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博 士 論 文

The evaluation of dentin-coating ability and photothermal antibacterial effect of graphene oxide
(象牙質の酸化グラフェンコーティングと近赤外線光による光熱抗菌効果)

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Abstract

Graphene oxide (GO) is a monolayer sheet of carbon with a thickness of 1 nm or less that has high hydrophilicity and dispersibility due to the presence of oxygen functional groups on its surface. Recent studies have revealed that GO exerts antibacterial properties, absorbs near-infrared (NIR) irradiation and generates heat. In this study, we assessed whether GO possessed the ability to cover the dentin. In addition, we investigated the photothermal and antibacterial effects of the GO film on *Streptococcus mutans*.

The dentin block was prepared using extracted human molars. The dentin block was immersed in GO dispersion (concentration: 0, 1 and 10 μg/mL). GO-coated dentin blocks were observed using scanning electron microscopy (SEM) and characterized using the dentinal tubule covering score. To assess the stability of the GO film, residual GO on the dentin block was observed after ultrasonic cleaning. Subsequently, the temperature increase of the GO-coated dentin surface following NIR irradiation was examined by thermography. Furthermore, antibacterial effects of the combination of GO film and NIR irradiation against *S. mutans* were evaluated by SEM observation, turbidity measurement, colony formation assessment and live/dead staining.

A thin GO film with a thickness of a few nanometers was successfully formed on

the dentin surface by immersion in GO solution. The dentinal tubule covering score increased in a GO concentration-dependent manner. Even after ultrasonic cleaning, GO residue was frequently observed on the dentin surface. When the GO-coated dentin block was irradiated with NIR light, the temperature of the dentin block surface increased in a GO concentration- and time-dependent manner. However, the temperature decreased as irradiation distance increased. Combination of GO application and NIR irradiation could heat the dentin surface to 58.5 ℃ after 30 seconds of irradiation. In antibacterial assessments, turbidity and colony formation were suppressed by GO and NIR irradiation. In addition, dead bacteria were detected by live/dead staining.

A stable GO film was successfully formed on the dentin surface by immersion in GO dispersion. Photothermal and antibacterial effects were remarkably exhibited by GO and NIR irradiation. The GO-NIR system would be beneficial for photothermal antibacterial therapy.

Key Words: antibacterial property, graphene oxide, near-infrared irradiation, photothermal therapy, *Streptococcus mutans*

1. Introduction

Graphene oxide (GO) exhibits a monolayer sheet of carbon with a thickness of 1 nm or less, which partly contains sp3 hybridized carbon atoms and oxygen functional groups, such as epoxy, carboxy and hydroxy groups. The presence of oxygen functional groups causes high hydrophilicity and dispersibility of GO. Recently, investigators have reported the antimicrobial properties of GO. Krishnamoorthy et al. showed that GO exerted antibacterial activity against *Pseudomonas aeruginosa* and *Streptococcus iniae*[1)]. Gurunathan et al. demonstrated that GO and reduced GO dose-dependently inhibited the growth of *P. aeruginosa* and suggested that oxidative stress of reactive oxygen species (ROS) attacked bacterial cells[2)]. Hu et al. reported that oxidized graphene sheets showed antibacterial activities, however, the cytotoxicity of GO sheets was slight[3)]. Hence, GO may be relevant for the treatment of infectious diseases in various therapeutic fields.

Photothermal therapy (PTT) comprises light irradiation of a photosensitizer, which subsequently damages bacterial cells or cancer cells by heat generation via photoexcitation[4, 5)]. PTT has been focused on in the field of drug delivery to locally provide biological substances to targets[6, 7)]. Various photosensitizers, such as gold nanoparticles[8, 9)] and nanocarbons[10, 11)], have been widely investigated. In particular, GO may be a strong candidate for PTT. Robinson et al. reported that GO could generate

fever after irradiation of near-infrared (NIR) light and successfully kill cancer cells[12]. In addition, chemo-photothermal therapy using GO loading of anticancer drugs was investigated[13]. Since long wavelengths showed low phototoxicity compared with short wavelengths such as UV light[14], we anticipate the application of NIR light is suitable for PTT using GO. We speculate that GO should adhere to the tooth surface and exhibit photothermal effects via NIR light irradiation. PTT using GO would likely exert strong bactericidal action against cariogenic bacterial cells. However, these hypotheses have not been investigated thus far. Accordingly, in this study, we evaluated whether GO films stably coated the surface of root dentin. In addition, we assessed the antibacterial effects of GO film irradiated by NIR light against an oral bacterium, *Streptococcus mutans.*

