Graphene Oxide Enables the Reosteogenesis of Previously Contaminated Titanium In Vitro

W. Qin, C. Wang, C. Jiang, J. Sun, C. Yu , and T. Jiao

Appendix

Materials and Methods

Preparation and Characterization of GO

A modified Hummers method was utilized to prepare GO nanosheets from natural graphite as previously described (Martinez Paino et al. 2017). Briefly, 3 g of natural graphite power, 40 mL of sodium nitrate and 230 mL of concentrated sulfuric acid were added to a three-necked flask in an ice bath; then, 18 g of potassium permanganate was introduced slowly, and the reaction mixture was stirred for 1 h. The ice bath was then removed, and the temperature of the suspension was brought to 35-40°C, which was maintained for 12 h. The reaction was then cooled, the product was poured onto ice, and 3 mL of hydrogen peroxide (30%) was introduced slowly with stirring until the color of the solution turned golden yellow. The solution was filtered, and the precipitate (GO) was washed with 5% (v/v) hydrochloric acid and distilled water until the pH approached 7. Finally, the GO was dispersed in water, sonicated for 8 h, and placed in a vacuum-drying oven until further use. All chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

To characterize the synthesized GO nanosheets, atomic force microscopy (AFM; Nanoscope IIIa, Digital Instrument, Boston, MA, USA) was used to determine the morphology and size of the GO. Fourier transform infrared spectroscopy (FTIR; Thermo, Waltham, MA, USA) and Raman spectroscopy (XploRA INV, HORIBA Scientific, PAR, FR) were used to further examine the surface functional groups on the GO and the carbon structure of the GO, respectively.

Bacterial Strains and Culture Conditions

The pathogenic bacterial strains used in this study, namely, *F. nucleatum* (ATCC 10953), *Streptococcus mutans* (*S. mutans*, UA 159) and *P. gingivalis* (ATCC 33277), were obtained from the Shanghai Key Laboratory of Stomatology, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). These strains were routinely seeded onto brain heart infusion (BHI; Becton Dickinson and Company, Franklin Lakes, NJ, USA) agar prepared with (*P. gingivalis* and *F. nucleatum*) or without (*S. mutans*) 5% sterile defibrinated sheep blood (Shanghai Kangrun Biological Technology Co., Ltd., Shanghai, China). Then, these plates were arranged in an anaerobic incubator (Don Whitley Scientific, West Yorkshire, UK) at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). After colony formation, single colonies were picked and transferred into BHI broth and for *P. gingivalis*, the culture was supplemented with 0.0005% hemin (Sigma, St. Louis, MO, USA) and 0.0001% menadione (Sigma, St. Louis, MO, USA); the cultures were incubated under the same conditions as those described above. Growth curves of the three bacteria were constructed using absorbance measurements for each.

Minimum Inhibitory Concentration (MIC) Determination

MICs were determined using a standard broth microtiter dilution method. Each bacterial inoculum was standardized to 0.5 McFarland standard in 96-well microtiter plates (Corning, NY, USA); no antimicrobials were added to the control well. The sterilized GO nanosheets were dispersed in BHI medium (supplemented with 0.0005% hemin and 0.0001% menadione) and sonicated for half an hour. Varying concentrations of GO from 512 to 16 μg/mL were obtained by 2-fold serial dilutions. The microtiter plates were incubated at 37°C under anaerobic conditions for 24 h for *S. mutans* and *F. nucleatum* and 48 h for *P. gingivalis*. MICs were determined by measuring the OD at 600 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). All of the tests were carried out in triplicate.

Appendix Table 1. Antibacterial effect of GO on *S. mutans*, *P. gingivalis* and *F. nucleatum*.

