博 士 論 文

Antibacterial coating of human dentin surface with surface pre-reacted glassionomer (S-PRG) nanofillers
(surface pre-reacted glassionomer (S-PRG) ナノフィラーによるヒト象牙質表面の抗菌コーティング)

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Title

Antibacterial coating of human dentin surface with surface pre-reacted glassionomer (S-PRG) nanofillers

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Running title

Characterization of S-PRG nanofiller

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Abstract

Surface pre-reacted glass ionomer (S-PRG) filler shows tooth remineralization and antibacterial effects via ion-releasing. In this study, we fabricated the nano- and micro-sized S-PRG fillers and assessed the adhesive properties of S-PRG fillers to the human dentin surface. In addition, the antibacterial effects of dentin coated with S-PRG fillers were examined.

The stock of S-PRG filler (average particle size; 5 μm) was fractionated to obtain the two types of dispersion; nano and micro scale particle types, and then labeled with nano- and micro-S-PRG, respectively. S-PRG fillers were characterized by scanning electron microscope (SEM), energy dispersive X-ray spectrometry (EDX), ion-releasing test and provided to antibacterial and cytotoxic assessments. Subsequently, we assessed the adhesion test of stock, nano- and micro-S-PRG to human dentin blocks. In addition, antibacterial effects of nano-S-PRG coated dentin was examined using *Streptococcus mutans* and *Actinomyces naeslundii*.

The results showed that the nano- and micro-S-PRG showed including of elements of F, Na, Al, Si and Sr, ion releasing ability comparable with the stock, antimicrobial activity and low cytotoxicity. SEM and EDX examinations revealed that S-PRG fillers uniformly covered the dentin surface after application of filler to dentin. After ultrasonically cleaning, nano-S-PRG predominantly remained on the dentin surface compared to stock and micro-S-PRG, suggesting that the nano-S-PRG could adhered to dentin. Nano-S-PRG treated dentin showed the sterilization effect and bacterial growth inhibition.

In conclusion, nano-S-PRG exhibited great adhesive and antimicrobial properties to human

dentin. S-PRG application to tooth would be beneficial for dental antibacterial treatment.

Introduction

The progression of oral infection diseases, such as dental caries and periodontitis, causes the tooth loss and diminishes the human health to decrease the function of mastication. Since antibiotics are slightly effective against infections associated with oral bacterial biofilm attached to tooth surface, the development of the anti-infective biomaterials is requested to attack the biofilm and to obtain the antibacterial tooth surface.

Ikemura et al. reported the effect of the novel ion-releasing filler; surface pre-reacted glass ionomer (S-PRG) filler[1]. fiThe surface of the core glass of the S-PRG filler possess multi-layered structure of thin glass ionomer to be able to slow-release of six types of ions; F^-, Na^+, BO_3^{3-}, Al^{3+}, SiO_3^{2-} and Sr^{2+}[2), 3)]. In particular, boric and fluoride ions are known to exhibit the antibacterial effects. Many investigators have reported that boric ion up-regulates the bacteriostatic effects, that is, suppression of bacterial growth and biofilm formation, against various bacterial strains[4), 5)]. Indeed, boric acid ophthalmic is clinically applied to irrigate the eyes[6)]. Fluoride ion also showed antibacterial properties like boric ion as well as tooth remineralization effects[7)]. Actually, elute of S-PRG fillers significantly inhibited the proliferation and subsequent sugar metabolism of *Streptococcus mutans* (*S. mutans*), major pathogen of human dental caries, and then promoted apatite induction for tooth repair[8), 9)]. Furthermore, S-PRG filler reportedly suppressed the growth of *Candida albicans* (*C. albicans*) related to oral candidiasis[10)]. Hence, we supposed that tooth surface coating with S-PRG fillers exerted to prevent the bacterial colony formation and the subsequent tooth substance degradation via ion-releasing from S-PRG fillers. In addition, dental caries and periodontal disease may be prevented by

S-PRG filler coating.

In general, nano-sized particles tend to be aggregated by attractive force because of its high surface area to volume ratio. We hypothesized that nanoparticulated S-PRG fillers could attach to the tooth surface due to its aggregation. However, the relation of particle size and tooth adhesive capability of S-PRG filler have not been investigated so far. In this study, we fabricated the S-PRG fillers with nano and micro particle size. The effect of particle size of S-PRG filler on adhesion to the human dentin surface was assessed. In addition, the antibacterial properties of dentin coated with S-PRG fillers were examined.

Materials and Methods

Fabrication and characterization of nano- and micro-S-PRG fillers

S-PRG filler (average particle size; 5 μm) prepared by Fujimoto's method was provided by SHOFU Inc. (Kyoto, Japan)[3)]. Glass frit was obtained by melted fluoroboroaluminosilicate glass and others and pulverized to produce irregularly shaped particles. The particles were subjected to a surface treatment with polyacrylic acid to obtain S-PRG fillers. Subsequently, S-PRG filler stock, namely, S-PRG fillers mixed with distilled water (DW), was fractionated by sedimentation procedure. After standing for 16 and 3.5 hours, the supernatant of aqueous dispersion of S-PRG filler was collected to obtain the two types of dispersion with S-PRG fillers; nano and micro scale particle types, and then labeled with nano- and micro-S-PRG, respectively. The particle sizes of stock, nano- and micro-S-PRG were measured by particle size distribution measuring instrument (Microtrac MT 3300 EX II, Microtrac BEL Inc., Osaka, Japan). Each S-PRG filler were characterized by energy dispersive X-ray spectrometry (EDX, JSM-6500F, JEOL Ltd., Tokyo, Japan) and scanning electron microscope (SEM, S-4000, Hitachi Ltd., Tokyo, Japan) with accelerating voltage of 10 kV after coating with a thin layer of Pt-Pd. In addition, the ion-releasing volumes of the elute of stock, nano- and micro-S-PRG were assessed. Fluoride ion was measured using the fluoride ion electrode method (Orion fluoride electrode, model 9609BN; Orion pH/ion meter, model 720A, Thermo Fisher Scientific, Waltham, MA, USA). The volumes of Na, B, Al, Si and Sr ions were measured using an inductively coupled plasma atomic emission spectrometer (ICP-AES, ICPS-8000, Shimadzu Corporation, Kyoto, Japan).

For assessments of antibacterial screening test of nano- and micro-S-PRG, 50 μL of filler

dispersion (1 wt%) were dispensed into 48well microplates. After drying for 24 hours, suspension of *S. mutans* ATCC35668 (final concentration: 5.5×10^6 colony-forming units (CFU)/mL) was seeded and incubated at 37°C for 24 hours with anaerobic condition. The suspension was incubated in brain heart infusion (BHI) broth (Pearlcore®, Eiken Chemical, Co., Ltd., Tokyo, Japan) supplemented with 0.1% antibiotic (gramicidin D and bacitracin, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 1% sucrose (FUJIFILM Wako Pure Chemical Corporation). After incubation for 24 h, the turbidity of each suspension was measured using a turbidimeter (CO7500 Colourwave, Funakoshi Co., Ltd, Tokyo, Japan) at 590 nm. Some samples were diluted 10-fold in fresh BHI broth, spread onto BHI agar plates (Eiken Chemical Co., Ltd, Tokyo, Japan) and incubated at 37°C for 24 hours to carry out the colony counts.