2. Material and Methods

2.1. Preparation of dentin blocks

Dentin blocks were prepared from extracted vital third molars of patients (20–40 years of age) in Hokkaido University Hospital. The use of human teeth in this study was approved by the Institutional Review Board of Hokkaido University Hospital for Clinical Research (approval no. 12-46). The tooth root was cut with a sterilized

diamond disc (87xFSI, Horico, Berlin, Germany) and sandpaper (#240 and #600) to prepare dentin blocks of 5 mm×5 mm size with 1-mm thickness. After ultrasonic cleaning twice with distilled water (DW) for 5 minutes, dentin blocks were sonicated with 3% ethylenediaminetetraacetic acid (EDTA) (SMEARCLEAN, Nippon Shika Yakuhin Co., Ltd., Shimonoseki, Japan) for 60 seconds. After ultrasonic cleaning with DW, dentin blocks were obtained for evaluation.

2.2. Fabrication and evaluation of GO film on dentin blocks

GO dispersion (nanoGRAX®, Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan) was produced by oxidation and chemical separation of graphite (Hummers-Offeman method[15]) (Figure 1A). Each dentin block was immersed in diluted GO dispersion (concentration: 0, 1 and 10 μg/mL) for 30 seconds and then immersed in DW for 5 minutes. To investigate GO film fabrication, some GO-coated dentin blocks were washed with ultrasonic waves (NS-100, Nippon Rikagaku Kikai Co., Ltd, Tokyo, Japan) for 20 seconds. Subsequently, dentin blocks were dehydrated in a graded ethanol series and then dried overnight. Dentin blocks were then coated with Pt-Pd and observed by scanning electron microscopy (SEM; S-4000, Hitachi Ltd., Tokyo, Japan) at 10 kV. Three area units (90 μm×120 μm) of SEM images were selected and then the ability of

the GO film to cover dentinal tubules was scored from 1 to 4 as described previously with some modification[16-18] (Table 1).

2.3. Assessment of surface temperature of dentin blocks after NIR irradiation

NIR irradiation of GO-coated dentin blocks was carried out using an NIR irradiation device (wavelength 800–1000 nm; LA-100 IR, Hayashi Watch-Works Co., Ltd., Tokyo, Japan). The irradiation distance was set to 1, 2 and 3 cm, and the irradiation time was set to 0, 10, 20 and 30 seconds. After a 5-second pause, the surface temperature was measured with thermography (FLIRix, FLIR Systems, Inc., Wilsonville, OR, USA). In addition, the increase in temperature (irradiation distance: 1 cm, irradiation time: 10 seconds) was repeatedly measured.

2.4. Antibacterial effects of GO film and NIR irradiation

A suspension of facultative anaerobic bacteria, *S. mutans* ATCC 35668, was cultured and kept frozen until analysis. Bacterial stocks were incubated in brain heart infusion broth (BHI; Pearlcore®, Eiken Chemical, Co., Ltd., Tokyo, Japan) supplemented with 0.1% antibiotic (gramicidin D and bacitracin, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1% sucrose (Wako Pure Chemical Industries, Ltd.).

GO-coated dentin blocks were placed into microplates and then the suspension of *S. mutans* (final concentration: 1.5×10^6 colony-forming units (CFU)/mL) was dispensed. After anaerobic incubation at 37 °C for 5 hours, dentin blocks were irradiated by NIR light for 30 seconds. Control specimens received no irradiation. Dentin blocks were then cultured again for 5 hours and the surface of the dentin blocks was observed by SEM. Some specimens were cultured for 24 hours after NIR irradiation to measure turbidity using a turbidimeter (CO7500 Colourwave, Funakoshi Co., Ltd, Tokyo, Japan) at 590 nm. In addition, *S. mutans* suspensions cultured for 12 hours after NIR irradiation were diluted 10-fold in fresh BHI broth, spread onto BHI agar plates (Eiken Chemical Co., Ltd.), and incubated at 37 °C for 48 hours to determine *S. mutans* colony counts.

Dentin blocks not incubated or incubated for 5 hours after NIR irradiation (10 or 30 seconds) were stained by the Live/Dead® BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Dentin blocks were observed using confocal laser scanning microscopy (Biorevo BZ-9000, Keyence Co., Osaka, Japan). Dead bacteria (red) and live bacteria (green) were then counted in three area units (40 μm×50 μm per unit) and the percentage of dead bacteria was calculated.