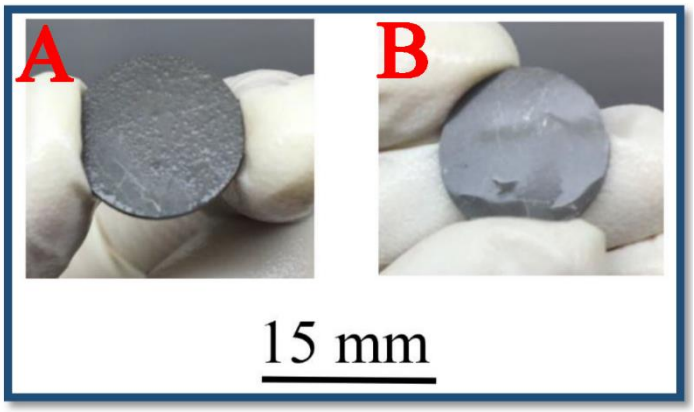
Bacteria Strain	***S. mutans***	***P. gingivalis***	***F. nucleatum***
MIC value (μg/mL)	**128-256**	**64-128**	**64-128**

Polymicrobial Biofilm Formation

The three strains were harvested when they reached the mid-exponential growth phase; at this phase, bacteria are in an optimal growth state that corresponds to a highly proliferative capability for adhesion development that favors the formation of mixed biofilms. A multispecies biofilm model was developed. In brief, sandblasted, large-grit and acid-etched (SLA; Baoji Noble Metal Co., Ltd., Shanxi, China) grade IV titanium plates (15 mm diameter, 1 mm thickness) were placed in the wells of 24-well microtiter plates (Corning, NY, USA), and 1.5 mL of mixed bacterial suspension containing 10^3 colony-forming units (CFU)/mL for *S. mutans*, 10^6 CFU/mL for *F. nucleatum* and *P. gingivalis* was added. The plates were placed in the anaerobic system for two days at 37°C for polymicrobial biofilm production. To observe the pathogens within these biofilms, the polymicrobial biofilms formed on the surface of titanium plates were scraped gently using a sterile inoculating loop (Corning, NY, USA) and a Gram staining reagent (BASO, Zhuhai Besso Biotechnology Co., Ltd., Zhuhai, China).

Management of Polymicrobial Biofilm

After two days of cultivation, the polymicrobial biofilms formed were visible on the surface of the titanium plates (Appendix Figure 1A). To evaluate the antimicrobial effect of GO on these polymicrobial biofilms formed by *S. mutans*, *F. nucleatum* and *P. gingivalis*, we designed four experimental groups: group B, biofilms were removed only with TePe Select™ slender-bristle toothbrushes (TePe, Malmö, SE); group G, biofilms treated with different concentrations of GO (64, 128, 256, and 512 μg/mL); group GB, combined treatments of group B and group G; and group C, untreated biofilms. Here, we chose to use a toothbrush instead of instruments such as a curette or a titanium rotary brush because we noted that the titanium plates were covered with polymicrobial biofilms without calculus. Therefore, the use of a toothbrush would be more convenient, thorough and economical; in addition, the damage to the surfaces of the titanium plates would be reduced.



Appendix Figure 1. Polymicrobial biofilm-contaminated SLA titanium plate before (A) and after (B) brushing treatment.

Specifically, the medium in 24-well microtiter plates was removed, and for groups C and G, the contaminated titanium plates were washed twice with phosphate-buffered saline (PBS, pH 7.4, HyClone, South Logan, UT, USA) to eliminate unattached bacteria and transferred to new 24-well plates. For groups B and GB, the polymicrobial biofilms on the surface of the titanium plates were cleaned with TePe Select™ slender-bristle toothbrushes with hand by brushing up and down, left and right, approximately 10 times, until the surface of the titanium plates were "clear", meaning no polymicrobial biofilms were visible (Appendix Figure 2B). The titanium plates were then washed twice with PBS and placed into new 24-well microtiter plates. Finally, fresh BHI medium with (groups GB and G) or without (groups C and B) different concentrations of GO was introduced into the wells. Based on the growth curves of these three bacteria and to roughly determine the time range of the antimicrobial effect of GO, we chose two observation points of 2 and 24 h.

Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was utilized to evaluate the bacterial viability of each group. In brief, after culturing for 2 and 24 h, the BHI cultures in 24-well microtiter plates were discarded, and 100 μL of MTT (Beyotime Biotechnology, Shanghai, China) was added to each well. The plates were placed in the dark for an additional 2 h. Subsequently, the unreacted MTT was removed, 400 μL of dimethyl sulfoxide (DMSO, Tong Cheng Chemical Agent Co., Ltd., Hangzhou, China) was added to dissolve the formazan crystals in each well, followed by shaking of the plates for 10 min. The DMSO-containing formazan was transferred into microcentrifuge tubes and centrifuged at 10,000 rpm for 1 min. The supernatant was collected and transferred to a 96-well plate, and OD at 490 nm was measured using the BioTek instrument (n = 3 for each group). Other subgroups were processed according to this analytical procedure.

Biofilm Biomass Assay

The biomasses of the bacterial biofilms on the SLA titanium plates were determined by a crystal violet (CV) staining assay. Briefly, after culturing for 2 and 24 h, the culture medium in 24-well microtiter plates was discarded, and each well was air dried completely. Then, 100 μL of 0.01% (v/v) CV (Sigma, St. Louis, MO, USA) solution was added to each well for 15 min at room temperature. The solution was removed, and each

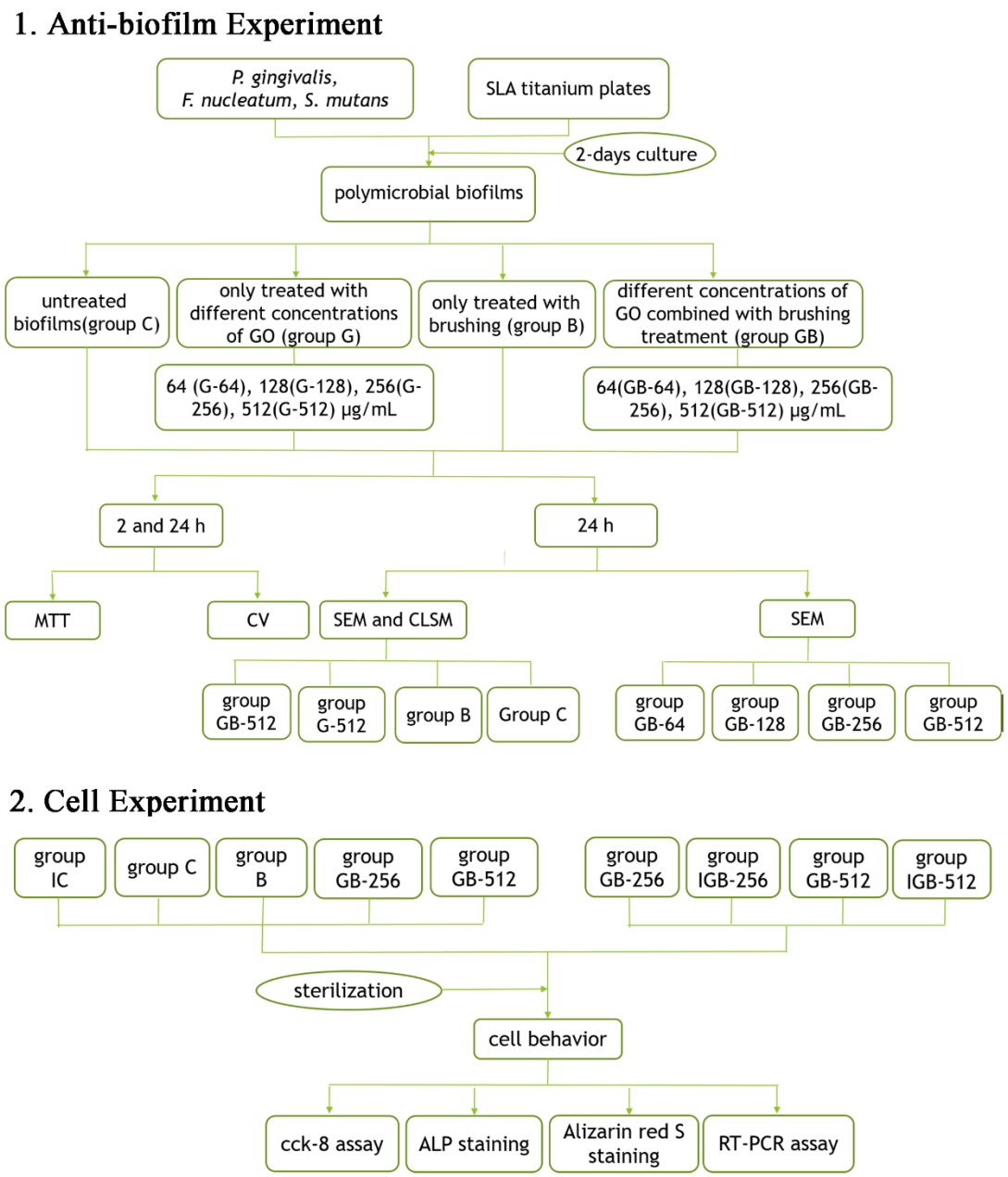
well was carefully washed twice with PBS to remove unabsorbed dye; 400 μL of 95% ethanol (v/v, Tong Cheng Chemical Agent Co., Ltd., Hangzhou, China) was added to each well for 15 min at room temperature. The solutions were transferred to a 96-well plate for estimation of the overall biomass using the BioTek instrument at 595 nm (n = 3 for each group). Samples from the other subgroups were also processed according to this analytical procedure.

