medium to mid-exponential phase with OD₆₀₀ value at 0.5. For S. mutans GO group, 250 μL of mid-exponential S. mutans was incubated with the GO solution (final concentration of 30 μg/mL). For ASvicR group, 200 ng of recombinant pDL278 ASvicR plasmid was added into 250 μL of mid-exponential S. mutans and cultured with the competence stimulating peptide (Sangon Biotech; final concentration of 1 μg/mL). For GO-PEI-ASvicR strains, 250 μL of mid-exponential S. mutans were incubated with prepared GO-PEI-ASvicR mixtures (final concentration of 30 μg/mL) including 200 ng of pDL278 ASvicR plasmid without competence stimulating peptide. All the S. mutans strains were grown at 37°C in a 5% CO₂ atmosphere for 1 h and then diluted into 5 mL of fresh BHI medium. The spent culture media were used for pH quantification.

Transfection efficiency of GO-PEI-ASvicR in vitro

The expression of ASvicR, vicR and vicK as well as virulent factors genes including gtfB, gtfC, gtfD and gpbB of all S. mutans strains were assessed by using real-time polymerase chain reaction (RT-PCR). And, the transcripts of acid production genes including ldh and ropA were also investigated. Total RNA was extracted from S. mutans strains of mid-phased planktonic growth in BHI medium using an RNA purification Kit (MasterPure, Epicentre, Madison, WI, USA) in accordance with the recommendations of the supplier. According to instructions from RT Reagent Kit (PrimeScript, Takara, Kyoto, Japan), quantitative RT-PCR assays were carried out with primers listed in Table 1 using a qPCR system (LightCycler 480, Roche, Basel, Switzerland) with gyrA rRNA gene as an internal control.

Evaluation the morphology of biofilm cells and acid production

Sterile glass coverslips were cocultured with different S. mutans strains suspensions in 24-well plates and the biofilms were established after 24 h. All the S. mutans biofilms were measured by scanning electron microscopy (Inspect F50, FEI, Hillsboro, OR, USA) and three randomly areas were selected from each sample. The biomass of S. mutans biofilms were evaluated by crystal violet (CV) assay. Briefly, the biofilms were stained with 0.1% (w/v) crystal violet for 15 min. The dye bound on the biofilms was solubilized with 1 mL de-staining solution (ethanol/acetone=8:2), then transferred into a new plate and measured at OD₅₅₀nm. The confocal laser scanning microscopy was used to assess the biomass of bacteria by staining samples with SYTO9 dye (Bacterial Viability Kit reagent; LIVE/DEAD, Invitrogen, Grand Island, NY, USA). The EPS matrix was labeled with an Alexa Fluor 647-labeled dextran conjugate (Alexa Fluor 647, Invitrogen). A three-dimensional reconstruction was conducted and analyzed by a software program (Imaris 7.0.0, Bitplane, Zurich, Switzerland). All procedures were repeated in triplicate. We rinsed S. mutans biofilms twice with PBS buffer and dried at room temperature. Then, AFM were measured using an AFM (SPM-9500J2, Shimadzu) in the contact mode. The probe was positioned over the biofilm surface and five randomly points were selected for adhesion forces assessment.

Anthrone method for polysaccharide measurement

All the S. mutans biofilms cells in 12-well plates after culturing for 24 h in BHI media, supplemented with 1% sucrose were collected by scraping, and were resuspended in PBS. Water-insoluble glucan (WIG) and water-soluble glucan (WSG) of S. mutans biofilms was isolated as previously described. The supernatant was filtered through a 0.22-μm pore size filter membrane (Corning, New York, NY, USA) and separated for WSG measurement using the anthrone method. For assessment,200 μL of WIG or WSG was mixed with 600 μL of anthrone reagent. The mixtures were heated at 95°C for 10 min and put on ice. The absorbance of each sample at 625 nm was measured using a microplate reader (ELX800, Gene).
Table 1 Sequences of primers used for RT-PCR analysis

