DENTAL MATERIALS 35 (2019) 673–685

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Chemical and biological properties of new sealant-use cement materials

Yuki Kondo[a], Shuichi Ito[b,*], Osamu Uehara[c], Yoshihito Kurashige[a], Yusuke Fujita[a], Takashi Saito[d], Masato Saitoh[a]

[a] Division of Pediatric Dentistry, Department of Oral Growth and Development, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido, 061-0293, Japan
[b] Division of Dental Education Development, Department of Integrated Dental Education, School of Dentistry, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido, 061-0293, Japan
[c] Division of Disease control & Molecular Epidemiology, Department of Oral growth and Development, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido, 061-0293, Japan
[d] Division of Clinical Cariology and Endodontology, Department of Oral Rehabilitation, Health Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido, 061-0293, Japan

ARTICLE INFO

Article history:
Received 5 October 2018
Accepted 7 February 2019

Keywords:
Glass ionomer cement
Sealant
Bioactive glass
Ion release
Fluoride recharge

ABSTRACT

Objectives. The aim of this study was to investigate the chemical and biological properties of newly developed bioactive cements, modified such that they are largely composed of calcium, phosphate and fluoride. We investigated whether newly developed bioactive cements have the potential to further protect surrounding hard tissue and enhance remineralization of demineralized tissue by additional ion release.

Methods. We developed four types of novel GIC based on Fuji VII, modified with phosphate and fluoride and calcium. Compressive strength tests were performed following JIS T6607 methods. Ion release of calcium, phosphate and fluoride after 24 h storage were determined using atomic absorption spectroscopy, colorimetry and an ion-specific electrode. Fluoride releases and recharge were measured at 1, 3, 6, 12, 24 and 168 h. Viability was determined by colony-forming units. Inhibitions of biofilm formation and cell proliferation activity were measured.

Results. The GIC groups showed no significant differences in compressive strength after 1 and 7 days. The rates of fluoride ion release from newly developed GICs were significantly greater than those of Fuji VII, Fuji III and BS. All materials except TM can be recharged with fluoride ions. Compared with the control group, which did not release fluoride ions, all materials showed significantly stronger antibacterial effects. The newly developed GICs and BS showed less biofilm formation than Fuji VII and Fuji III.

Significance. Three of four newly developed GICs modified with calcium, phosphate and fluoride ions were found to be superior to other sealant materials.

* *Corresponding author.*
E-mail address: shu@hoku-iryo-u.ac.jp (S. Ito).
https://doi.org/10.1016/j.dental.2019.02.014

1. Introduction

In pediatric dentistry, fissure sealants effectively prevent caries and are therefore frequently used in clinical settings [1–5]. Pits and fissures are not easily self-cleansed and are difficult to clean by brushing due to their complex anatomy. Consequently, pits and fissures are susceptible to dental caries, and bacteria invade into fissures immediately after molar eruption [6–8]. Because enamel calcification is incomplete at the fissures of immature permanent teeth, Polk et al. [9] reported that sealants provide the most effective protection for immature permanent teeth by preventing caries as well as preventing early caries from further decay. Thus, fissure sealants are essential in prophylactic treatment [10]. Another option for preventing caries at pits and fissures is to apply fluoride, but the effects of fluoride do not easily reach pits and fissures. Given that fissure sealants are highly beneficial [11–13], they will continue to be a widely used treatment.

Glass ionomer cement (GIC) and resin composite are major sealant materials used at present, and resin composite is widely used in clinical settings because of its good mechanical strength and handleability [14–18]. However, several problems have clearly demonstrated that the prognosis depends on the drying conditions, and the required tooth surface treatment with strong acid decalcifies the tooth surface unnecessarily [19–21]. Meanwhile, recently developed bioactive resin-based sealant materials have become clinically available. BeautiSealant® (Shofu Inc., Kyoto, Japan) is a unique and novel bioactive resin based on surface pre-reacted glass (S-PRG) filler technology. This sealant releases several ions (e.g., borate, strontium, and silicate) in addition to fluoride ions, allowing fluoride ions to recharge and exert antibacterial and buffering effects [22–24]. However, Surintanasarn et al. reported that GIC's ability to both release and recharge ions is more important than resin-based sealants that release fluoride transiently [25]. GIC-based sealants are unique because they adhere to the tooth substance, are highly biocompatible, and have bioactive functions (i.e., both antibacterial and anticariogenic effects based on fluoride ion release) [26–29]. However, GIC-based sealants are less widely used as resin-based sealants because of their poorer handleability. Nevertheless, GIC-based sealant materials remain essential because resin-based sealants cannot be used for immature permanent teeth because they require moisture exclusion. Conventional GIC-based sealant materials effectively prevent caries through their antibacterial and anticariogenic properties, but whether they have a remineralization effect on early caries remains unreported. GIC releases calcium and phosphate ions as well as fluoride ions, which benefits tooth substance strengthening and remineralization. Thus, along with sealants, GIC may be widely applicable in restoring teeth and preventing root caries. Further modifications of GIC's bioactive functions are anticipated [30,31].

GIC with favorable properties is expected to provide more benefits in strengthening the tooth substance and exerting antibacterial effects. In this study, novel sealant-use cement materials were prepared that release calcium, phosphate, and more fluoride ions than conventional materials, and their chemical and biological properties were investigated.

Table 1 – Ultimate analysis by XPS.