Live/Dead Fluorescent Staining

As MTT and CV staining results showed that the antibacterial effect of GO was dose and time dependent, we chose the subgroups with the highest concentration of GO (512 μg/mL) in group G (G-512) and group GB (GB-512) to identify the viability and structure of the polymicrobial biofilms by using LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) after 24 h. Briefly, after culturing for 24 h, the titanium plates in group C, group B, group G-512 and group GB-512 were washed gently with PBS, and equal volumes of SYTO 9 and PI solutions were added to a clean microcentrifuge tube; 1.5 μL of the dye mixture was diluted in 500 μL of double-distilled water, and 50 μL of the resulting solution was used to stain each plate. After 15 min of staining in the dark at room temperature, the plates with stained biofilms were observed by confocal laser scanning microscopy (CLSM) (Leica TCS SP5, Wetzlar, Germany). The excitation/emission wavelength of the two dyes was 488 nm for SYTO 9 and 543 nm for PI.

Biofilm Integrity Detection

After the titanium plates were subjected to the various treatments for 24 h, the residual polymicrobial biofilms in group C, group B, group G-512 and group GB-512 were observed by scanning electron microscopy (SEM). To confirm the appropriate concentrations of GO, GB subgroups treated with concentrations of 64, 128, 256, and 512 μg/mL GO were also observed by SEM to evaluate the antimicrobial effect of different GO concentrations after brushing. Specifically, the plates were rinsed gently with PBS, and glutaraldehyde (2.5% (v/v); pH 7.4, Sangon Biotech Co., Ltd., Shanghai, China) was used to fix the samples at room temperature for 2-3 h. An ethanol concentration gradient (v/v) consisting of 50, 70, 80 and 90% ethanol was sequentially used to dehydrate the specimens for 15-20 min per step, ending with 3 changes of 100% ethanol within 30 min. Next, a 1:1 mixture of tertiary butyl alcohol (Tong Cheng Chemical Agent Co., Ltd., Hangzhou, China) and ethanol followed by pure tertiary butyl alcohol was applied, each for 15 min, to achieve metathesis of ethanol in the cells. Before visualization, the SLA titanium plates were kept under vacuum to ensure that the samples were moisture free; subsequently, the plates were sputter-coated with gold. Images at high magnification were taken at different locations on each plate by SEM (JSM-7600F, JEOL, Brno, Tokyo, Japan).