Preparation of dentin blocks

Dentin blocks were prepared from extracted vital third molars of 20-40 years patients 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. 17-222). The tooth root was shaped with a diamond disk (Horico diamond disk 87xFSI, Horico, Berlin, Germany) and sandpaper (# 600 and # 2000) to fabricate the dentin block (5 x 5 x 1 mm). After that, dentin blocks were sonicated with 3% ethylenediaminetetraacetic acid (EDTA, smear clean, Nippon Shika Yakuhin Co., Ltd. Shimonoseki, Japan) for 5 minutes to remove the smear layer. After ultrasonic cleaning with DW, the dentin block was obtained for evaluation.

Evaluation of nano- and micro-S-PRG coating to dentin block

The dentin block was immersed in stock, nano- and micro-S-PRG (1 wt%) for 3 minutes and then washed with PBS for 10 seconds. Filler-applied block was dehydrated with ethanol series in a conventional method, and then dried overnight. After Pt-PD coating, dentin block surface was observed by SEM and characterized by EDX. To evaluate the invasion of S-PRG filler into dentinal tubules, the area units (30 μm×40 μm) of SEM images of dentin block surface were selected and then the score from 1 to 3 was determined as described in previous reports[11, 12]. The average score of each sample was calculated.

Score definition;

1 Dentinal tubules were fully opened.

2 Less than 50% of dentinal tubules were closed by S-PRG fillers.

3 More than 50% of dentinal tubules were closed by S-PRG fillers.

To investigate S-PRG filler adhesion to dentin, S-PRG filler-applied dentin block was washed with DW in ultrasonic waves (45 kHz, VS-100 III, AS ONE Corporation., Ltd, Osaka, Japan) for 1 and 10 seconds. After that, dentin block surface was characterized by SEM and EDX. To evaluate the amount of S-PRG filler remained on the surface of dentin, the area units (5 μm×5 μm) of SEM images were selected and the number of the particle over 200 nm thickness was counted.

As the pseudo-pellicle test, dentin block was immersed in solution of 5% albumin (from Bovine Serum pH5.2, FUJIFILM Wako Pure Chemical Corporation) or 5% lysozyme (from Egg White,

FUJIFILM Wako Pure Chemical Corporation) for 10 sec[13]. After drying, dentin block was immersed in nano- and micro-S-PRG (1 wt%) for 3 minutes, washed with PBS for 10 seconds and observed by SEM. To evaluate the amount of S-PRG filler remained on protein-applied dentin surface, the number of particles over 200 nm thickness in the area units (5 μm×5 μm) of SEM images were counted.

The ions releasing test was performed for nano-S-PRG-applied dentin. Treated dentin block was immersed into DW over 24 hours. Fluoride and other ions containing supernatant were measured using the fluoride ion electrode method and ICP-AES, respectively.

Antibacterial properties of S-PRG filler coated dentin block

Dentin block coated with nano-S-PRG (1 wt%) was placed into 48 well plate. The suspensions of *S. mutans* ATCC35668 (final concentration: 5.5×10^6 colony-forming units (CFU)/mL) and *Actinomyces naeslundii* (*A. naeslundii*) ATCC27039 (final concentration: 1.1×10^8 CFU/mL) were seeded on the nano-S-PRG-applied dentin and cultured for 24 hours in brain heart infusion (BHI) broth (Pearlcore®, Eiken Chemical, Co., Ltd., Tokyo, Japan) supplemented with 0.1% antibiotic (gramicidin D and bacitracin, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 1% sucrose (FUJIFILM Wako Pure Chemical Corporation) for *S. mutans*; and actinomyces broth (BBL™ Actinomyces Broth, Dickinson and Company, NJ, USA) for *A. naeslundii*. After culture periods, dentin block was washed with medium using vibrator to collect the attached bacterial cells. The number of collected bacteria was calculated by colony count.

In addition, the samples incubated with *S. mutans* were observed by SEM and stained by the

LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific). Live bacteria were stained with SYTO9 to produce green fluorescence and bacteria with compromised membranes were stained with propidium iodide to produce red fluorescence. Observation was carried out using fluorescence microscope (Biorezo BZ-9000, Keyence Corp., Osaka, Japan).

Assessment of cytotoxicity of S-PRG fillers

To assess the cytotoxicity of S-PRG filler, 20 μL of nano- and micro-S-PRG were dispensed into 96 well microplates. After drying for 24 hours, 5×10^3 fibroblastic NIH3T3 cells (RIKEN BioResource Center, Tsukuba, Japan) were seeded and incubated at 37°C with 5% CO_2 using culture medium (MEM alpha, GlutaMAX-I, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Qualified FBS, Thermo Fisher Scientific) and 1% antibiotics (Penicillin-Streptomycin, Thermo Fisher Scientific). The cytotoxicity after incubation for 1, 3, 5 and 7 days was determined using the WST-8 assay (Cell Counting Kit-8, Dojindo Laboratories, Mashiki, Japan) and lactate dehydrogenase (LDH) assay (Cytotoxicity LDH Assay Kit-WST, Dojindo Laboratories). The absorbance at 450 nm (WST-8) and 490 nm (LDH) was measured on a microplate reader (ETY-300, Tokyo Sokki, Yokohama, Japan).

Some samples incubated for 24 h were washed with PBS and fixed with 3.5% formaldehyde. The samples were immersed in dissolving phalloidin (1.5 μg, Actistain 555 Fluorescent Phalloidin, Cytoskeleton Inc., Denver, CO, USA) and 4', 6-diamidino-2-phenylindole (2 μg, Dojindo Laboratories) in 500 μL of a bovine serum albumin solution (7.5 w/v%, Albumin Dulbecco's-PBS (-)

Solution from bovine serum, FUJIFILM Wako Pure Chemical Corporation) overnight at 4°C. After washing with PBS, the stained cells were observed using a fluorescence microscope.

Statistical analysis

Statistical analysis was performed by Scheffe's test and Student's *t* test. *P* values < 0.05 were considered statistically significant. All statistical procedures were performed using a software package (SPSS 11.0, IBM Corporation, Armonk, NY, USA)

Results

Characterization of nano- and micro-S-PRG.