	0 YG	5.4 YG	8.9 YG	10.7 YG	Fuji VII	atom% Fuji III
F	24.93	24.82	24.06	25.51	21.00	21.12
Ca	0.00	3.98	6.96	7.39	0.00	0.00
P	6.27	9.41	7.09	8.07	4.57	4.02
Al	30.51	27.60	27.36	27.81	30.90	29.77
Si	23.81	21.61	23.35	22.85	34.91	33.68
Sr	5.81	3.48	2.27	0.00	7.16	6.13
Na	7.94	7.92	7.91	7.26	1.47	4.10
K	0.73	1.18	1.00	1.11	0.00	1.19

2. Materials and methods

2.1. Materials

Four new samples were prepared using the powder component (fluoroaluminosilicate glass) of the commercially available GIC sealant, Fuji VII® (GC Corp., Tokyo, Japan), to have higher phosphorus and fluorine content with varying calcium and strontium content: 0 YG (calcium 0 mol %, strontium 10.6 mol %); 5.4 YG (calcium 5.4 mol %, strontium 5.3 mol %); 8.9 YG (calcium 8.9 mol %, strontium 1.6 mol %); and 10.7 YG (calcium 10.7 mol %, strontium 0 mol %). Commercially available sealant materials were used as controls: the GIC-based sealants Fuji VII and Fuji III® (GC Corp.) and the resin-based sealants Teethmate F-12.0® (TM; Kuraray Noritake Dental Inc., Tokyo, Japan) and BeautiSealant® (BS; Shofu Inc.). Fuji VII and Fuji III were calcium-free but contained 12.9 mol % and 10.9 mol % strontium, respectively (estimates by X-ray photoelectron spectroscopy (XPS) analysis, Table 1. The phosphorus and fluorine contents were approximately 1.7 and 1.1 times higher in the new materials than in Fuji VII and Fuji III, respectively (estimates by XPS analysis). Fuji III is used solely as a sealant, while Fuji VII is also used as a material for fillers, bases, and agents for dental hypersensitivity. The ingredients, lot numbers, and typical powder/fluid ratios of the commercial products used are shown in Table 2.

2.2. X-ray photoelectron spectroscopy (XPS)

An X-ray photoelectron spectrometer (ESCA-850, Shimadzu Corp., Kyoto, Japan) was used for quantitative and qualitative analyses. Al Kα (1486.6 eV) was used as an X-ray source, and narrow scans were performed with an accelerating voltage of 7 kV in a vacuum of $1.0 \times 10\text{-}6$ Pa.

2.3. Measurement of compressive strength

JIS T6607 was used to measure the compressive strengths of 0 YG, 5.4 YG, 8.9 YG, 10.7 YG, and the controls (Fuji VII, Fuji III, TM, and BS).

Samples were prepared using a brass cylindrical mold (6 mm diameter, 12 mm height) and a pressurizer. New materials were mixed using a fluid provided with Fuji VII at a powder/fluid ratio of 1.3/1.0. Samples were moved to an incubator (37 ± 1 °C, relative humidity 95–100%) 2.5 min after beginning mixing, then left to stand for 60 min. They were then

Table 2 – Formulations of the test materials.

Product	Manufacture	Lot No.	Composition	Powder/liquid ratio(g/g)
Fuji VII	GC	1502031	Powder: fluoroaluminosilicate glass Liquid: polyacrylic acid, distilled water, polybasic carboxylic acid	1.8/1.0
Fuji III	GC	1506011	Powder: fluoroaluminosilicate glass Liquid: polyacrylic acid, distilled water, polybasic carboxylic acid	1.2/1.0
Teethmate F-1 2.0	Kuraray	0023BA	monomer(TEGDMA, HEMA, MDP) MMA-MF copolymer, photo catalyst, colorant	
BeautiSealant	Shohu	41553	glass powder, UDMA, TEGDMA, Particulate silicic acid	

immersed in warm distilled water (37 °C) for 1 day, 1 week, or 5 weeks. A material testing machine (AG-IS20kN, Shimadzu) was used to test compressive strength under the following conditions: load cell capacity, 20 kN; crosshead speed, 1.0 mm/min; and compressive strain in the long-axis direction (n = 6).

2.4. *Measurement of released ion amounts*

Test samples were prepared using a silicone mold tray (20 mm diameter, 2 mm height) (Dosaka EM Co., Ltd., Kyoto, Japan). Samples were moved to an incubator (37 ± 1 °C, relative humidity 95–100%) 2.5 min after beginning mixing, then left to stand for 60 min. After incubating an additional 24 h at room temperature, disks were immersed in 15 mL pH-adjusted ultrapure water (pH 6.9), 50 mM lactic acid solution (pH 5.0), or 50 mM citric acid solution (pH 5.0) for 1 week without shaking, and the ion release into the individual solutions was measured.

2.4.1. *Measurement of released calcium ion levels*

Ultrapure water, lactic acid and citric acid solutions containing released ions were filtered through a membrane filter (pore size, 0.45 μm) made of cellulose nitrate-cellulose acetate esters (Merck Millipore, Billerica, MA, USA). Each eluate (3 mL) was mixed with a solution containing 0.1% mol/L hydrochloric acid and 0.25% lanthanum oxide (3 mL), and the calcium ion levels were quantified using an atomic absorption spectrometer (AAnalyst 5100, Perkin-Elmer, Waltham, MA, USA) (n = 6).