2.5. Statistical analysis

Statistical analysis was performed by Scheffe's test. P-values <0.05 were considered statistically significant. All statistical procedures were performed using a software package (Statistical Package for the Social Sciences 11.0, IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Evaluation of GO film fabrication on dentin blocks

The color of the dentin blocks did not change after GO application (Figure 1B and 1C). In the SEM observation, dentin block surfaces showed open dentinal tubules after EDTA treatment (Figure 2A). After immersion in GO solution, a thin wrinkled film of nanoscale thickness was observed on the dentin surface that sealed dentinal tubules (Figure 2B–2D). The mean dentinal tubule covering score was 1.02, 3.02 and 3.78 after GO coating using 0 (control), 1 and 10 μg/mL GO solution, respectively (Table 2). GO coating increased the covering score, and the covering ability of 10 μg/mL GO was significantly greater than that of 1 μg/mL GO (p<0.05). After ultrasonic cleaning, a part of the GO film disappeared and open dentinal tubules were frequently observed (Figure 2F and 2G). However, residual GO film was observed on the

intertubular dentin. After ultrasonic washing, the mean dentinal tubule covering score was 1.75 and 2.10 for 1 and 10 μg/mL GO, respectively (Table 2). High concentrations of GO possessed greater coating capability compared to low concentrations of GO.

3.2. Assessment of the temperature of the dentin surface after NIR irradiation

The temperature of the GO-coated dentin surface increased after NIR irradiation. The dentin surface temperature increased in a GO concentration- and time-dependent manner. In addition, NIR irradiation decreased surface temperature in distance-dependent manner. NIR irradiation at a distance of 1 cm significantly increased the surface temperature of GO-coated dentin blocks compared with untreated dentin blocks (p<0.05). In contrast, irradiation at a distance of 2 and 3 cm tended to decrease the degree of temperature rise. For NIR irradiation of 30 seconds at a distance of 1 cm, the surface temperature was 39.9, 47.1 and 58.5 °C on 0 (control), 1 and 10 μg/mL GO-coated dentin blocks, respectively. The temperature of 10 μg/mL GO-coated dentin blocks was about 1.5-fold higher than that of untreated dentin blocks (Figure 3A).

We next confirmed the stability of GO film after repeated NIR irradiation of GO-coated dentin blocks. Three rounds of repetitive NIR irradiation every 10 seconds consistently heated the GO-coated (10 μg/mL) dentin surface to about 50 °C (data not

shown). SEM images show GO films exhibited no morphological change. Furthermore, dentinal tubules were completely sealed by GO film even after repetitive NIR irradiation (Figure 3B and 3C).

3.3. Antibacterial evaluation of GO film and NIR irradiation

In SEM observation, bacterial aggregation on dentin block surfaces was remarkable in control specimens receiving no GO or NIR irradiation (Figure 4A). In contrast, slight *S. mutans* growth appeared on GO-coated dentin blocks (Figure 4B and 4C). Furthermore, GO and NIR light application strongly suppressed bacterial growth and colonization (Figure 4C).

Assessment of *S. mutans* turbidity and colony count was carried out to determine the antibacterial capability of GO film and NIR irradiation. The turbidity of 10 μg/mL GO-coated dentin samples was significantly suppressed compared with untreated dentin samples ($p<0.05$). NIR irradiation clearly reduced *S. mutans* turbidity of GO-coated dentin samples. In 1 and 10 μg/mL GO-coated dentin samples, turbidities were decreased by about 0.47- and 0.10-fold that of control, respectively (Figure 4D). The number of *S. mutans* colonies of 10 μg/mL GO-coated dentin samples was decreased compared with that of untreated dentin samples. In addition, the combination of GO and

NIR irradiation strongly inhibited the formation of bacterial colonies (Figure 4E).

To assess the bactericidal effects of GO film and NIR irradiation, live/dead staining of *S. mutans* was carried out. GO coating and NIR irradiation increased the number of dead bacteria after 0 and 5 hours of incubation (Figures 5A–5I and 6A–6I). The percentage of dead bacteria was approximately 80% and 100% on 1 and 10 μg/mL GO-coated dentin blocks incubated for 5 hours, respectively. In the absence of NIR irradiation, the percentage of dead bacteria on both 1 and 10 μg/mL GO-coated dentin blocks was only about 20% (Figure 6J).

4. Discussion

SEM observation of GO-coated dentin revealed that the wrinkled film structure sealed dentinal tubules on dentin block surfaces. The cross-sectional SEM image of GO-coated dentin revealed a very thin film on the dentin. In contrast, control specimens showed that dentinal tubules were fully opened. Thus, dispersed GO easily aggregated to form thin GO films on the dentin, consequently sealing dentinal tubules. Regarding the concentration of the GO dispersion, a high concentration of GO (10 μg/mL) remarkably covered the dentin surface compared with a low concentration of GO (1 μg/mL), suggesting that GO coating covered dentinal tubules in a

concentration-dependent manner. Ultrasonic cleaning of GO-coated dentin resulted in partial removal of the GO film to open dentinal tubules. However, as compared with the control, GO-coated dentin showed a high covering ratio of dentinal tubules, and GO film strongly remained on the intertubular dentin after ultrasonic cleaning. Therefore, GO film can stably cover the surface of root dentin. Some investigators showed that GO possesses protein absorption capability[19, 20]. GO has a large amount of hydrophilic oxygen functional groups on its surface because of oxidation processes[21]. GO-dentin binding force may be enhanced by covalent bonding between epoxy groups of GO and dentin protein exposed by EDTA treatment[22]. Due to these attractive forces, GO film would readily form on the dentin surface by only the immersion process.