Appendix Figure 2. Technology Roadmap of the entire experiment. SLA, sandblasted, large-grit and acid-etched; GO, graphene oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CV, crystal violet; SEM, scanning electron microscopy; CLSM, confocal laser scanning microscopy; cck-8, cell counting kit-8; ALP, alkaline phosphatase; RT-PCR, reverse transcription-polymerase chain reaction. Group B, biofilms removed only with brushing; group C, untreated; group G, treated with different GO concentrations; group GB, combined treatments from groups B and G; group IC, intact clean titanium; group IGB, intact clean titanium treated with brushing and GO.

Cell Isolation and Culture

Rat bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from the femurs and tibias of

4-week-old Sprague-Dawley rats (Ninth People's Hospital Animal Center, China) according to the method previously described (Darabi et al. 2013) and cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 100 μg/mL penicillin/streptomycin (HyClone, South Logan, UT, USA) at 37°C in an atmosphere of 5% CO_2. Cells from passages 2-4 and 80-90% confluence were used for experiments.

BMSC Viability Assessment

According to the results described in the anti-biofilm section, it seems that only groups GB-256 and GB-512 can significantly decrease residual bacteria and prevent the re-formation of polymicrobial biofilms. Thus, we chose these two groups for further cell experiments. Before cellular experiments, titanium plates of groups GB-256, GB-512, B, and C were sterilized with an ethylene oxide sterilizer; that is, only dead bacteria/biofilm remained on the surface of titanium plates for each group. Intact clean titanium plates (group IC) were used as a non-contaminated control group. These titanium plates were placed in 24-well microtiter plates. BMSC viability was analyzed using a cell counting kit (CCK)-8 assay (AbD Serotec, Oxford, UK) at 1, 3 and 7 days after initially seeding 1×10^4 BMSCs onto the surfaces. CCK-8 solution (10 μL) was added to each well and incubated for 2 h, and absorbance at 450 nm was measured using a BioTek instrument (n = 3 for each group).

BMSC Osteogenic Differentiation Assay

BMSCs were seeded onto the surfaces of titanium plates in groups GB-256, GB-512, B, C and IC in 24-well plates at a density of 5×10^4 cells/well. Osteogenic differentiation of BMSCs was performed by changing the culture medium to differentiation medium composed of DMEM supplemented with 100 nM dexamethasone, 10 mM sodium β-glycerophosphate and 50 nM ascorbic acid-2-phosphate (Thermo Fisher Scientific, Waltham, MA, USA).

After incubation for 7 and 14 days, alkaline phosphatase (ALP) staining was carried out. In brief, the samples were rinsed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then, the samples were washed three times with PBS and stained using a BCIP/NBT ALP Colour Development Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer’s instructions.

Alizarin red S staining was performed to detect mineralization nodules of the BMSCs on the titanium plates in each group after incubation for 21 days. In brief, the samples were rinsed three times with PBS and fixed with 4% paraformaldehyde for 30 minutes; the samples were washed three times with PBS, stained with 0.5% alizarin red S solution (Sigma-Aldrich, St. Louis, MO, USA) and photographed.

Expression of osteogenesis-related genes in BMSCs was determined after culturing each group of titanium plates for 7 and 14 days. Total RNA of adherent BMSCs was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed into cDNA using Primescript RT Reagent Kit (Takara Bio Inc., Tokyo, Japan). Expression of Runx-2, β-catenin, OCN, and COL1-a1 was determined by reverse transcription-polymerase chain reaction (RT-PCR) (LightCycler 480, Roche-Diagnostics, Basel, Switzerland) using a QuantiTest SYBR Green Kit (Takara Bio Inc., Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control; a negative control without a cDNA template was

included in each assay (n = 3 for each group). The data were analyzed by relative expression analysis ($2^{-\Delta\Delta Ct}$). The primer sequences are displayed in Appendix Table 2.