2.4.2. *Measurement of the released phosphate ion levels*

Ultrapure water and solutions of lactic acid and citric acid containing released ions were filtered through a membrane filter (pore size, 0.45 μm), and the eluates were subjected to colorimetric measurement of the phosphate ion levels. Absorbance at 750 nm was measured using the Phospha C-test Wako Inorganic kit (Wako Pure Chemical Corp., Osaka, Japan) and a plate reader (SpectraMax 340 PC, Molecular Devices, Sunnyvale, CA, USA) (n = 6).

2.4.3. *Measurement of released fluoride ion levels*

Eluates of lactic acid solutions (10 mL) similarly obtained were mixed with 1 mL of a total ionic strength adjuster (TISAB III, Thermo Fisher Scientific, Waltham, MA, USA) and quantified using an ion meter (TiN-510 for fluoride ion measurement, Toko Chemicals Inc., Tokyo, Japan) (n = 6).

2.5. *Measurement of released fluoride ion levels before and after recharge*

Disks (20 mm diameter, 2 mm height) were similarly prepared and immersed in 10 mL of the lactic acid solution (pH 5.0), and the levels of fluoride ions released after 1-, 3-, 6-, 12-, 24-, and 168-h immersions were quantified. The lactic acid solution was replenished at each time point. After the 168-h immersion, the disks were removed, washed with ultrapure water, and left to stand in distilled water for 15 min. After wiping the excess water off the surface, the disks were immersed in 5 mL of acidulated phosphate fluoride (APF) solution (9000 ppm F) for 5 min. The disks were washed with ultrapure water and left to stand in distilled water for 15 min. After recharge immersion in fresh lactic acid solution, the levels of fluoride ions released were similarly measured (n = 6).

2.6. *Measurement of antibacterial effect*

Streptococcus mutans JCM 5705 cells (Riken, Tsukuba, Ibaraki, Japan) were cultured in tryptone yeast (TY) medium containing 30 g/L tryptic soy broth and 5 g/L yeast extract (both from BD Diagnostic Systems, Holzheim, Germany). Disk-shaped samples were similarly prepared and directly immersed in the TY medium containing *S. mutans* cells and incubated anaerobically at 37 °C for 24 h. Supernatant was seeded on a TY-medium agar plate, and the viable bacteria (colony-forming units, CFU) were counted after 24-h of incubation (n = 6). In addition to the sealant control samples, disks made from fluorine-free borosilicate glass (20 mm diameter, 2 mm height), which does not release fluoride ions, were tested for comparison.

2.7. *Measurement of the inhibitory effect on experimental biofilm formation*

S. mutans cells were seeded on 60-mm glass dishes (Corning, Corning, NY, USA) containing a medium supplemented with 0.5% sucrose. Similarly prepared disks were immersed with cell strainers (Corning) and incubated anaerobically at 37 °C for 24 h. After the cell strainers and disks were removed, the biofilm was stained with calcein-AM (Promo Kine, Heidelberg, Germany) and observed under a confocal laser microscope (TE2000-E, Nikon, Tokyo, Japan). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the colony area by binarizing image data followed by area extraction (n = 6). In addition to the sealant control samples, glass disks that do not release fluoride ions were also tested for comparison.

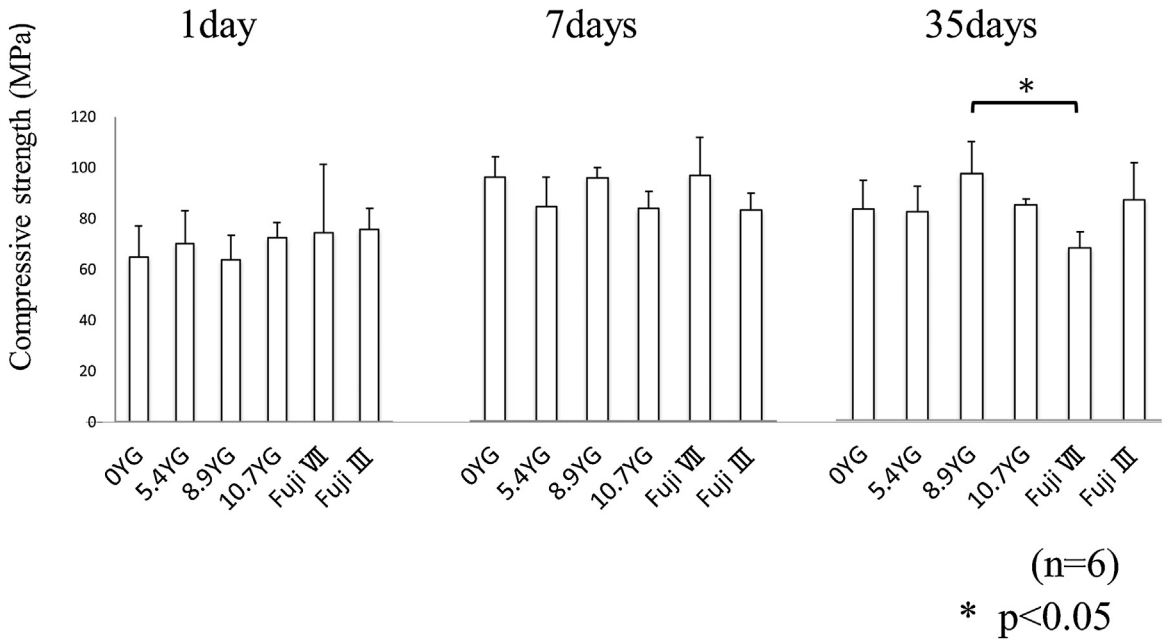

Fig. 1 – Mean values and standard deviations of compressive strength of each materials. Among the GIC-based materials, the compressive strengths after 1-day and 1-week immersions did not significantly differ between the new and the commercial materials (Fuji VII and Fuji III) ($p > 0.05$).