The temperature of GO-coated dentin surfaces time-dependently increased via NIR light irradiation, however, dentin without GO coating exhibited no temperature rise after NIR irradiation. It was suggested that both application of GO film and NIR irradiation increased the surface temperature of dentin. Robinson et al. detected the absorption spectrum of GO and revealed the NIR absorption ability of GO[12]. Li et al. showed that the temperature of a solution containing GO was increased by NIR irradiation[23]. Taken together, GO film formed on dentin possesses NIR absorption ability and releases thermal energy to generate heat after NIR irradiation. In the present

study, the morphology of GO film remained unchanged after repetitive (3 rounds) NIR irradiation (Figure 3B and 3C). It is considered that the GO film stably covered the dentin even by heat generation.

Live/dead staining showed that dead bacteria were significantly observed on GO-coated dentin that received subsequent NIR irradiation. In addition, *S. mutans* turbidity and colony formation were significantly reduced by GO and NIR irradiation. It was suggested that the GO-NIR system provided thermal damage to *S. mutans* and consequently facilitated bacterial sterilization. Ma et al. demonstrated that the growth of mesophilic bacteria, such as *S. mutans*, occurs at 30–47 °C[24]. In the present study, the surface temperature of GO-coated dentin via NIR irradiation was higher than 50 °C. Therefore, *S. mutans* would likely receive profound damage, inhibiting bacterial proliferation. The GO-NIR system conclusively exhibited great antibacterial effects against *S. mutans*.

Although NIR irradiation was not performed, *S. mutans* was sparsely observed on dentin blocks immersed in 10 μg/mL GO dispersion compared with biofilms grown on control specimens in SEM images. In addition, GO application with no irradiation reduced *S. mutans* turbidity and colony formation compared with the control. These findings suggested that GO-coated dentin independently exhibited antibacterial effects.

The antimicrobial properties of GO against various bacteria, such as *P. aeruginosa*, *Escherichia coli* and *S. iniae*, have been investigated[1, 2]. Some investigators concluded that the antimicrobial properties of GO were exerted by ROS production, which caused bacterial cell damage[2]. It was considered that GO should gradually and continuously produce ROS because of GO reduction and release the oxygen functional groups by temporal degradation. Accordingly, GO films on the tooth surface would exert a long-term antibacterial effect.

5. Conclusions

We investigated the dentin-coating ability of GO and the photothermal antibacterial effects of GO via NIR irradiation against an oral bacterium, *S. mutans*. The stable, thin GO film formed on the dentin surface to cover dentinal tubules in a concentration-dependent manner. Furthermore, the combination of GO film and NIR irradiation remarkably exhibited photothermal and bactericidal effects. The GO-NIR system is anticipated to be an effective photothermal antibacterial therapy against oral infections.

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lactic-acid bacteria. Antonie Van Leeuwenhoek 72 : 91-100, 1997.

Table 1 Scoring system to evaluate the coatablity of GO film

Score	Definition
1	Dentinal tubules were clearly opened.
2	Less than 50% of dentinal tubules were closed.
3	More than 50% of dentinal tubules were closed.
4	Dentinal tubules were completely closed.

Table 2 Mean dentinal tubule covering scores (n=4)

	0 μg/mL GO (control)	**1 μg/mL GO**	**1 μg/mL GO (after UC)**	**10 μg/mL GO**	**10 μg/mL GO (after UC)**
Mean score	1.02[b]	3.02 [ab]	1.76 [ab]	3.78 [a]	2.10 [ab]

[a] p<0.05, statistically significant difference compared with 0 μg/mL GO (control).

[b] p<0.05, statistically significant difference compared with 10 μg/mL GO.

GO, graphene oxide; UC, ultrasonic cleaning.