Appendix Table 2. Primer sequences used in real-time PCR.

Genes	Forward	Reverse
OCN	ATTGTGACGAGCTAGCGGAC	GCAACACATGCCCTAAACGG
RUNX-2	ACCAGCAGCACTCCATATCTCTAC	CTTCCATCAGCGTCAACACCATC
COL1-α1	TGTTGGTCCTGCTGGCAAGAATC	GTCACCTTGTTCGCCTGTCTCAC
BSP	AGCGGAGGAGACAACGGAGAAG	GTCGTGGTGCCATAACTGGTCAG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCC

Effect of GB-256/512 treatments on Cell Behavior

To determine the effects of the previous contamination by polymicrobial biofilms and the GB-256/512 treatments, intact clean titanium plates that had not been previously incubated with bacteria but had been treated with GB-256 (group IGB-256) and GB-512 (group IGB-512) were used for the comparison with GB-256 and GB-512. According to the methods described above, BMSCs were seeded onto titanium plates in groups GB-256, GB-512, IGB-256 and IGB-512, and then the cell proliferation of BMSCs in each group was evaluated by a CCK-8 assays. The osteogenic differentiation of BMSCs in each group was analyzed by ALP staining, Alizarin red S staining and RT-PCR.

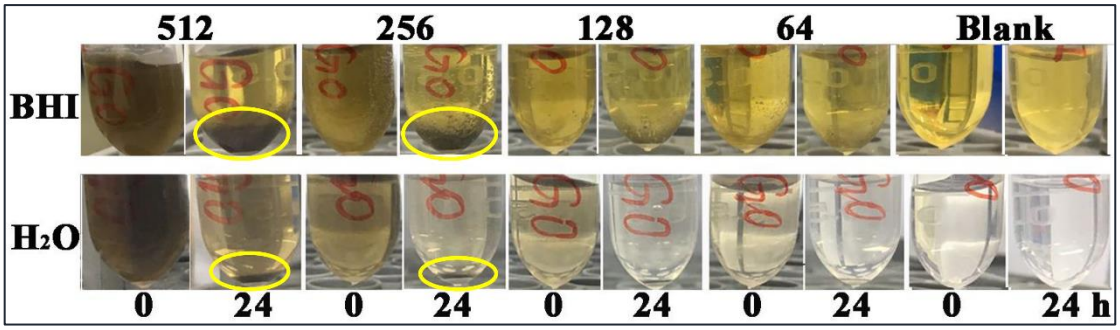
Statistical Analysis

Data are presented as the mean ± standard deviation, and values were compared. Differences between two mean values were calculated using the 2-tailed paired Student's t-test. One-way analysis of variance with Student's test was employed to analyze significant differences between groups. Differences at $P < 0.05$ were considered statistically significant.

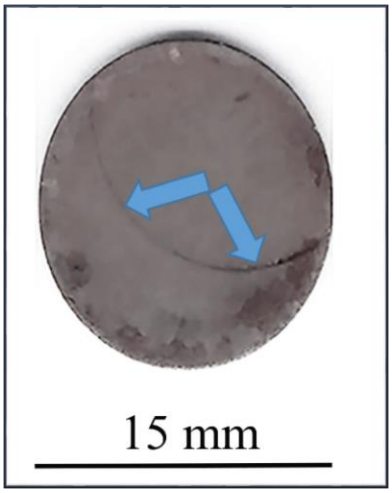
Appendix Table 3. Sequence results of pathogenic bacteria in the polymicrobial biofilm by cloning and Sanger sequencing.