| Primers | sequence 5′-3′ (Forward/Reverse) | Amplification size (bp) |
|---------|---------------------------------|-------------------------|
| RT-qPCR |                                 |                         |
| gyrA    | 5′-ATTGTTGCTCGGGCTCTTCCAG-3′/    | 105                     |
|         | 5′-ATGCGGCTGTGTCAGGAACCACCAG-3′ |                         |
| vicR    | 5′-CGCAGTGCTGAGAAAATG-3′/        | 157                     |
|         | 5′-ACCTGTGTGTCAGGAATGTGAG-3′    |                         |
| vicK    | 5′-CAGTGGCTGAGGAAAATG-3′/        | 102                     |
|         | 5′-ACCTGTGTGTCAGGAATGTGAG-3′    |                         |
| vicX    | 5′-TGCTCAACCACACAGTTTACCG-3′/    | 127                     |
|         | 5′-GGACCTCAATCAGTAACCACATCAG-3′ |                         |
| gtfB    | 5′-ACACTTTCGCTTGGCTTG-3′/        | 127                     |
|         | 5′-GCTTAGATGTGTTCTGGTTG-3′      |                         |
| gtfC    | 5′-CCAAAATGTTATGCTGGCTGTCG-3′/   | 136                     |
|         | 5′-TGAGTTCTCTATCAAGTAACGCAG-3′  |                         |
| gtfD    | 5′-AATGGAATTCTCGAGCCGACTTGGAG-3′ | 245                     |
|         | 5′-TTAGCCGTAGCTGGCTCTATTGTA-3′  |                         |
| gbpB    | 5′-AGCAACAGAAGACACACATCAG-3′/    | 150                     |
|         | 5′-CCACCATTACCCAGTCTTTTAC-3′    |                         |
| ropA    | 5′-TGATGGTCGGTACTCAGCCGA-3′/     | 152                     |
|         | 5′-TCACTTCAATCGTGTCATCCATC-3′   |                         |
| ldh     | 5′-GGACCGCTTGGATCTTGATATG-3′/    | 235                     |
|         | 5′-GGTTAGACGAGCAGAGGAG-3′       |                         |

Primers sequence 5′-3’ (Forward/Reverse) Relevant purpose

Antisense RNA detection

| Primers | sequence 5′-3’ (Forward/Reverse) | Relevant purpose          |
|---------|---------------------------------|---------------------------|
| AS1     | 5′-GCCCTTTGTATGGCCGTG-3′        | First strand cDNA synthesis |
| PCR1    | 5′-CCAACACCCGCCTCGGTC-3′       | RT-PCR analysis           |

Data analysis
The Bartlett’s test was performed to assess the homogeneity of data variances and Shapiro-Wilk test was conducted to determine the normal distribution of data. One-way analysis of variance was used to compare the data, followed by pairwise multiple comparisons by a software program (SPSS version 20, IBM, Armonk, NY, USA).

RESULTS

Cytotoxicity and characterization of GO-PEI-ASvicR
The cytotoxicity of GO-PEI-ASvicR was assessed by CCK-8 assays. After 48 or 72 h of incubation with GO-PEI-ASvicR, the viability of 3T3 fibroblasts cells significantly decreased at concentrations of 60 μg/mL or higher. The results showed that the GO-PEI-ASvicR complexes were not toxic with concentrations no more than 60 μg/mL when compared with the control group (Fig. 3A).

DLS measurements were performed in cell culture medium to show the size distributions of GO, GO-PEI and GO-PEI-ASvicR. The Z-average sizes of the GO were 383 nm. For GO-PEI and GP-PEI-ASvicR, Z-average sizes of 185 and 390 nm were obtained respectively which indicated that the size of GP-PEI-ASvicR was slightly larger than GO (Fig. 3B). By using zeta potential measurement, the values of surface charge of GO were approximately −24.4 mV indicating its inability to form a complex with DNA strains. However, when combined with cationic polymer PEI, GO-PEI and GO-PEI-ASvicR complexes showed the positive surface charges at approximately 13.7 and 35.4 mV respectively (Fig. 3C). The AFM results showed the roughness of GO-PEI-ASvicR nanosheets increased to 6.8 nm compared to GO 3.3 nm and GO-PEI 3.8 nm (n=10, p<0.05; Figs. 3D and E).