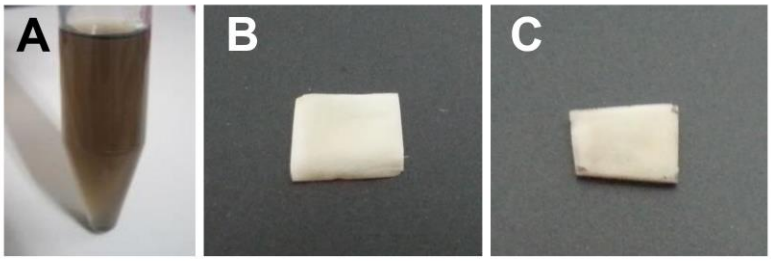

Fig. 1

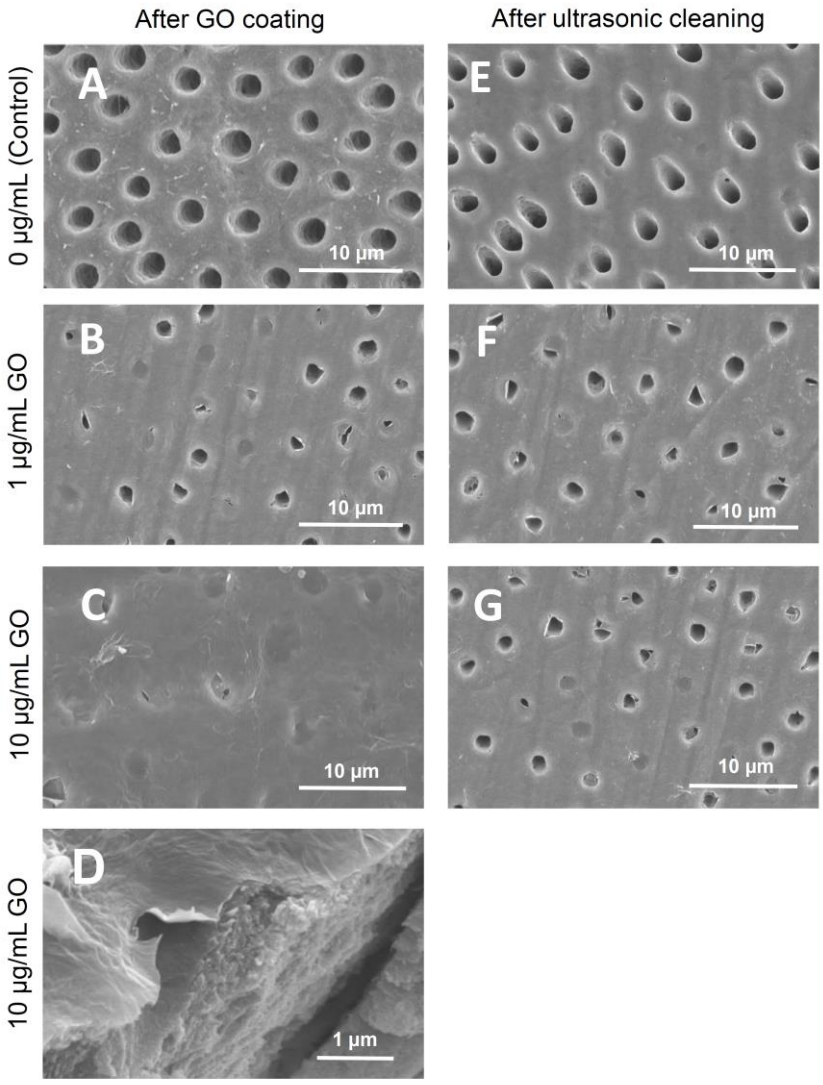
After GO coating
After ultrasonic cleaning
0 µg/mL (Control)
1 µg/mL GO
10 µg/mL GO
10 µg/mL GO
A
B
C
D
E
F
G
10 µm
10 µm
10 µm
1 µm
10 µm
10 µm
10 µm