Sequence results	Corresponding bacterial strain
TGGTTACCTTGTTACGACTTAGCCCCAGTCACCGGTATTACCCTAGTGCGCCCCTTGCGGTTACGCCCTTCAGGTACCCCCGACTCCCATGGCTT GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGCCATGGCTGATGCGCGATTACTAGCGAATCCAGCTTCACGGAGTCGAGTTGC AGACTCCGATCCGAACTGGGGAAGGGTTTAGAGATTCGCATCCGGTCGCCCGGTAGCTGCCCTTTGTCCCTCCCATTGTAACACGTGTGTCGC CCCGGATGTAAGGGCCGTGCTGATTTGACGTCATCCACACCTTCCTCGCGCCTTACGACGGCAGTCTCGGTAGAGTCCTCAGCGAAAACTGTT AGCAACTACCGATGTGGGTTGCGCTCGTTATGGCACTTAAGCCGACACCTCACGGCACGAGCTGACGACAACCATGCAGCACCTACATAGACG CCCCGAAGGGAAGACGGTTTTCACCATCCGTCATCTACATTTCAATCCCGGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGTTCCTCC GCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCACCGTTGCCGGCGTACTCCCCAGGTGGATTACTTAACGCTTTCGCTGTGGAAGCTTGAC GGTATATCGCAAACTCCTAGTAATCATCGTTTACTGCGTGGACTACCAGGGTATCTAATCCTGTTTGATACCCACGCCTTCGTGCTTCAGTGTCA GTCGCAGTATGGCAAGCTGCCTTCGCAATCGGAGTTCCTCGTGATATCTATGCATTTCACCGCTACACCACGAATTCCGCCTGCCGCCACTGAA CTCAAGCCCGGCAGTTTCAACGGCAGGCTGAACGTTGAGCGCTCAGGTTTCACCGCTGACTTACCGAACAACCTACGCACCCTTTAAACCCA ATAAATCCGGATAACGCTCGCATCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTTATTCTTACGGTACATTCAATGCAATACT CGTATCGCCCGTTATTCCCGTATAAAAGAAGTTTACAATCCTTAGGACTGTCTTCCTTCACGCGACTTGGCTGGTTCAGGCTCTCGCCCATTGAC CAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCTCAGTACCAGTGTGGGGGATAAACCTCTCAGTTCCCCTACCCATCGTCGC CTTGGTGAGCCGTTACCTCACCAACCAGCTAATGGGACGCATGCCTATCTTACAGCTATAAATATTTCCTTGTAATATCATGCAATAATACAAGTG TATGCGGTTTTAGTCCGTCTTTCAACGGGTTATCCCCCTCTGTAAGGCAAGTTGCATACGCGTTACGCACCCGTGCGCCGGTCGCCATCAACCTT AGCAAGCTAAGATCATGCTGCCCCTCGACTTGCATGTGTTAAGCCTATCGCTAGCGTTCATCCTGAGCCATGATCAAACTCTA	*P. gingivalis*
TAGAGTTTGATCCTGGCTCAGGATGAACGCTGACAGAATGCTTAACACATGCAAGTCAACTTGAACTTCGGTTTGGGTGGCGGACGGGTGAGT AACGCGTAAAGAACTTACCTCACAGATAGGGACAACATTTGGAAACGAATGCTAATACCTAATATTATGATTTTAGGGCATCCTAGGATTATGAA AGCTATATGCGCTGTGAGAGAGCTTTGCGTCCCATTAGCTAGTTGGAGAGGTAACGGCTCACCAAGGCGATGATGGGTAGCCGGCCTGAGAGG GTGATCGGCCACAAGGGGACTGAGACACGGCCCTTACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGACCAAGAGTCTGATCCA GCAATTCTGTGTGCACGATGAAGTTTTTCGGAATGTAAAGTGCTTTCAGTTGGGAAGAAAAAAATGACGGTACCAACAGAAGAAGTGACGGC TAAATACGTGCCAGCAGCCGCGGTAATACGTATGTCACAAGCGTTATCCGGATTTATTGGGCGTAAAGCGCGTCTAGGTGGTTATGTAAGTCTGA TGTGAAAATGCAGGGCTCAACTCTGTGTTGCGTTGGAAACTGTGTAACTAGAGTACTGGAGAGGTAAGCGGAACTACAAGTGTAGAGGTGAA ATTCGTAGATATTTGTAGGAATGCCGATGGGGAAGCCAGCTTACTGGACAGATACTGACGCTGAAGCGCGAAAGCGTGGGTAGCAAACAGGAT	*F. nucleatum*