2.8. *Determination of cell proliferative activity*

The cell proliferation reagent WST-1 (Roche Applied Science, Penzberg, Germany) was used for assessment. Human gingival epithelial progenitors (HGEP, CELLnTEC Advanced Cell Systems, Bern, Switzerland) were cultured in CnT-Prime Epithelial Culture Medium (CELLnTEC Advanced Cell Systems) at 37 °C under 5% CO2. After 3–4 passages using trypsin ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 0.53 mM EDTA, Gibco, Thermo Fisher Science, Tokyo, Japan), HGEP (4.0×104 cells/well) were seeded into flat-bottom 96-well plates (Asahi Glass, Tokyo, Japan) to culture for 24 h. After washing 3 times with phosphate-buffered saline, 100 μL of a cell culture medium preincubated with a disk for 24 h was added, and the plates were incubated for another 24 h. WST-1 was added, and the plates were incubated at 37 °C for 1 h before reading the absorbance at 450 nm using a microplate reader (model 680, Bio-Rad, Hercules, CA, USA).

2.9. *Statistical analysis*

One-way analysis of variance was used for comparison, and Tukey's test was performed, with $p < 0.05$ set as the statistical significance level.

3. Results

3.1. *XPS*

The XPS results are shown in Table 1. The presence of calcium and higher phosphorus and fluorine contents were confirmed in 5.4 YG, 8.9 YG, and 10.7 YG compared with Fuji VII and Fuji II.

The strontium contents in Fuji VII and Fuji III were 12.9 mol % and 10.9 mol %, respectively. The phosphorus and fluorine contents were approximately 1.7 and 1.1 times higher in the new materials than in Fuji VII and Fuji III, respectively.

3.2. *Measurement of compressive strength*

Fig. 1 shows the compressive strengths of the hardened cement samples. Among the GIC-based materials, the compressive strengths after 1-day and 1-week immersions did not significantly differ between the new and the commercial materials (Fuji VII and Fuji III) ($p > 0.05$). After the 5-week immersion, 8.9 YG showed higher compressive strength (93.48 ± 11.99 MPa) than Fuji VII (65.39 ± 5.97 MPa); however, differences between the other pairs were not significant ($p > 0.05$). The compressive strengths of TM and BS after 1 day of immersion were 93.62 ± 29.49 MPa and 125.62 ± 3.01 MPa, respectively; BS showed a significantly higher compressive strength than the GIC-based materials.

3.3. *Measurement of released ions*

3.3.1. *Measurement of released calcium ion levels*

Fig. 2 shows the released calcium ion levels. The 10.7 YG sample released 1.42 ± 0.77 ppm calcium into the ultrapure water during the 1-h immersion, which was significantly higher than the levels released from 5.4 YG and 8.9 YG under the same conditions ($p < 0.05$). When immersed in the lactic acid solution, the level of calcium ions released from 8.9 YG (1.76 ± 0.30 ppm) was significantly higher than that released from 5.4 YG (0.37 ± 0.12 ppm), and the level released from 10.7 YG (2.39 ± 0.16 ppm) was significantly higher than that released from 5.4 YG and 8.9 YG ($p < 0.05$). When immersed in