The particle size distribution measurements showed that the average particle sizes of nano- and micro-S-PRG was 0.44 nm and 1.2 μm, respectively. SEM images of S-PRG fillers were shown in Fig. 1A. Stock of S-PRG filler contained the particles with various sizes, especially over 5 μm thickness was remarkable. After particle size adjusting, nano-S-PRG contained fine particles in 300-800 nm size range, and micro-S-PRG contained nano and microscale fillers in 500-1500 nm size range. EDX analysis showed that nano- and micro-S-PRG possessed elements, Al, Si, Sr, Na, and F, which were identified in the stock of S-PRG filler. The result of ion releasing test was shown in Fig. 1C. Na, B, Al, Si, Sr and F were detected in the supernatant of nano- and micro S-PRG, as well as stock of S-PRG filler. The amounts of released ions of nano- and micro S-PRG were comparable to the stock dispersion.

To assess the antibacterial properties related to particle size of S-PRG filler, measurements of turbidity and CFU of *S. mutans* for 24 hours incubation were carried out (Fig. 1D and E). The turbidity of *S. mutans* was reduced by S-PRG filler application when compared to control (no application). In particular, nano-S-PRG significantly suppressed bacterial growth. In addition, nano- and micro-S-PRG significantly decreased the CFUs of *S. mutans* when these results suggest that nano- and micro-SPRG have the bactericidal activity against *S.mutans* compared to control.

S-PRG filler coating to dentin surface

The dentin blocks, following immersion into nano- and micro-S-PRG, were assessed by SEM observation (Fig. 2A). In control (no application), many dentinal tubules were cut to open on the block surface. Nano-S-PRG received sample showed that the fine particles of S-PRG filler uniformly covered the dentin surface and frequently invaded into the dentinal tubules. In contrast, samples applied with micro-S-PRG and stock exhibited to sparsely cover the dentin and decreased the invasion of fillers into dentinal tubules when compared to nano-S-PRG. The score of filler invading into dentinal tubules was 1.0, 2.4, 1.5 and 1.4 in control, nano-S-PRG, micro-S-PRG and stock, respectively (Table1). The score of nano-S-PRG was significantly greater in all groups. Further, score of micro-S-PRG significantly increased when compared to control.

In EDX analysis (Fig. 2B), C, P and Ca associated with dentin substrate were detected in all samples and the specific elements of F, Na, Al, Si and Sr regarding S-PRG filler were identified on the dentin applied with S-PRG fillers. Stock exhibited the remarkable intensity of the elements related to S-PRG filler. However, the elements intensity of nano- and micro-S-PRG-applied dentin was size-dependently reduced. The results of element mapping of Si (blue) is shown in Fig. 3. Si was not detected in control sample, in contrast, consistently detected on the particles on the dentin in S-PRG applied groups.

The adhesion test revealed that nano-S-PRG was remained on the dentin surface after ultrasonic cleaning for 1 and 10 s. The residual particle after 10 s ultrasonic cleaning was 300-500 nm size range, indicating they were smaller than nano-S-PRG (300-800 nm size range). In contrast, microscale fillers containing micro-S-PRG and stock were slight on the dentin after ultrasonic cleaning. Count of S-

PRG filler revealed that there was no significant reference between pre- and after ultrasonic cleaning of nano-S-PRG-applied dentin. However, micro-S-PRG and stock showed significant decrease of particles after 10 s cleaning (Fig. 4B). In addition, EDX analysis demonstrated that intensity of Si of the dentin applied with nano-S-PRG was equivalent between before and after ultrasonic cleaning. However, dentin treated micro-S-PRG and stock exhibited low intensity of Si after ultrasonic cleaning when compared to pre-cleaning (Fig. 4C).

Ion releasing test showed that the elements of B, F, Si and Sr was released from dentin treated with nano-S-PRG (Fig. 5A). As pseudo-pellicle test resembling acquired pellicle on the tooth surface, dentin pre-coating with protein; albumin and lysozyme, was performed before S-PRG filler application. SEM images of albumin coating revealed that both nano- and micro-S-PRG was frequently observed on the dentin, however, lysozyme coating tended to reduce the fillers when compared to albumin (Fig. 5B). Lysozyme significantly diminished the filler number on the dentin when compared to albumin (Fig. 5C).

Antibacterial test of S-PRG coated dentin

Since nano-S-PRG exerted great adhesion properties to dentin, the antibacterial tests employed nano-S-PRG-applied dentin. SEM images of the dentin seeded with *S. mutans* at 24 h incubation were shown in Fig. 6A. In control (no application), marked colonization of *S. mutans* was observed on the dentin. In contrast, nano-S-PRG-applied sample showed slight bacterial accumulation and biofilm formation. CFU of *S. mutans* and *A. naeslundii* were significantly reduced by S-PRG filler application

when compared to control (Fig. 6B). In LIVE/DEAD BacLight staining, we frequently found *S. mutans* stained in red, indicating dead cells, in the presence of nano-S-PRG. In contrast, control rarely showed *S. mutans* stained in red. (Fig. 6C). Quantify of LIVE/DEAD staining resulted in a significant decrease in green fluorescence emissions of live cells and significant increase in dead cell when applied with nano-S-PRG (Figure 6D).

Cytotoxic assessments of S-PRG fillers

All groups revealed that WST-8 activity of NIH3T3 cells were time-dependently increased thorough the experimental periods. WST-8 activity of nano-S-PRG was significantly lower than micro-S-PRG and control (no application). In contrast, LDH activity of nano- and micro-S-PRG was significantly low through the experimental period when compared to control. LDH activity of nano-S-PRG group was comparable to micro-S-PRG. Immunostaining showed that the expression of actin and vinculin associated with cell adhesion and spreading was observed in all groups.

Discussion

The characterization of S-PRG filler demonstrated that fractionated nano- and micro-S-PRG possessed the elements comparable to stock dispersion of S-PRG filler in EDX and ion releasing assessments. Many researches have revealed the antibacterial effect of boric and fluoride ions and recalcified effect of fluoride ion[4), 5), 7)]. Hence, nano- and micro-S-PRG would be anticipated for the bioactive effects on dental disease via ion release. In the results of screening turbidity examinations of *S. mutans*, nano-S-PRG exhibited the great antibacterial effect compared to micro-S-PRG. In general, the interface of nanoparticles can be effective because the surface area per unit weight is significantly increased by nanoparticulate formulation. Nano-S-PRG may stimulate to suppress the bacterial growth via rapidly ion-releasing. Xianping et al. revealed that the corrosion of nanoparticles and microparticles of Cu was different, and Cu^{2+} transformation of nanoparticles was rapid compared to microparticles[14)]. In addition, nanoparticles penetrates cell membrane via interaction force, such as electrostatic, van der Waals, hydrophobic forces and ligand–receptor binding[15)]. Further study will be needed to elucidate the nanoscale behavior of nano-S-PRG.