GO-PEI-ASvicR enhancement ASvicR transformation and inhibition virulent-associated gene expressions
In current study, ASvicR plasmids were applied to inhibit vicR expression, and RT-PCR showed quantitatively ASvicR gene expressions in both ASvicR and GO-PEI-ASvicR strains increased significantly by 2.5 and
4.6 folds respectively when compared to *S. mutans* UA159 strain (n=10, p<0.05; Fig. 4A). Consistently, the expressions level of *vicR* were significantly reduced in ASvicR and GO-PEI-ASvicR strains (n=10, p<0.05). Furthermore, the results showed that the expressions of *gtfB*, *gtfC*, *gtfD* and *gbpB* genes were inhibited most in the GO-PEI-ASvicR strain (n=10, p<0.05). These results indicated the potential of GO-PEI-ASvicR to develop transfection efficiency of antisense *vicR* genes. And, the transcripts of acid production genes including *ldh* and *ropA* were also decreased in ASvicR and GO-PEI-ASvicR strains (n=10, p<0.05).

**GO-PEI-ASvicR reduced bacterial growth and acid production**

The growth curves of strains were compared in three independent experiments. The entry into log phase was significantly delayed in GO-PEI-ASvicR strains compared to the *S. mutans* UA159, GO, and ASvicR strains (Fig. 4B). The effects of GO, ASvicR and GO-PEI-ASvicR on acid production by the strains were also investigated. A progressive decrease of pH values was observed after 9 h of incubation with or without GO, ASvicR and GO-PEI-ASvicR, initially close to 7.4 and terminated at 5.5 or lower. However, decrease of pH value in GO-PEI-ASvicR strains was highest (Fig. 4C).

**GO-PEI-ASvicR suppressed biofilm formation and adhesion force**

Crystal violet microtiter assays demonstrated that decreased biofilms biomass in ASvicR, GO and GO-PEI-ASvicR cells when compared to that of *S. mutans* UA159. Quantitatively, GO-PEI-ASvicR strain exhibited the lowest OD values of biofilm biomass (n=10, p<0.05, Fig. 5A). The adhesion force of biofilms among the bacterial cells was measured by AFM and it was considerable in
**Fig. 4** GO-PEI-ASvicR increased ASvicR transformation and affected virulence gene expressions. (A) Quantitative RT-PCR analysis showed gene transcription in untreated *S. mutans* and ASvicR-, GO- and GO-PEI-ASvicR-treated strains; *S. mutans* gene expression was quantified relatively using *gryA* as an internal control and calculated based on untreated *S. mutans* UA159 expression, which was set as 1.0 (n=10, *p*<0.05); (B) *S. mutans* and ASvicR-, GO- and GO-PEI-ASvicR-treated strains were grown in BHI medium, and growth was monitored every hour; (C) Effect of ASvicR-, GO- and GO-PEI-ASvicR on acid production which were determined by measuring the pH values every hour.

**Fig. 5** GO-PEI-ASvicR suppressed *S. mutans* biofilm aggregation. (A) Biomass was quantified by crystal violet staining; Optical densities at 550 nm were measured (n=10, *p*<0.05); (B) The values of adhesion force of *S. mutans* biofilms obtained from atomic force microscopy (AFM) experiments (n=10, *p*<0.05); (C) SEM of untreated *S. mutans* and ASvicR-, GO- and GO-PEI-ASvicR-treated strains for 24 h biofilms; Scale bar for 5,000× magnification, 20 μm. Scale bar for 20,000× magnification, 5 μm.
S. mutans UA159 biofilms (7.5±0.8 nN; Fig. 5B) while the same was significantly decreased in the GO-PEI-AS\textit{vic}R biofilms (2.45±0.22 nN; Fig. 5B). SEM observation demonstrated that S. \textit{mutans} UA159 cells were most densely packed with extracellular matrix, when compared with the AS\textit{vic}R and GO-PEI-AS\textit{vic}R strains which showed biofilms reduction with extracellular matrix interspersed among “blank” areas (Fig. 5C). Meanwhile EPS-enmeshed cell clusters were greatly destructed in the GO-PEI-AS\textit{vic}R strains formed biofilms compared to the other groups, and the very few small microcolonies were randomly spread (Fig. 5C).