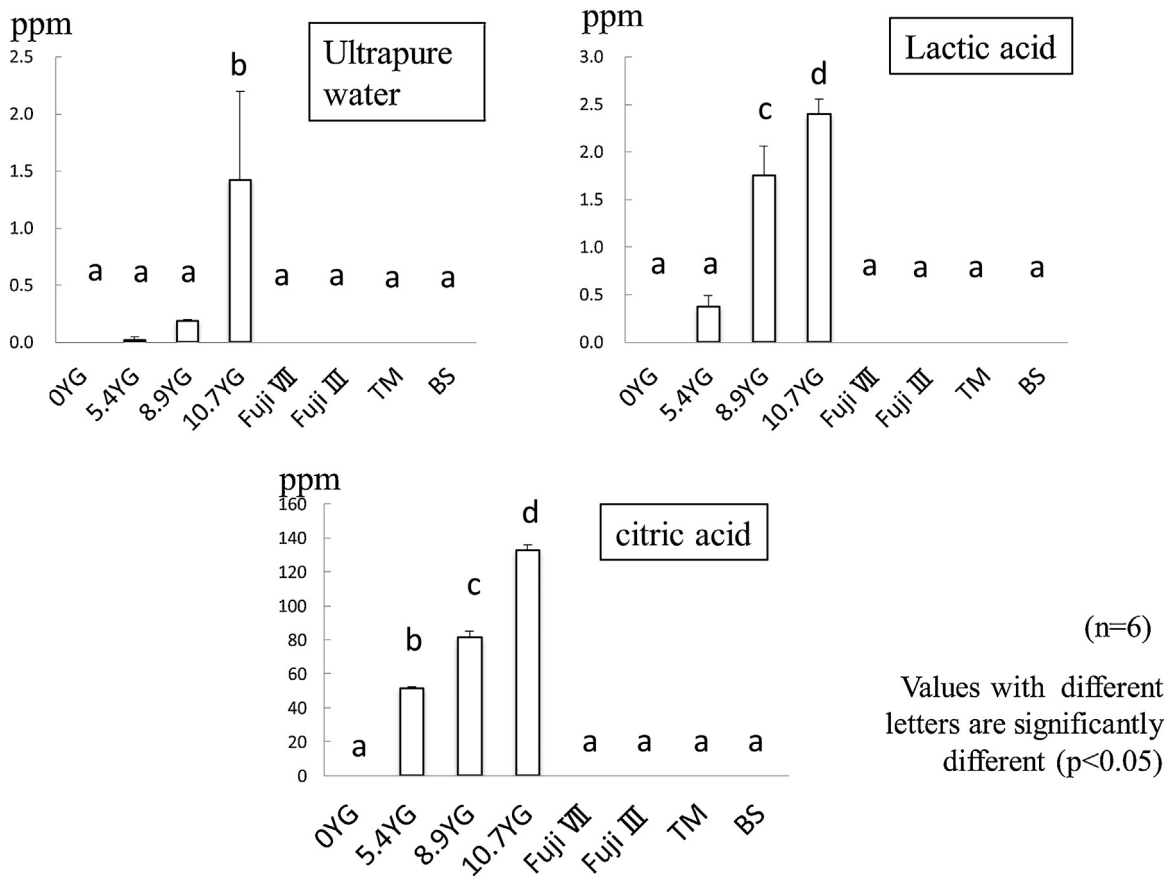

Fig. 2 – Mean level of calcium ion release of each materials (1 week immersion).
The 10.7YG sample released 1.42 ± 0.77 ppm calcium into the ultrapure water during the 1-h immersion, which was significantly higher than the levels released from 5.4YG and 8.9YG under the same conditions ($p < 0.05$). No calcium ion release was detected in any solution after 0YG, Fuji VII, Fuji III, TM, and BS were immersed.

the citric acid solution, the level of calcium ions released from 8.9 YG (81.66 ± 3.64 ppm) was significantly higher than that released from 5.4 YG (51.70 ± 0.77 ppm), and the level released from 10.7 YG (132.94 ± 3.05 ppm) was significantly higher than that released from 5.4 YG and 8.9 YG ($p < 0.05$). No calcium ion release was detected in any solution after 0 YG, Fuji VII, Fuji III, TM, and BS were immersed.

3.3.2. *Measurement of released phosphate ion levels*

Fig. 3 shows the levels of phosphate ions released. No phosphate ion release was detected in the ultrapure water or lactic acid solution after immersing 0 YG and 5.4 YG. When immersed in ultrapure water, the level of phosphate ions released from 10.7 YG (0.56 ± 0.24 ppm) was significantly higher than that from any other sample tested ($p < 0.05$). When the samples were immersed in the lactic acid solution, the phosphate ion levels released from 10.7 YG (0.65 ± 0.19 ppm), 8.9 YG (0.35 ± 0.36 ppm), and BS were significantly higher than those from 0 YG, 5.4 YG, Fuji VII, Fuji III, and TM ($p < 0.05$). When the samples were immersed in the citric acid solution, the phosphate ion levels released from 0 YG (7.66 ± 0.41 ppm), 5.4 YG (7.81 ± 0.38 ppm), 8.9 YG (10.01 ± 1.28 ppm), and 10.7 YG (11.81 ± 0.67 ppm) were significantly higher than those released from the controls ($p < 0.05$). No phosphate ion release from TM was detected in any solution. The phosphate ion levels released from BS into the ultrapure water, lactic acid solution, and citric acid solution were 0.35 ± 0.07 ppm, 0.46 ± 0.15 ppm and 0.42 ± 0.12 ppm, respectively; these values did not significantly differ.

3.3.3. *Measurement of released fluoride ion levels*

Fig. 4 shows the levels of fluoride ions released. When the samples were immersed in ultrapure water, the fluoride ion levels released from 0 YG, 5.4 YG, 8.9 YG, 10.7 YG, Fuji VII, Fuji III, and BS were 32.07 ± 0.75 ppm, 29.99 ± 5.23 ppm, 33.93 ± 2.48 ppm, 32.68 ± 3.82 ppm, 18.45 ± 1.29 ppm, 17.71 ± 1.04 ppm, and 12.77 ± 0.40 ppm, respectively. The fluoride ion level released from the resin-based sealant material TM was 88.03 ± 7.20 ppm, which was significantly higher than the levels released from the other materials ($p < 0.05$). Among GIC-based sealant materials, the levels of fluoride ions released from the new materials were significantly higher than those from Fuji VII and Fuji III ($p < 0.05$). No significant differences were found in the levels of released fluoride ions among the new sealant materials ($p > 0.05$).