In SEM observation, S-PRG fine particles covered the dentin surface and partly invade into dentinal tubules. Subsequently, we carried out the ultrasonic cleaning test after S-PRG filler application to observe the fine particles adhered to the dentin. The results showed that the nanofiller remained on the dentin surface, however most of the microscale fillers was disappeared after receiving ultrasonic waves for 10 seconds. Accordingly, it was considered that the nano-S-PRG could adhere to the dentin surface. In general, nanoscale particles tend to be aggregated by attractive forces[16)]. Some

researches explained the nanoscale substrate for drug delivery systems exerted the bioadhesive capability using attractive force[17-19]. We challenged to elucidate the mechanism of nano-S-PRG adhesion to dentin. However, it was impossible to examine the zeta potential of S-PRG fillers because of great ion releasing effects, therefore, pseudo-pellicle test was carried out. Since albumin charge negatively and lysozyme charge positively, surface charge of the target substrate is speculated by the adsorption behavior of the substrate against these proteins[20]. In this work, albumin pre-coated dentin exhibited the trend of well-adhesion of nano-S-PRG. Weerkamp et al. showed that the enamel, dentin and saliva coated tooth substrate were negatively charged[21]. It was suggested that nano-S-PRG had the affinity against negative surface charge, and consequently showed the great property of adhesion to tooth surface.

Dentin applied with nano-S-PRG showed antibacterial activity against *S. mutans* and *A. naeslundii*. It was suggested that boric and fluoride ions released from nano-S-PRG adhered to dentin exhibited the antibacterial effects. *A. naeslundii* is known as primary colonizer of the tooth surface to obtain the dental biofilm[22]. S-PRG application therapy may provide the bactericidal or bacteriostatic action for tooth surface and periodontal pocket. Iwamatsu-Kobayashi et al. demonstrated that S-PRG eluate prevented the tissue destruction in a mouse ligature-induced periodontal disease[23]. In addition, Han et al. reported that S-PRG fillers containing product could sustain the ion release for 90 days, therefore, nano-S-PRG-applied dentin may acquire the long-term preventive effect on bacterial adhesion, demineralization and subsequent dental disease such as dental caries and periodontitis[24].

Since the antibacterial biomaterials frequently exhibits the cytotoxicity, the activity of WST-8

and LDH of S-PRG fillers was investigated in this study. Although NIH3T3 cells could growth on the S-PRG filler application, degree of cell proliferation of nano-S-PRG was significantly reduced when compared to micro-S-PRG and control. However, LDH activity, vinculin expression and cell morphology by nano-S-PRG application were comparable to micro-S-PRG, suggesting that nano-S-PRG have great antibacterial effects and minimum cytotoxicity. Additional in vitro and in vivo investigations must be conducted to facilitate biosafety of S-PRG therapy.

Conclusion

We assessed the effect of particle size of S-PRG filler on adhesion to the dentin surface. In addition, the antibacterial and cytotoxic effects of S-PRG fillers were examined in vitro. SEM and EDX images showed that the stable tooth coating with nano-S-PRG was obtained, when compared to micro-S-PRG. Application of S-PRG fillers significantly inhibited the growth of oral bacterial cells. In addition, S-PRG fillers exhibited low cytotoxicity in fibroblastic cells. Therefore, nano-S-PRG are expected to be beneficial for dental antibacterial therapy.

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Tables

Table1. The score of the invasion of S-PRG filler into dentinal tubules (n=4).

Groups	Control	Nano-S-PRG	Micro-S-PRG	Stock
Score	1	2.4*	1.5†	1.4

*$P < 0.05$ vs control, micro-S-PRG and stock, †$P < 0.05$ vs control

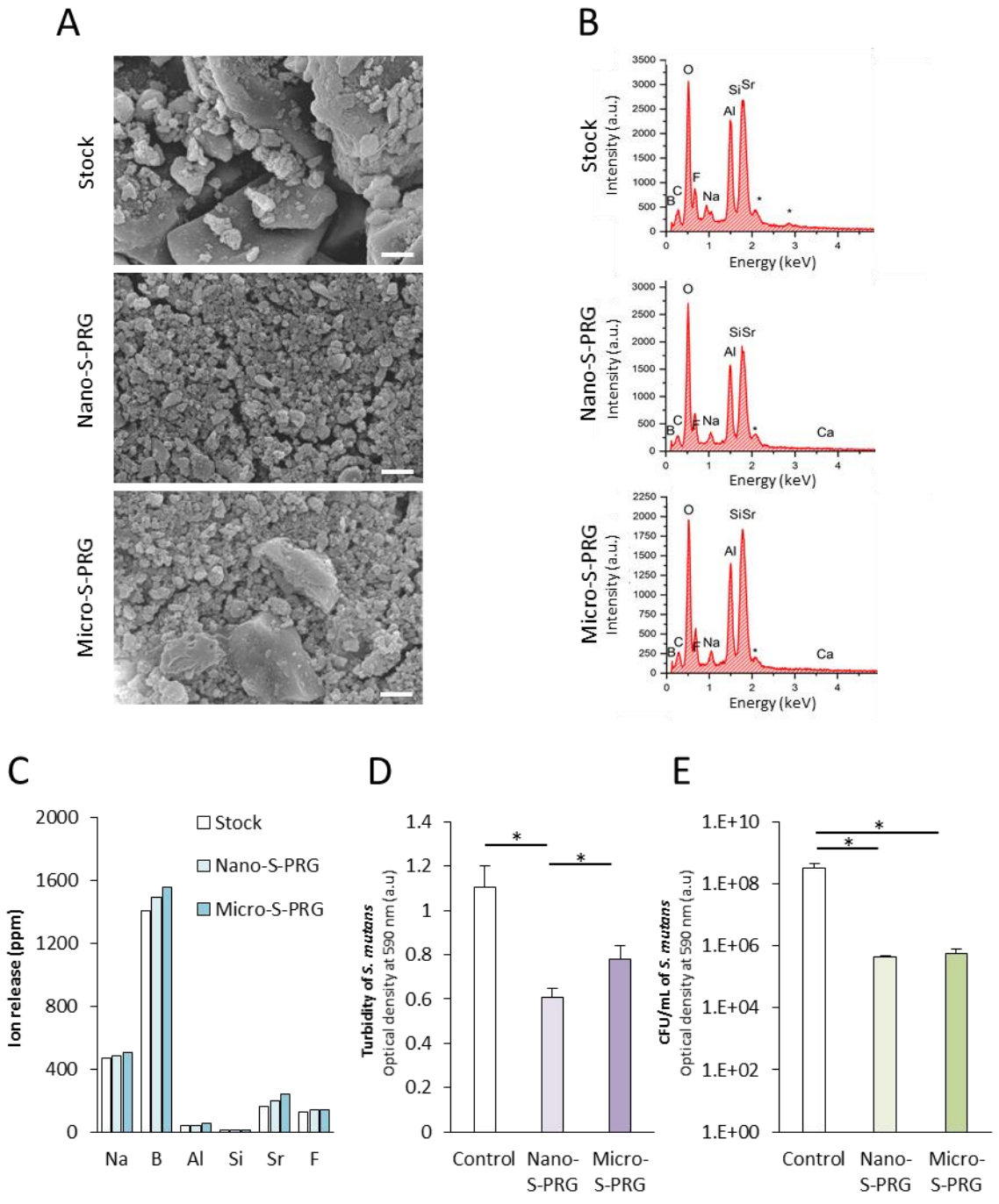
A
Stock
Nano-S-PRG
Micro-S-PRG
B
Stock
Nano-S-PRG
Micro-S-PRG
Intensity (a.u.)
Energy (keV)
O
Si
Sr
Al
C
F
Na
B
Ca
C
Ion release (ppm)
Stock
Nano-S-PRG
Micro-S-PRG
Na
B
Al
Si
Sr
F
D
Turbidity of S. mutans
Optical density at 590 nm (a.u)
Control
Nano-S-PRG
Micro-S-PRG
E
CFU/mL of S. mutans
Optical density at 590 nm (a.u)
Control
Nano-S-PRG
Micro-S-PRG