**DISCUSSIONS**

Until recently, we identified a \textit{vic}R antisense RNA (AS\textit{vic}R) and expression of VicR protein correlated inversely with different levels of AS\textit{vic}R transcript. It also has been suggested that the biofilm biomass decreased in the AS\textit{vic}R overexpression mutant\textsuperscript{3}. Due to the RNA instability, antisense RNAs are difficult for preservation, therefore we used recombinant pDL278 AS\textit{vic}R overexpression plasmid for transformation. However, few nucleic acid molecules or plasmids could enter bacterial cells without an effective vector. Therefore, there is a need to develop effective and relatively stable vectors which can deliver nucleic acid molecules\textsuperscript{20}. In present study, a GO based antisense RNA transformation method has been developed using electrostatic interacted GO-PEI complexes loaded with recombinant pDL278 AS\textit{vic}R plasmid vector.

When ionically bonded to cationic polyethyleneimine (PEI) polymers, GO is reported that be used to deliver genes efficiently\textsuperscript{21}. These positive surface charges could favor the interaction with the negatively charged cellular surface and facilitate cellular transfection\textsuperscript{13}. Therefore, we used this relatively stable GO-PEI complexes to load AS\textit{vic}R RNA to enhance the efficiency in bacterial transformation and acquired a more efficiency than the competence stimulating peptide method. It has been showed that the concentrations of GO-PEI lower than 50 μg/mL had no toxic effects on cellular apoptosis rate\textsuperscript{15}. In present study, it has been showed that synthesized GO-PEI-AS\textit{vic}R was not toxic until the concentration reached to 60 μg/mL or higher (Fig. 3A). Therefore, the GO-PEI-AS\textit{vic}R of 30 μg/mL was adopted as the working concentration for the subsequent studies. The surface roughness of materials may influence the colonization of micro-organisms, and

**Fig. 6** GO-PEI-AS\textit{vic}R suppressed architecture of the EPS matrix.

(A) Volume ratio of the EPS matrix to the bacterial biomass in the biofilms (n=10, *p<0.05); (B) WIG and WSG of samples from different strains, as measured using the anthrone method (n=10, *p<0.05); (C) Double labeling of the biofilms in the untreated \textit{S. mutans} and AS\textit{vic}R-, GO- and GO-PEI-AS\textit{vic}R-treated strains. Green, bacteria (SYTO 9); red, EPS matrix (Alexa Fluor 647); scale bars, 100 μm. The three-dimensional reconstruction of the biofilms was performed using Imaris 7.0.0.
the greater roughness provides greater cells adhesion\textsuperscript{22}. By AFM observations, the surface roughness of GO-PEI-AS\textit{vicR} nanosheets increased compared to GO and GO-PEI membrane films (Fig. 3D). The surface parameter of the GO-PEI-AS\textit{vicR} material films likely increased the roughness which probably contributes to enhanced adhesion force due to the thicker the multilayers\textsuperscript{23}.

The successful construction of the GO-PEI-AS\textit{vicR} delivery system carrying this AS\textit{vicR} was evidenced by the efficient uptake by bacterial cells and quantitative AS\textit{vicR} expressions of \textit{S. mutans}. The quantitative RT-PCR showed expressions of AS\textit{vicR} RNA in GO-PEI-AS\textit{vicR} strain significantly increased by 4.6 folds compared to \textit{S. mutans} UA159 strain (Fig. 4A). Particularly, the fold change of GO-PEI-AS\textit{vicR} strain was about two folds in AS\textit{vicR} transformed by competence stimulating peptide indicating the GO-PEI-AS\textit{vicR} probably act more efficiently than the conventional competence stimulating peptide strategy as delivery system. Regarding the results of AFM observations, we speculated that improved delivery efficiency of the GO-PEI-AS\textit{vicR} may be attributed to altered surface charge and higher surface roughness\textsuperscript{24}.