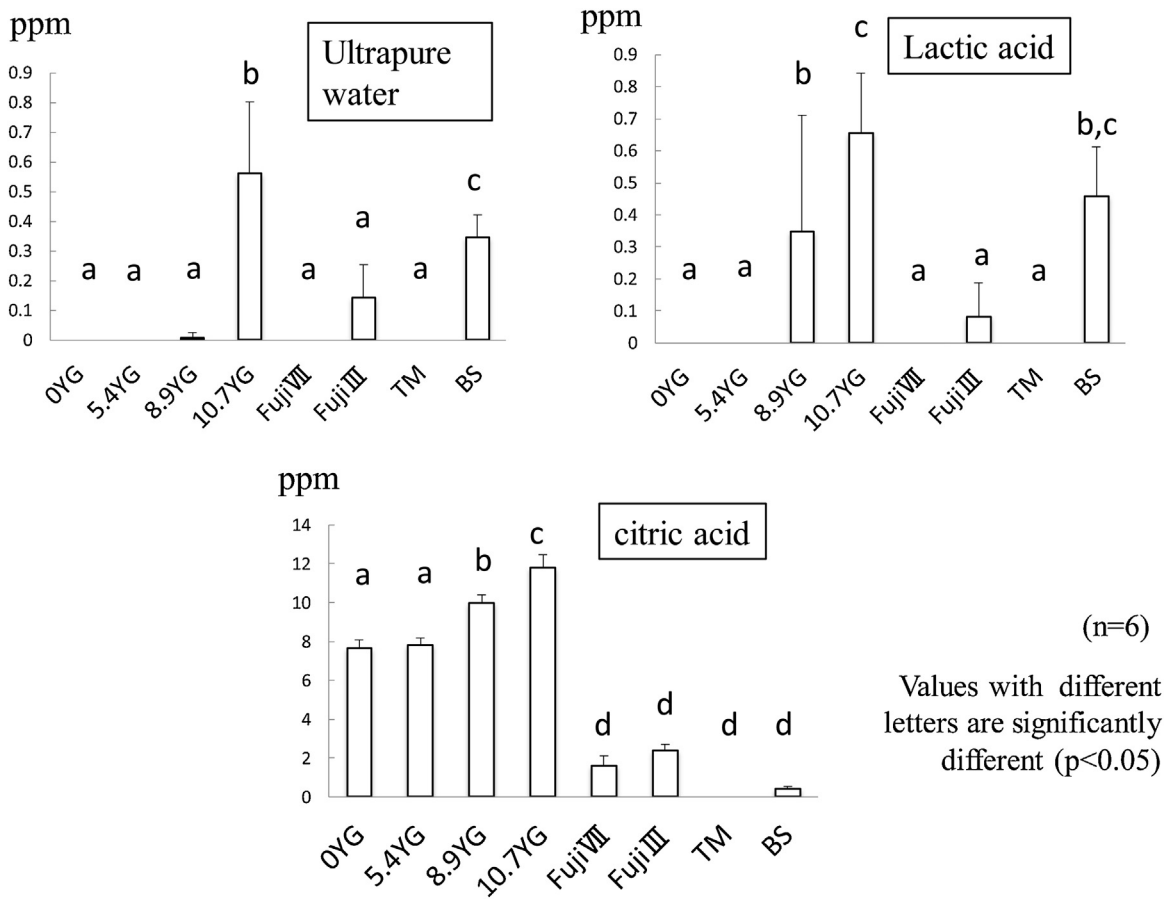

Fig. 3 – Mean level of phosphate ion release of each materials (1 week immersion).
When immersed in ultrapure water, the level of phosphate ions released from 10.7YG (0.56 ± 0.24 ppm) was significantly higher than that from any other sample tested ($p < 0.05$). No phosphate ion release from TM was detected in any solution.

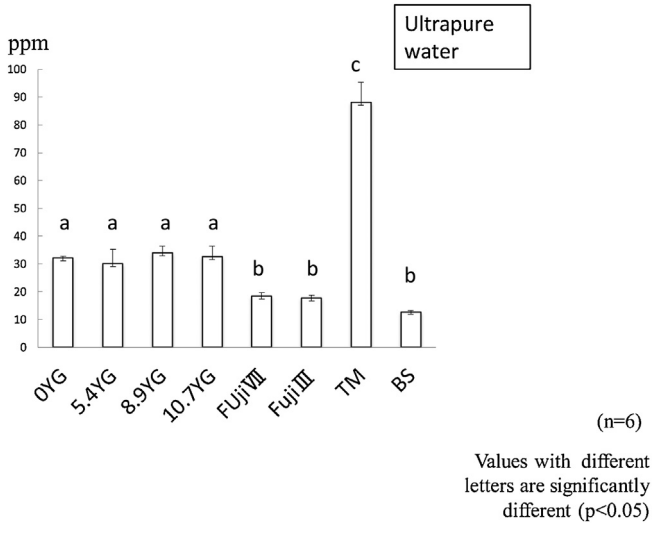

Fig. 4 – Mean level of fluoride ion release of each materials (1 week immersion).
The fluoride ion level released from TM was 88.03 ± 7.20 ppm, which was significantly higher than the levels released from the other materials ($p < 0.05$). No significant differences were found in the levels of released fluoride ions among the new sealant materials ($p > 0.05$).

3.4. *Measurement of fluoride ion levels released before and after recharge*

Tables 3 and 4 show the time courses of the changes in the levels of released fluoride ions immediately after preparing the samples (before recharge) and after recharge using a solution containing a high level of fluoride (an APF solution), respectively. TM released the highest levels of fluoride ions before recharge but released no fluoride ions after recharge.

Figs. 5 and 6 show the fluoride ion release rates during the 1-, 3-, 6-, 12-, 24-, and 168-h immersions before and after recharge, respectively. The fluoride ion release rate was defined as the hourly fluoride ion release into the immersion fluid, which was replenished with fresh immersion fluid at each time point. When the lactic acid solution was used as an immersion fluid, the fluoride ion release rate peaked during the 1-h immersion, then gradually decreased in a time-dependent manner. TM and BS showed the highest and lowest rates of sustained fluoride ion release, respectively. The fluoride ion release rates did not differ among the GIC-based sealant materials.