Fig. 1

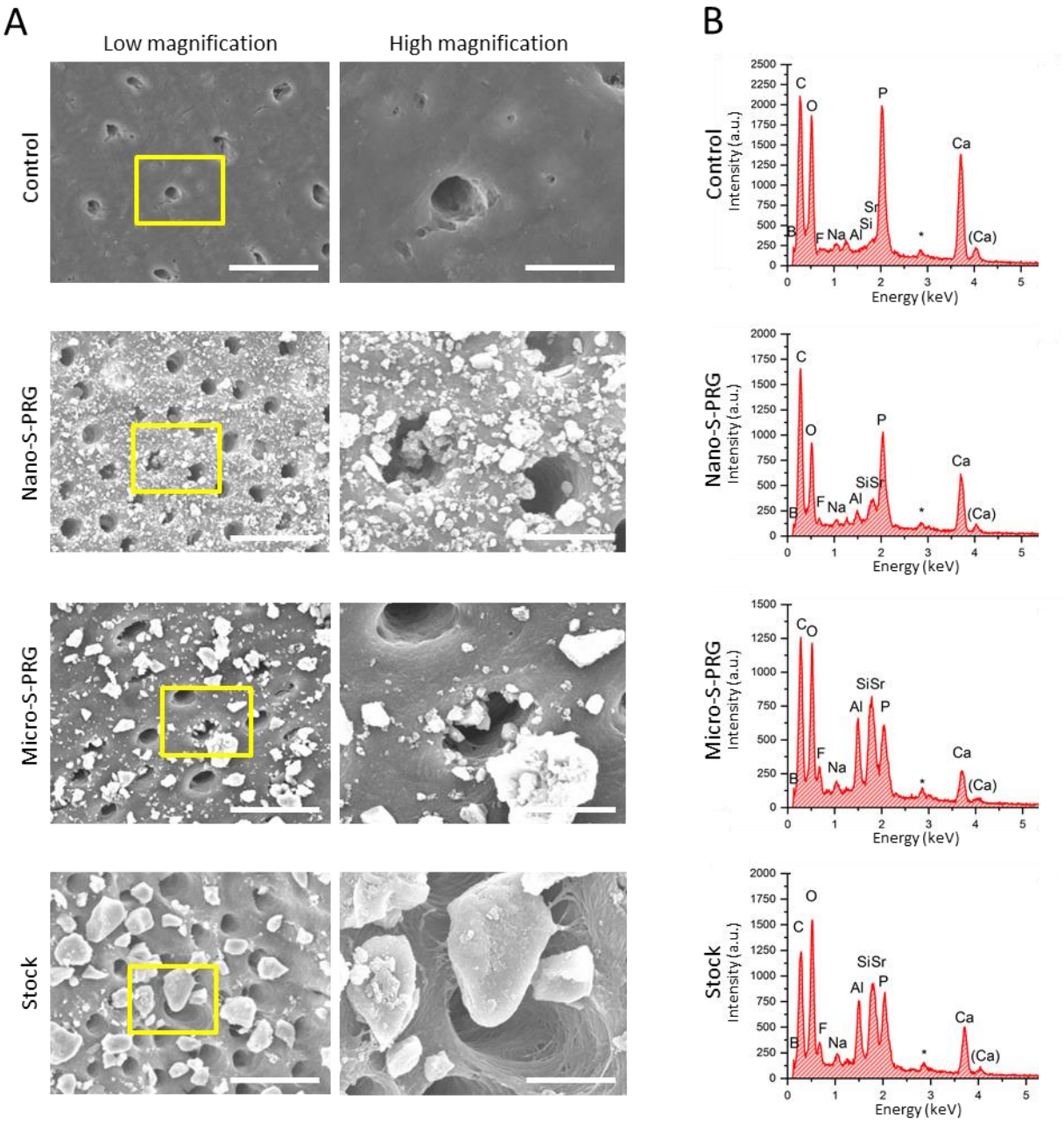
A
Low magnification
High magnification
Control
Nano-S-PRG
Micro-S-PRG
Stock
B
Control
Nano-S-PRG
Micro-S-PRG
Stock
Intensity (a.u.)
Energy (keV)
C
O
P
Ca
(Ca)
B
F
Na
Al
Si
Sr
SiSr
*