TAGATACCCTGGTAGTCCACGCCGTAAACGATGATTACTAGGTGTTGGGGGTCGAACCCCAGCGCCCAAGCAAACGCGATAAGTAATCCGCCT GGGGAGTACGTACGCAAGTATGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAG GAACCTTACCAGCGTTTGACATCTTAGGAATGAGACAGAGATGTTTCAGTGTCCCTTCGGGGAAACCTAAAGACAGGTGGTGCATGGCTGTCG TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTTCGTATGTTACCATCATTAAGTTGGGGACTCATGCGATACT GCCTGCGATGAGCAGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATACGCTGGGCTACACACGTGCTACAATGGGTAGTACAGA GAGTCGCAAAGCCGTGAGGTGGAGCTAATCTCAGAAAACTATTCTTAGTTCGGATTGTACTCTGCAACTCGAGTACATGAAGTTGGAATCGCT AGTAATCGCGAATCAGCAATGTCGCGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTGGTTGCACCTGAAGTA GCAGGCCTAACCGTAAGGAGGGATGCTCCGAGGGTGTGATTAGCGATTGGGGTGAAGTCGTAACAAGGTAACCA	
TAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTGGGACGCAAGGAAACACACTGTGCTTGCACACCGTG TTTTCTTGAGTCGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTATTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAATATT AATTATTGCATGATAATTGATTGAAAGATGCAAGCGCATCACTAGTAGATGGACCTGCGTTGTATTAGCTAGTTGGTAAGGTAAGAGCTTACCAA GGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT CTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGTGT GTGAGAGTGGAAAGTTCACACAGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG CGTTGTCCGGATTTATTGGGCGTAAAGGGAGCGCAGGCGGTCAGGAAAGTCTGGAGTAAAAGGCTATGGCTCAACCATAGTGTGCTCTGGAA ACTGTCTGACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAAGCG GCTCTCTGGTCTGTCACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC TAGGTGTTAGGCCCTTTCCGGGGCTTAGTGCCGGAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGG AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGATGCTATTC TTAGAGATAGGAAGTTACTTCGGTACATCGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC GAGCGCAACCCTTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAA ATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGTCGGTACAACGAGTTGCGAGCCGGTGACGGCAAGCTAATCTCTGAAAG CCGATCTCAGTTCGGATTGGAGGCTGCAACTCGCCTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCC CGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGGGCCAGCCGCCTAAGGTGG GATGGATGATTGGGGTGAAGTCGTAACAAGGTAACCA	*S. mutans*



Appendix Figure 3. The dispersibility of GO nanosheets (64 to 512 μg/mL) in BHI and H_2O at 0 and 24 h.



Appendix Figure 4. A deposition line of GO nanosheets on the surface of a titanium plate after 24 h coincubation with bacteria/biofilm in BHI.

Appendix References

Darabi S, Tiraihi T, Ruintan A, Abbaszadeh HA, Delshad A, Taheri T. 2013. Polarized neural stem cells derived from adult bone marrow stromal cells develop a rosette-like structure. In Vitro Cellular & Developmental Biology Animal. 49(8):638-652.

Martinez Paino IM, Santos F, Zucolotto V. 2017. Biocompatibility and toxicology effects of graphene oxide in cancer, normal, and primary immune cells. Journal of Biomedical Materials Research Part A. 105(3):728-736.