After recharge, the fluoride ion release rates from all samples except TM peaked during the 1-h immersion and were higher than the corresponding rates before recharge. Release of fluoride ions from recharged TM was undetectable.

Table 3 – Values of fluoride ions before recharge.

Time(h)	0 YG	5.4 YG	8.9 YG	10.7 YG	Fuji VII	Fuji III	TM	pmm BS
1	4.03 (1.29)	4.46 (0.74)	3.90 (1.29)	4.31 (1.09)	3.68 (1.44)	2.74 (0.51)	9.68 (0.14)	0.50 (0.11)
3	4.93 (0.91)	4.45 (1.62)	3.99 (0.95)	4.59 (0.67)	4.22 (0.56)	3.93 (0.87)	10.85 (0.39)	1.72 (0.07)
6	6.11 (1.97)	5.39 (1.81)	5.33 (2.35)	7.42 (2.41)	4.28 (0.28)	5.18 (1.45)	8.39 (0.11)	2.94 (0.05)
12	7.10 (1.00)	8.37 (2.03)	7.67 (1.31)	8.65 (1.67)	8.83 (0.73)	10.19 (0.66)	12.22 (0.43)	6.54 (1.13)
24	9.05 (1.70)	9.36 (1.81)	7.97 (2.22)	8.30 (2.20)	8.95 (1.64)	9.25 (0.87)	10.43 (0.28)	6.65 (0.84)
168	24.7 (2.02)	27.2 (2.87)	27.5 (3.36)	27.7 (2.45)	26.9 (0.74)	26.8 (7.41)	77.7 (4.15)	31.0 (2.12)

Table 4 – Values of fluoride ions after recharge.

Time(h)	0 YG	5.4 YG	8.9 YG	10.7 YG	Fuji VII	Fuji III	TM	pmm BS
1	37.61 (1.71)	35.71 (2.17)	38.20 (1.61)	36.49 (3.31)	34.78 (4.77)	40.11 (2.22)	0.24 (0.09)	37.63 (5.06)
3	30.54 (1.64)	27.73 (2.25)	35.08 (1.78)	27.02 (2.99)	26.89 (3.72)	27.61 (2.88)	0.14 (0.01)	30.30 (2.81)
6	30.17 (1.97)	27.32 (2.47)	29.71 (1.42)	27.37 (2.37)	24.99 (1.99)	21.04 (1.92)	0.20 (0.01)	23.83 (2.26)
12	20.09 (2.06)	21.32 (1.42)	19.66 (0.85)	21.64 (2.93)	18.43 (2.81)	20.52 (0.56)	0.33 (0.08)	17.45 (1.83)
24	2.92 (2.63)	2.86 (2.20)	3.15 (1.52)	2.98 (1.94)	3.31 (1.75)	1.98 (2.82)	0.63 (0.09)	2.98 (2.76)
168	5.21 (0.47)	5.64 (0.83)	5.39 (0.82)	5.81 (0.96)	1.55 (0.34)	0.75 (0.27)	0.04 (0.21)	1.13 (0.30)

Values are given as mean (SD).

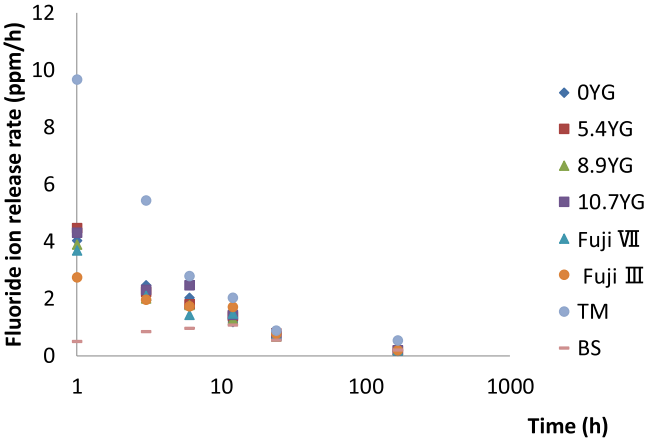

Fig. 5 – Fluoride ion release late before recharge. When the lactic acid solution was used as an immersion fluid, the fluoride ion release rate peaked during the 1-h immersion, then gradually decreased in a time-dependent manner. TM and BS showed the highest and lowest rates of sustained fluoride ion release, respectively.

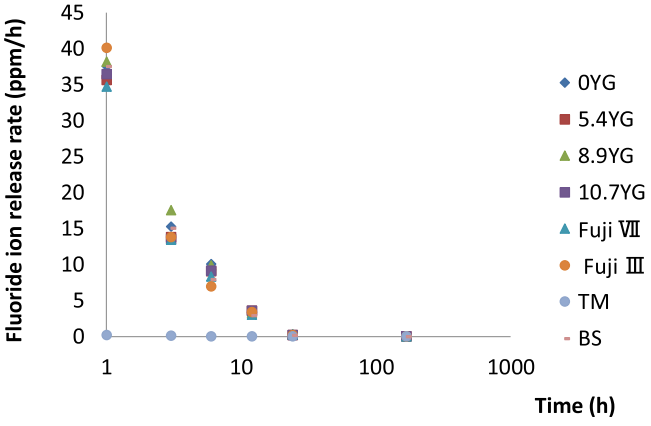

Fig. 6 – Fluoride ion release late after recharge. Fluoride ion release rates from all samples except TM peaked during the 1-h immersion and were higher than the corresponding rates before recharge. Release of fluoride ions from recharged TM was undetectable.