Fig. 2

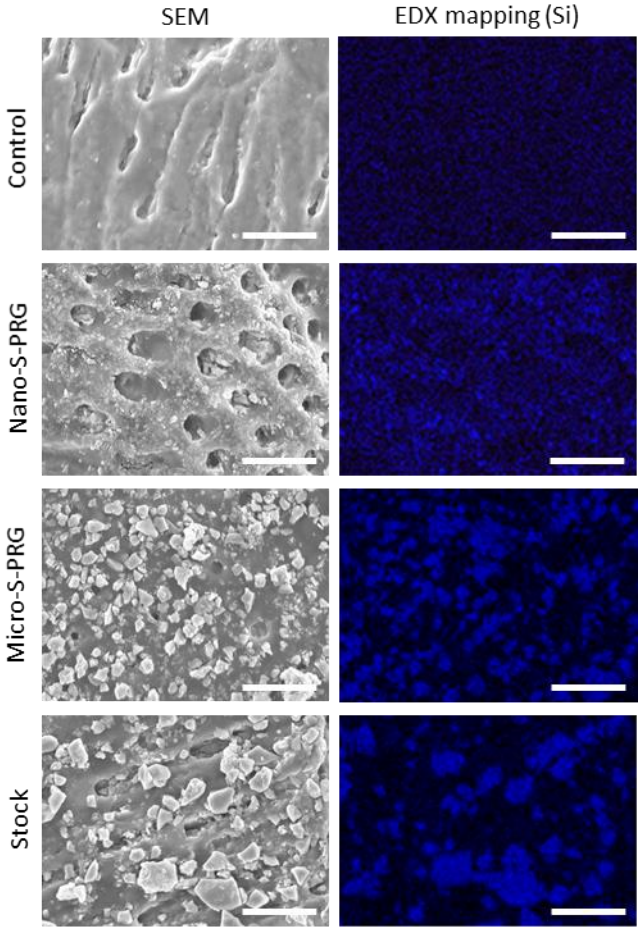

Fig. 3

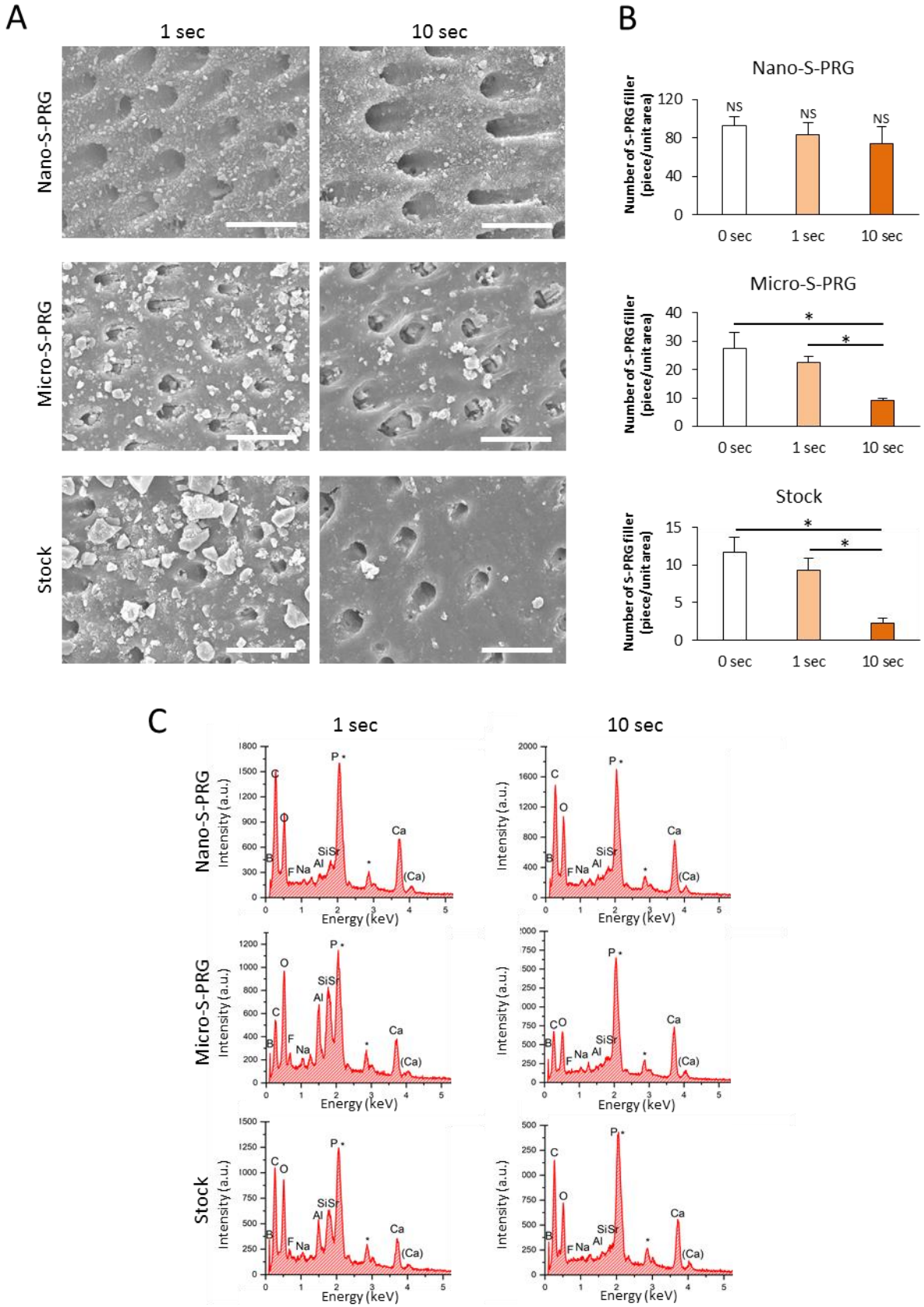
A
1 sec
10 sec
Nano-S-PRG
Micro-S-PRG
Stock
B
Nano-S-PRG
Number of S-PRG filler (piece/unit area)
NS
0 sec
1 sec
10 sec
Micro-S-PRG
*
Stock
C
Intensity (a.u.)
Energy (keV)
P
C
O
Ca
(Ca)
B
F
Na
Al
Si
Sr

Fig. 4

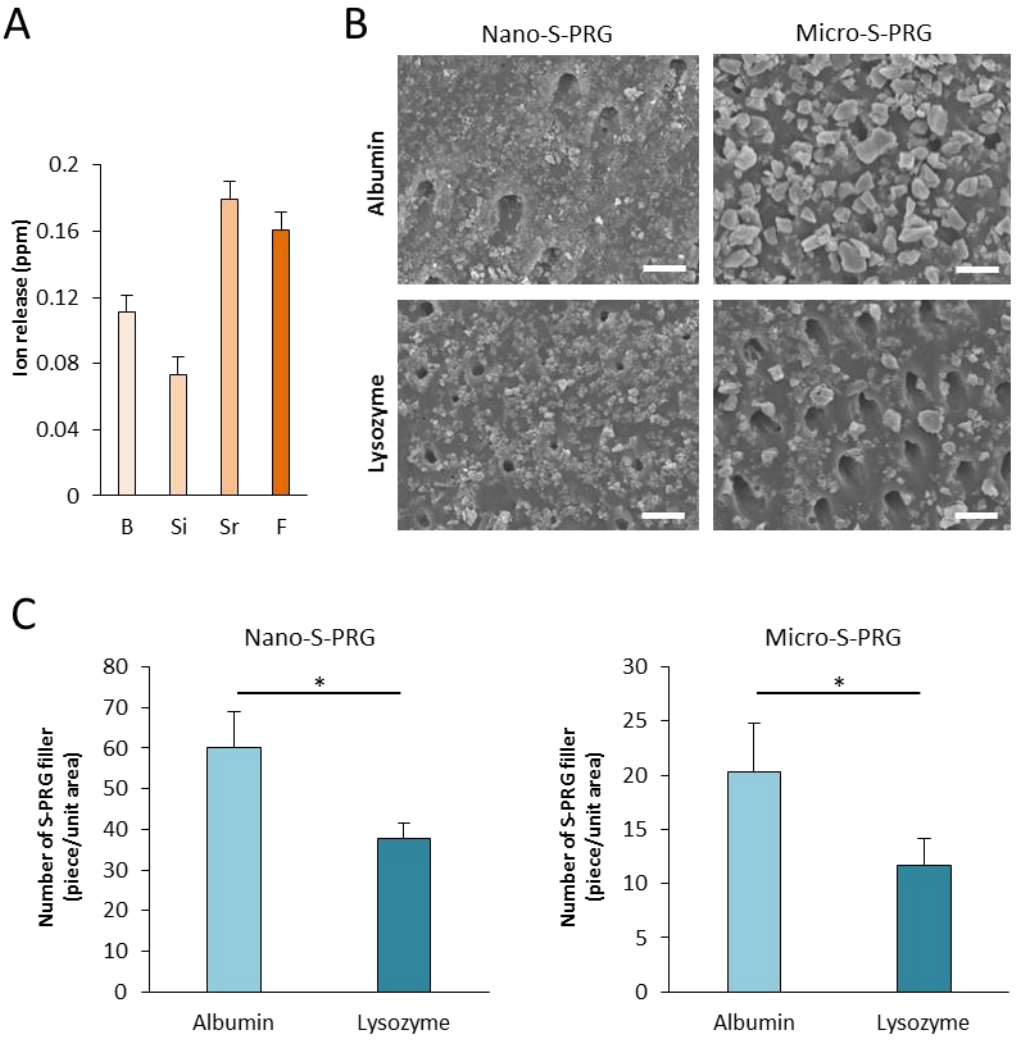
A
Ion release (ppm)
0.2
0.16
0.12
0.08
0.04
0
B
Si
Sr
F
B
Nano-S-PRG
Micro-S-PRG
Albumin
Lysozyme
C
Nano-S-PRG
Micro-S-PRG
Number of S-PRG filler (piece/unit area)
80
70
60
50
40
30
20
10
0
*
Albumin
Lysozyme
30
25
20
15
10
5
0