Fig. 2

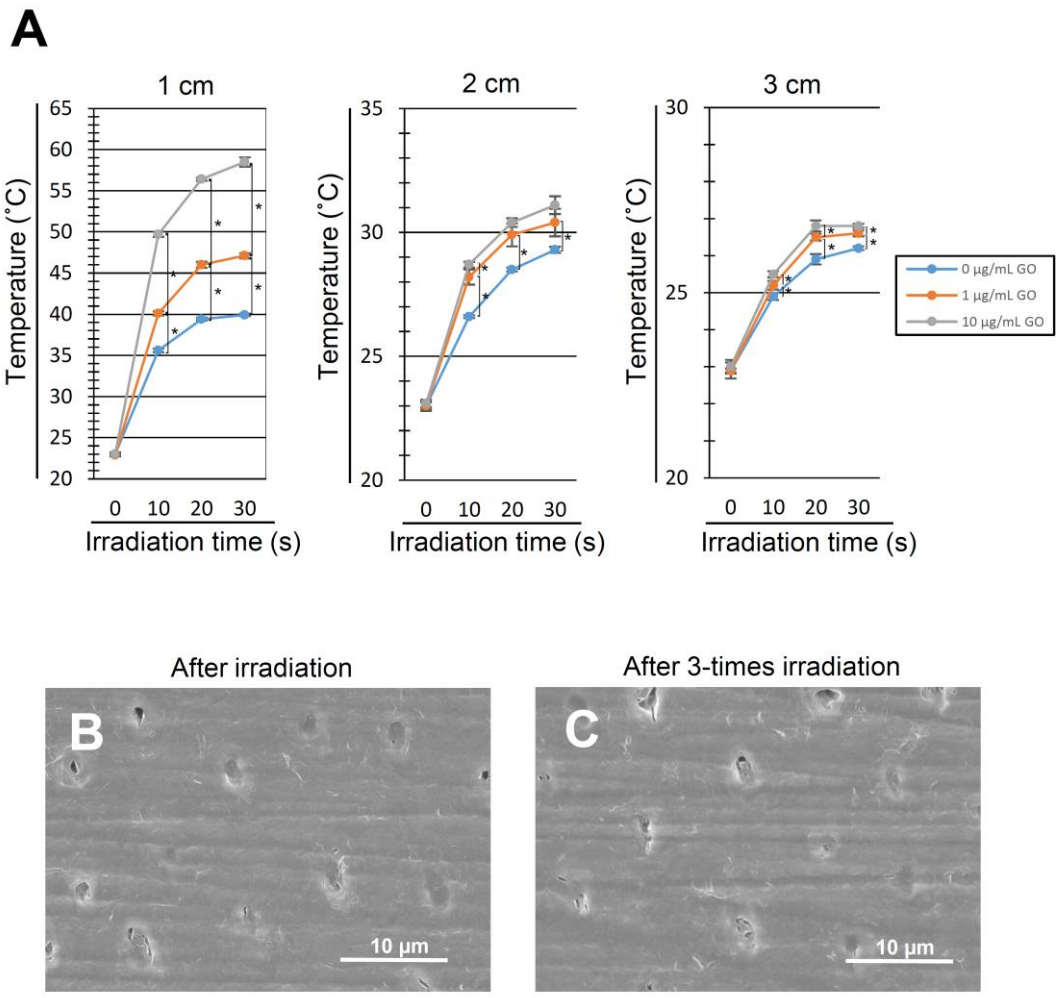
A
1 cm
2 cm
3 cm
Temperature (°C)
Irradiation time (s)
0 µg/mL GO
1 µg/mL GO
10 µg/mL GO
After irradiation
After 3-times irradiation
B
C
10 µm
10 µm

Fig. 3

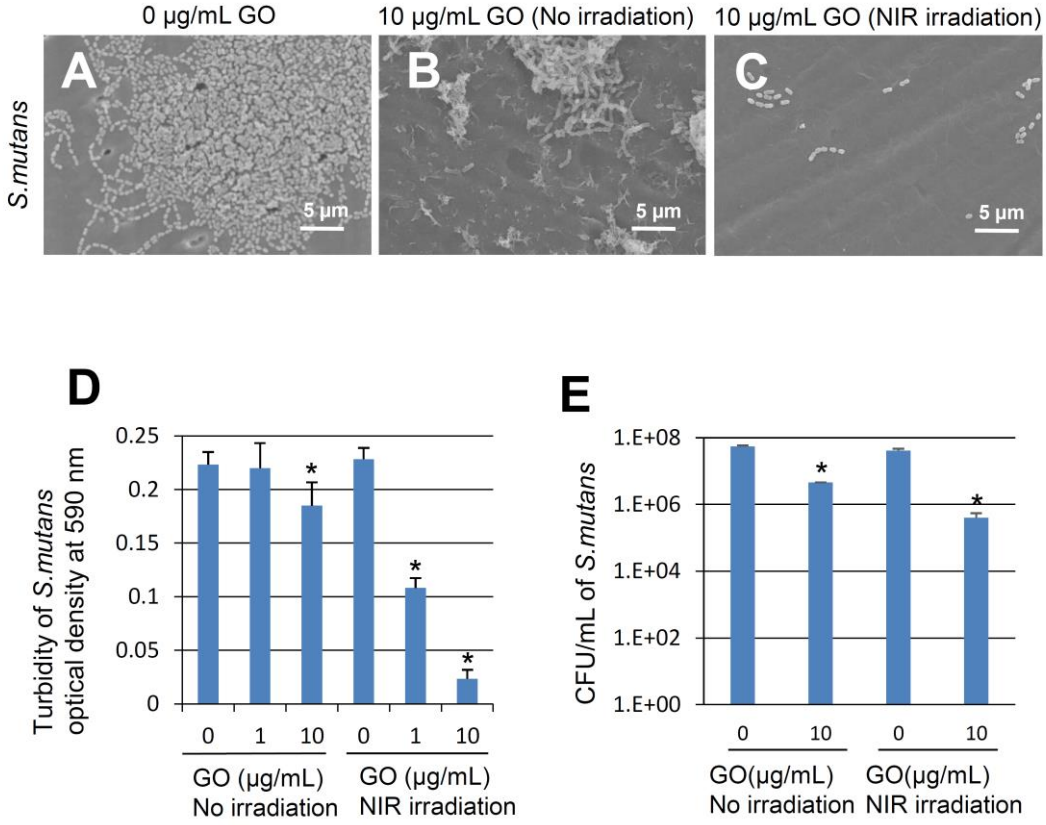
0 μg/mL GO
10 μg/mL GO (No irradiation)
10 μg/mL GO (NIR irradiation)
S.mutans
A
B
C
5 μm
5 μm
5 μm
D
Turbidity of S.mutans optical density at 590 nm
0.25
0.2
0.15
0.1
0.05
0
0 1 10 0 1 10
GO (μg/mL) No irradiation
GO (μg/mL) NIR irradiation
E
CFU/mL of S.mutans
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
0 10 0 10
GO(μg/mL) No irradiation
GO(μg/mL) NIR irradiation