There are three kinds of Gtfs produced by \textit{S. mutans}: GtfB, which synthesizes mostly insoluble glucan; GtfD, which mostly synthesizes soluble glucan; and GtfC, which synthesizes a mixture of insoluble and soluble glucans, and the deletion of gtfC drastically reduces \textit{S. mutans} adherence to smooth surfaces\textsuperscript{25,26}. Our data revealed that the transcriptional levels of gtfB, gtfC, and gtfD, were significantly decreased in the GO-PEI-AS\textit{vicR} and AS\textit{vicR} strains after 24 h incubation for biofilm formation, suggesting an AS\textit{vicR}-mediated negative influence on glucan synthesis and initial adherence. Also, it has been established that the depletion of gbpB (glucan binding protein B) resulted in altered cell surface properties and adhesion ability in \textit{S. mutans}\textsuperscript{27}. Based on present data, a significant reduction of gbpB expression was found in GO-PEI-AS\textit{vicR} strains which indicated impaired initial phases of sucrose-dependent biofilm formation by AS\textit{vicR} interference.

Growth curve analysis revealed that entrance into the log phase of the GO-PEI-AS\textit{vicR} and AS\textit{vicR} strains were delayed, suggesting that AS\textit{vicR} transcripts affected the growth rate (Fig. 4B). On the other hand, lactate dehydrogenase (ldh) plays a critical role in production of acid during metabolism of carbohydrates (acidogenicity), which is one of the main virulence factors of \textit{S. mutans}\textsuperscript{28}. Report demonstrated that deletion of ropA gene resulted in altered biofilm architecture and increased sensitivity to acid and oxidative stress\textsuperscript{29}. The GO-PEI-AS\textit{vicR} and AS\textit{vicR} strains appeared decreased acid productions (Fig. 4C), which could likely be attributed to the suppressed transcriptional levels of \textit{ldh} and \textit{ropA}. Whether response regulator VicR activities are directly associated with \textit{ldh} and \textit{ropA} expressions also warrants further investigation.

To provide further confirmation, we evaluated the biomass of the 24 h biofilm, and the results showed that the biomass of GO-PEI-AS\textit{vicR} strains was significantly decreased compared to those of UA159 and AS\textit{vicR} overexpressing strains. Thus, these analyses of biofilm formation again confirmed that improved delivery efficiency of the GO-PEI-AS\textit{vicR} and AS\textit{vicR} RNA function in biofilm formation by \textit{S. mutans}. After 24 h biofilm establishment, the results revealed that GO-PEI-AS\textit{vicR} most suppressed biofilm aggregation and reduced the EPS synthesis (Fig. 5A). EPSs are crucial components of the protective shelter in \textit{S. mutans} biofilms\textsuperscript{30}. Glucans, including WIG and WSG, are mostly synthetized the effects of glucosyltransferases (Gtfs)\textsuperscript{31}. Particularly, insoluble glucan promotes the accumulation and binding of microorganisms to the tooth surface\textsuperscript{32}. By double staining of CLSM observation and the anthrone method, the glucans synthesis in the GO-PEI-AS\textit{vicR} and AS\textit{vicR} strains significantly declined compared to the UA159 strain (Fig. 6). We speculated that the inhibited EPS, especially WIGs, synthesis of the GO-PEI-AS\textit{vicR} and AS\textit{vicR} strains biofilms are primarily due to the reduced expression of gtfC/D. In the current study, the GO-PEI-AS\textit{vicR} strain exhibited the lowest EPS accumulation and the virulent-associated gene expressions of \textit{S. mutans} were markedly decreased by AS\textit{vicR} interference. Future directions needed to extend the applications of GO-PEI-AS\textit{vicR} strategy as a potential substitutive therapy for dental caries management.