Fig. 5

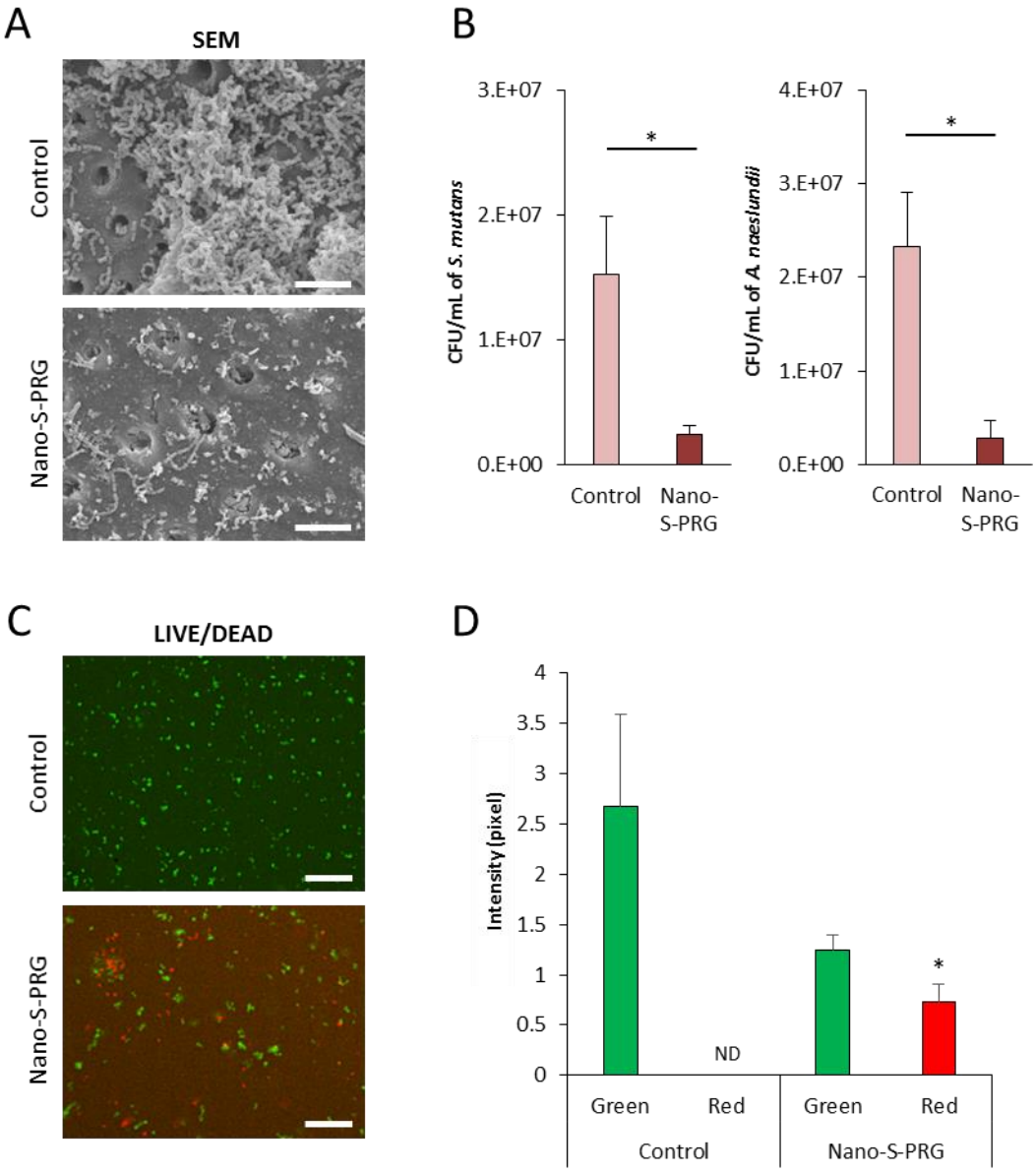
A
SEM
Control
Nano-S-PRG
B
CFU/mL of S. mutans
3.E+07
2.E+07
1.E+07
0.E+00
*
Control
Nano-S-PRG
CFU/mL of A. naeslundii
4.E+07
3.E+07
2.E+07
1.E+07
0.E+00
*
Control
Nano-S-PRG
C
LIVE/DEAD
Control
Nano-S-PRG
D
Intensity (pixel)
4
3.5
3
2.5
2
1.5
1
0.5
0
ND
*
Green
Red
Green
Red
Control
Nano-S-PRG