Fig. 4

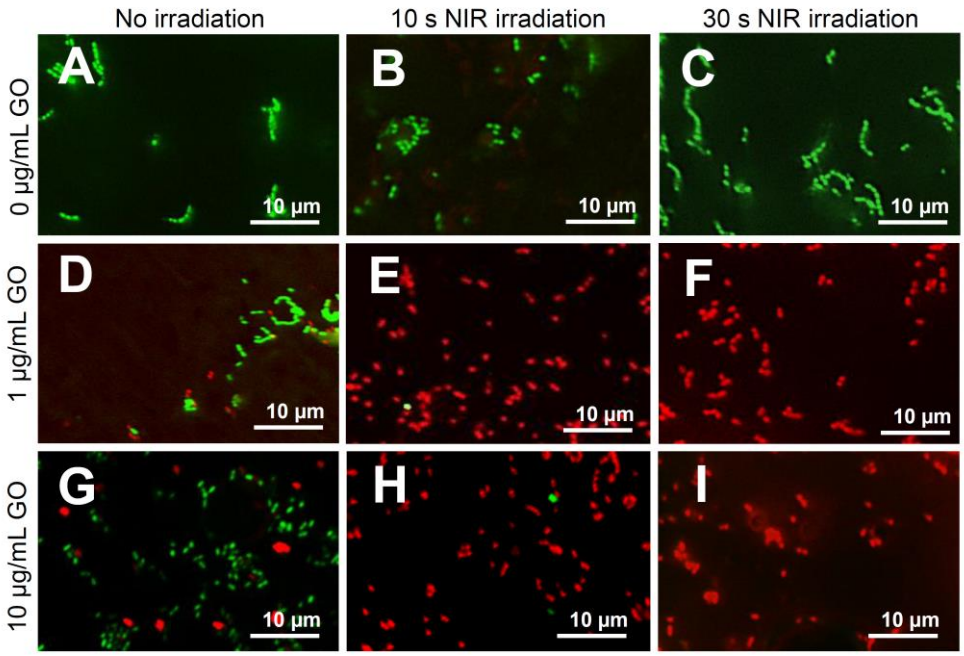
No irradiation
10 s NIR irradiation
30 s NIR irradiation
0 μg/mL GO
1 μg/mL GO
10 μg/mL GO
A
B
C
D
E
F
G
H
I
10 μm