CONCLUSIONS

In summary, a graphene-based plasmid transformation system was developed using electrostatic interacted GO-PEI complexes loaded with antisense vic\textit{R} plasmid (GO-PEI-AS\textit{vicR}). Our reports demonstrated that GO-PEI could efficiently deliver AS\textit{vicR} plasmid into \textit{S. mutans} cells with excellent transcripts of AS\textit{vicR}. GO-PEI-AS\textit{vicR} significantly reduced virulent-associated gene expressions, suppressed biofilm aggregation and inhibited EPS accumulation. The results of current study revealed that preserving nano-GO with antisense vic\textit{R} RNA will be a more effective a strategy as a potential substitutive therapy for dental caries management.

ACKNOWLEDGMENTS

We are most grateful to Huiqi Xie for her excellent technical assistance. This work was supported in part by the National Natural Science Foundation of China (Grant No. 81800964), Sichuan Provincial Natural Science Foundation of China (Grant No. 2018SZ0125).

CONFLICTS OF INTEREST

No conflicts of interest.

REFERENCES

1) Nyvad B, Crielaard W, Mira A, Takahashi N, Beighton D. Dental caries from a molecular microbiological perspective. Caries Res 2013; 47: 89-102.
2) Marquis RE. Oxygen metabolism, oxidative stress and acid-
3) Lei L, Stipp RN, Chen T, Wu SZ, Hu T, Duncan MJ. Activity of streptococcus mutans VicR is modulated by antisense RNA. J Dent Res 2018; 97: 1477-1484.

4) Klein MI, Hwang G, Santos PH, Campanella OH, Koo H. Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms. Front Cell Infect Microbiol 2015; 5: 10.

5) Mattos-Graner RO, Duncan MJ. Two-component signal transduction systems in oral bacteria. J Oral Microbiol 2017; 9: 1400858.

6) Song L, Sudhakar P, Wang W, Conrads G, Brock A, Sun J, et al. A genome-wide study of two-component signal transduction systems in eight newly sequenced mutants streptococci strains. BMC Genomics 2012; 13: 128.

7) Fabret C, Hoch JA. A two-component signal transduction system essential for growth of Bacillus subtilis: implications for anti-infective therapy. J Bacteriol 1998; 180: 6375-6383.

8) Cideciyan AV, Jacobson SG, Drack AV, Ho AC, Charng J, Garafalo AV, et al. Effect of an intravitreal antisense oligonucleotide on vision in Leber congenital amaurosis due to a photoreceptor cilium defect. Nat Med 2019; 25: 225-229.

9) Wolfe JM, Fadzen CM, Holden RL, Yao M, Hanson GJ, Pentelute BL. Perfluoroaryl bicyclic cell-penetrating peptides for delivery of antisense oligonucleotides. Angew Chem Int Ed Engl 2018; 57: 4756-4759.

10) Ghosal A, Vitali A, Stach JE, Nielsen PE. Role of SbmA in the uptake of peptide nucleic acid (PNA)-peptide conjugates in E. coli. ACS Chem Biol 2013; 8: 360-367.

11) Liu Y, Yuan C, Cheng Y, Yao G, Xie L, Xu B. Graphene oxide affects growth and resistance to sclerotinia sclerotiorum in Brassica napus L. J Nanosci Nanotechnol 2018; 18: 8345-8351.

12) Putnam D, Gentry CA, Pack DW, Langer R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc Natl Acad Sci U S A 2001; 98: 1200-1205.

13) Feng L, Zhang S, Liu Z. Graphene based gene transfection. Nanoscale 2011; 3: 1252-1257.

14) Yin D, Li Y, Lin H, Guo B, Du Y, Li X, et al. Functional graphene oxide as a plasmid-based Stat3 siRNA carrier inhibits mouse ovarian cancer. Adv Sci (Weinh) 2017; 5: 1700578.

15) Tacconelli E, Sifakis F, Harbarth S, Schrijver R, van Mourik JH, Grande-Tovar CD. Synthesis and application of scaffolds of chitosan-graphene oxide by the freeze-drying method for tissue regeneration. Molecules 2018; 23: E2651.