Fig. 6

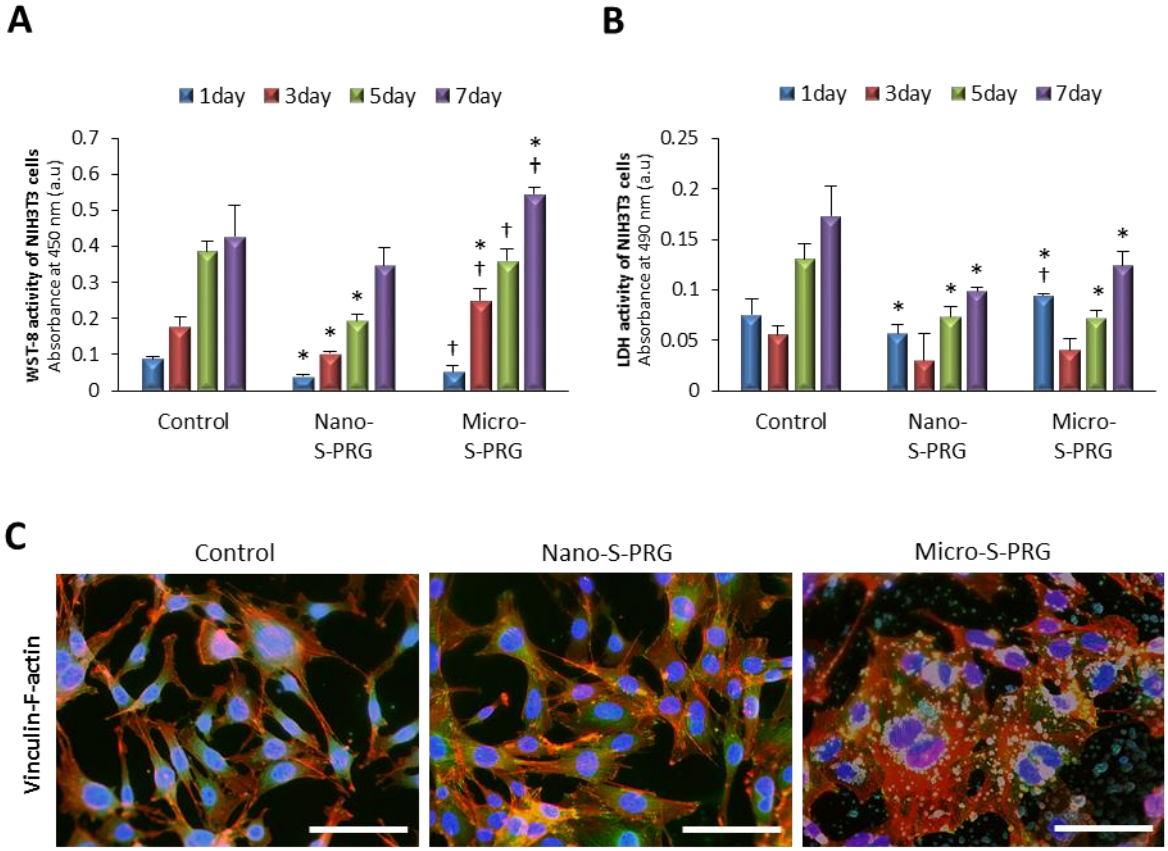
A
1day
3day
5day
7day
WST-8 activity of NIH3T3 cells
Absorbance at 450 nm (a.u)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Nano-S-PRG
Micro-S-PRG
B
1day
3day
5day
7day
LDH activity of NIH3T3 cells
Absorbance at 490 nm (a.u)
0.25
0.2
0.15
0.1
0.05
0
Control
Nano-S-PRG
Micro-S-PRG
C
Control
Nano-S-PRG
Micro-S-PRG
Vinculin-F-actin

Fig. 7

Figure legends

Fig. 1. Characterization of nano- and micro-S-PRG

(A) SEM micrographs of stock, nano- and micro-S-PRG. Scale bar represents 10 μm. (B) EDX analysis of stock, nano- and micro-S-PRG. (C) The amounts of released ions from stock, nano- and micro-S-PRG. (D) Turbidity of *S. mutans* applied with nano- and micro-S-PRG after 24 h incubation. (n=6, mean ± SD) (E) CFU of *S. mutans* applied with nano- and micro-S-PRG after 24 h incubation (n=3, mean ± SD); *, $P<0.05$.

Abbreviations: CFU, colony-forming units; EDX, energy dispersive X-ray spectrometry; SD, standard deviation; SEM, scanning electron microscope; *S. mutans*, *Streptococcus mutans*; S-PRG, surface pre-reacted glass ionomer filler.

Fig. 2. Characterization of S-PRG coated dentin

(A) SEM micrographs of dentin surface in control, nano-S-PRG, micro-S-PRG and stock groups. High magnification images related to flamed area in low magnification images. Scale bar represents 10 μm (low magnification) and 3 μm (high magnification). (B) EDX analysis of dentin in control, nano-S-PRG, micro-S-PRG and stock groups.

Abbreviations: EDX, energy dispersive X-ray spectrometry; SEM, scanning electron microscope; S-PRG, surface pre-reacted glass ionomer filler.

Fig. 3. EDX mapping of S-PRG coated dentin

SEM micrographs and EDX mapping (Si) of dentin surface in control, nano-S-PRG, micro-S-PRG and stock groups. Scale bar represents 10 μm.

Abbreviations: EDX, energy dispersive X-ray spectrometry; SEM, scanning electron microscope; S-PRG, surface pre-reacted glass ionomer filler.

Fig. 4. S-PRG filler adhesion test

(A) SEM micrographs of dentin surface after ultrasonic cleaning for 1 and 10 sec. Scale bar represents 10 μm. (B) Number of S-PRG filler on the dentin surface related to ultrasonic cleaning time (n=3, mean ± SD); *, $P<0.05$. (C) EDX analysis of dentin surface after ultrasonic cleaning for 1 and 10 sed.

Abbreviations: EDX, energy dispersive X-ray spectrometry; NS, not significant; SD, standard deviation; SEM, scanning electron microscope; S-PRG, surface pre-reacted glass ionomer filler.

Fig. 5. Ion releasing and pseudo-pellicle test

(A) The amounts of released ions from nano-S-PRG coated dentin. (B) SEM micrographs of dentin surface with albumin and lysozyme pre-coating. Scale bar represents 5 μm. (C) Number of S-PRG filler on the dentin surface related to albumin and lysozyme pre-coating (n=3, mean ± SD); *, $P<0.05$.

Abbreviations: nano-S-PRG, nano size surface pre-reacted glass ionomer; NS, not significant; SD, standard deviation; SEM, scanning electron microscope; S-PRG, surface pre-reacted glass ionomer.

Fig. 6. Antibacterial assessments of nano-S-PRG coated dentin

(A) SEM micrographs of *S. mutans* after 24 h incubation. Scale bar represents 10 μm. (B) CFU of *S. mutans* and *A. naeslundii* (n=4, mean ± SD); *, $P<0.05$. (C) LIVE/DEAD BacLight staining of *S. mutans* after 24 h incubation. Scale bar represents 20 μm. (D) Number of live (green) and dead (red) bacteria (n=3, mean ± SD). *, $P<0.05$ vs control (red).

Abbreviations: *A. naeslundii*, *Actinomyces naeslundii*; CFU, colony-forming units; ND; not detected; SD, standard deviation; SEM, scanning electron microscope; *S. mutans*, *Streptococcus mutans;* S-PRG, surface pre-reacted glass ionomer filler.

Fig. 7. Cytotoxic assessments of S-PRG fillers

(A) WST-8 activity of NIH3T3 cells. (n=5, mean ± SD) (B) LDH activity of NIH3T3 cells (n=5, mean ± SD); *, $P<0.05$ vs control, †, $P<0.05$ vs nano-S-PRG. (C) Vinculin-F-actin double staining of NIH3T3 cells. Scale bar represents 100 μm.

Abbreviations: LDH, lactate dehydrogenase; SD, standard deviation; S-PRG, surface pre-reacted glass ionomer filler; WST-8, water-soluble tetrazolium salts-8.