Fig. 5

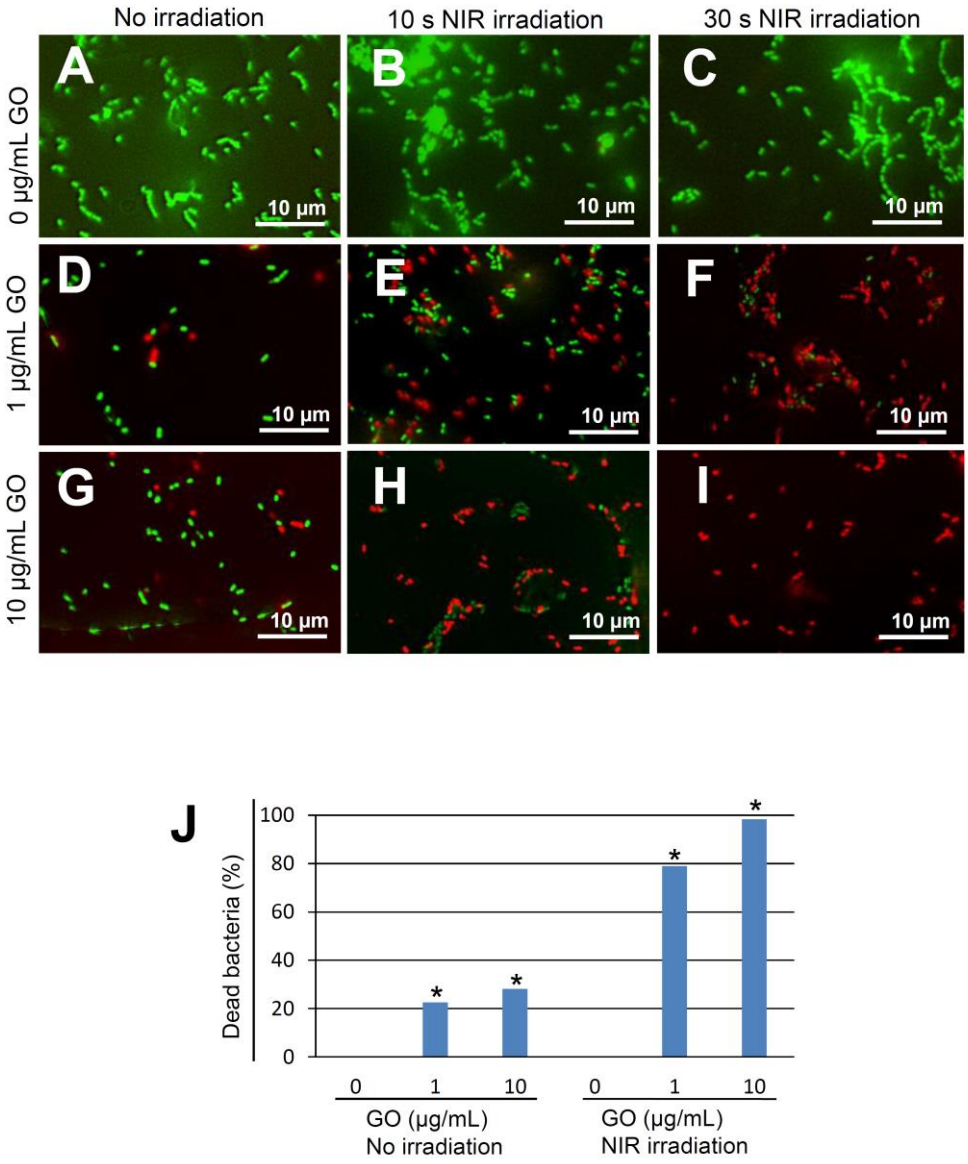
No irradiation
10 s NIR irradiation
30 s NIR irradiation
0 µg/mL GO
1 µg/mL GO
10 µg/mL GO
A
B
C
D
E
F
G
H
I
10 µm
J
Dead bacteria (%)
100
80
60
40
20
0
0
1
10
GO (µg/mL)
No irradiation
GO (µg/mL)
NIR irradiation
*

Fig. 6

Figure legends

Fig. 1.

Digital photographs. (A) GO dispersion (10 μg/mL). (B) Untreated dentin block. (C) GO-coated dentin block (10 μg/mL GO).

Fig. 2.

SEM images of the dentin block surface. (A–C) Images of the dentin block surface after immersion in 0, 1 and 10 μg/mL GO dispersion, respectively. (D) Cross-sectional image of a dentin block coated in 10 μg/mL GO. (E–G) Images of GO-coated dentin blocks after ultrasonic cleaning. GO, graphene oxide.

Fig. 3.

Assessment of the temperature of the dentin surface after NIR irradiation. (A) Measurement of the temperature of the GO-coated dentin surface (n=4, mean ± SD). *p<0.05. (B) SEM image of a GO-coated dentin block after one and three rounds of NIR irradiation. GO, graphene oxide.

Fig. 4.

Antibacterial evaluation of GO film and NIR irradiation. (A–C) SEM images of the GO-coated dentin block surface after *S. mutans* seeding and NIR irradiation. (A) 0 μg/mL GO. (B) 10 μg/mL GO and no irradiation. (C) 10 μg/mL GO and NIR irradiation. (D) Turbidity of *S. mutans* (n=6, mean ± SD). *p<0.05, vs 0 μg/mL GO. (E) CFU/mL of *S. mutans* (n=2, mean ± SD). *p<0.05, vs 0 μg/mL GO (No irradiation). GO, graphene oxide.

Fig. 5.

Live/dead BacLight staining of *S. mutans* after NIR irradiation. (A–C) 0 μg/mL GO. (D–F) 1 μg/mL GO. (G–I) 10 μg/mL GO. GO, graphene oxide.

Fig. 6.

Live/dead BacLight staining of *S. mutans* after NIR irradiation and 5 hours of subsequent culture. (A–C) 0 μg/mL GO. (D–F) 1 μg/mL GO. (G–I) 10 μg/mL GO. (J) Percentage of dead bacteria (n=3). *p<0.05, vs 0 μg/mL GO. GO, graphene oxide.