3.5. *Measurement of antibacterial effects*

Viable bacterial counts after the 24-h incubation were significantly lower when the new materials, Fuji VII, Fuji III, TM, or BS were present in the culture than when the glass control, which does not release fluoride ions, was present (2.3×109 CFU/mL) ($p < 0.05$). The bacterial count was lowest when TM was present in the culture ($p < 0.05$). The bacterial counts in the presence of the new materials were lower than those in the presence of Fuji VII or Fuji III (Fig. 7).

3.6. *Measurement of the inhibitory effect on experimental biofilm formation*

Fig. 8 shows confocal laser microscopy images of calcein-AM stained experimental biofilms. New materials inhibited biofilm formation more than the glass that did not release fluoride ions (Fig. 8, Control). Image analysis showed that the new materials reduced the biofilm formation more significantly than did the glass control, Fuji VII, and Fuji III (Fig. 9) ($p < 0.05$).

3.7. *Determination of cell proliferative activity*

Fig. 10 shows the cell proliferative activity as determined using WST-1. The resulting absorbance values suggested that adding the sealant material-exposed medium to the cell culture did not significantly change the cell proliferative activity (control, 1.15 ± 0.20; 0 YG, 1.44 ± 0.32; 5.4 YG, 1.14 ± 0.20; 8.9 YG, 1.01 ± 0.17; 10 YG, 1.00 ± 0.08; Fuji VII, 1.22 ± 0.13; Fuji III, 1.24 ± 0.17; TM, 0.85 ± 0.13; and BS, 1.24 ± 0.15 [$p > 0.05$]).

4. Discussion

4.1. *Mechanical properties*

This study formulated new sealant-use cement materials that release more calcium, phosphate, and fluoride ions than commercial GIC-based sealant materials to enhance tooth substance strengthening and provide stronger antibacterial effects. Conventional GIC-based sealant materials have advantages over resin-based materials in both fluoride release and moisture tolerance, allowing the use of GIC-based sealant materials in treating immature permanent teeth. Many previous studies have shown that adding Ag [32] or antibacterial agents and bioactive glass [33–36] to improve flexural strength and biological properties, respectively, reduced the compressive strength of GIC-based sealants. Additives to enhance certain properties may worsen other properties, such as decreasing the compressive strength [37,38].

This study found no differences in compressive strength among the GIC-based materials tested (the new materials and Fuji VII and Fuji III) after 1 day and 1 week of immersion in water, and 8.9 YG had significantly higher compressive strength than did Fuji VII after a 5-week immersion, suggesting that immersing the new materials in a neutral aqueous solution had a negligible effect on their mechanical strength. However, the new materials released more ions than Fuji VII and Fuji III did under acidic conditions, suggesting that the new materials had lower compressive strengths than did Fuji VII and Fuji III. Several studies reported solubilities of chemically cured GIC in organic acid solutions [39,40]. Wada demonstrated that exposing hardened cement to an acidic environment more rapidly degraded the polyacrylate matrix, thus more prominently affecting mechanical strength after exposure to a lactic acid solution than to distilled water [41]. This study's main objective was the clinical application of new sealant materials. Given that desorption can be detected and refilling can be performed during routine examinations in clinical settings, new materials that release more ions than existing sealant materials will be beneficial for their anticariogenic and tooth substance-strengthening effects, even with decreased mechanical strength. Additionally, in the oral cavity, various ions will be released upon exposure to lactic acid,

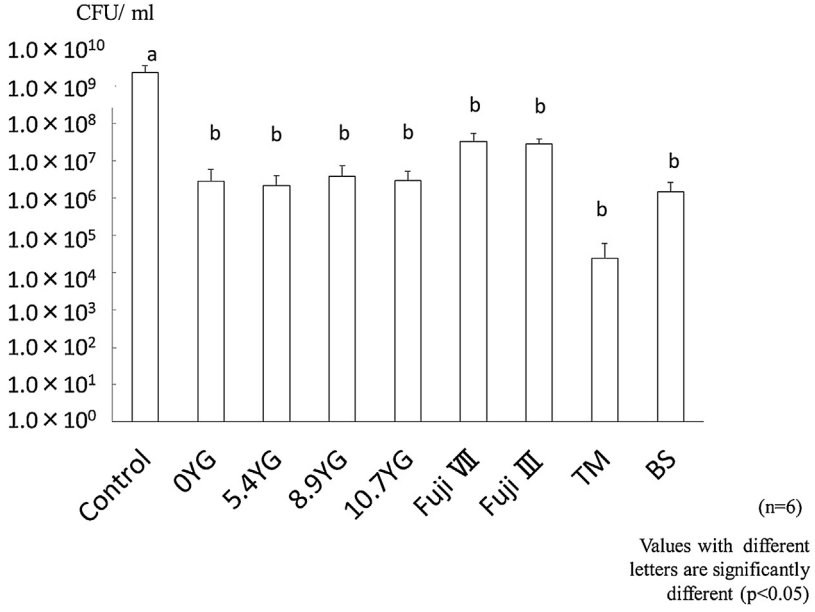

Fig. 7 – Measurement of antibacterial effects.
The bacterial count was lowest when TM was present in the culture ($p < 0.05$). The bacterial counts in the presence of the new materials were lower than those in the presence of Fuji VII or Fuji III.

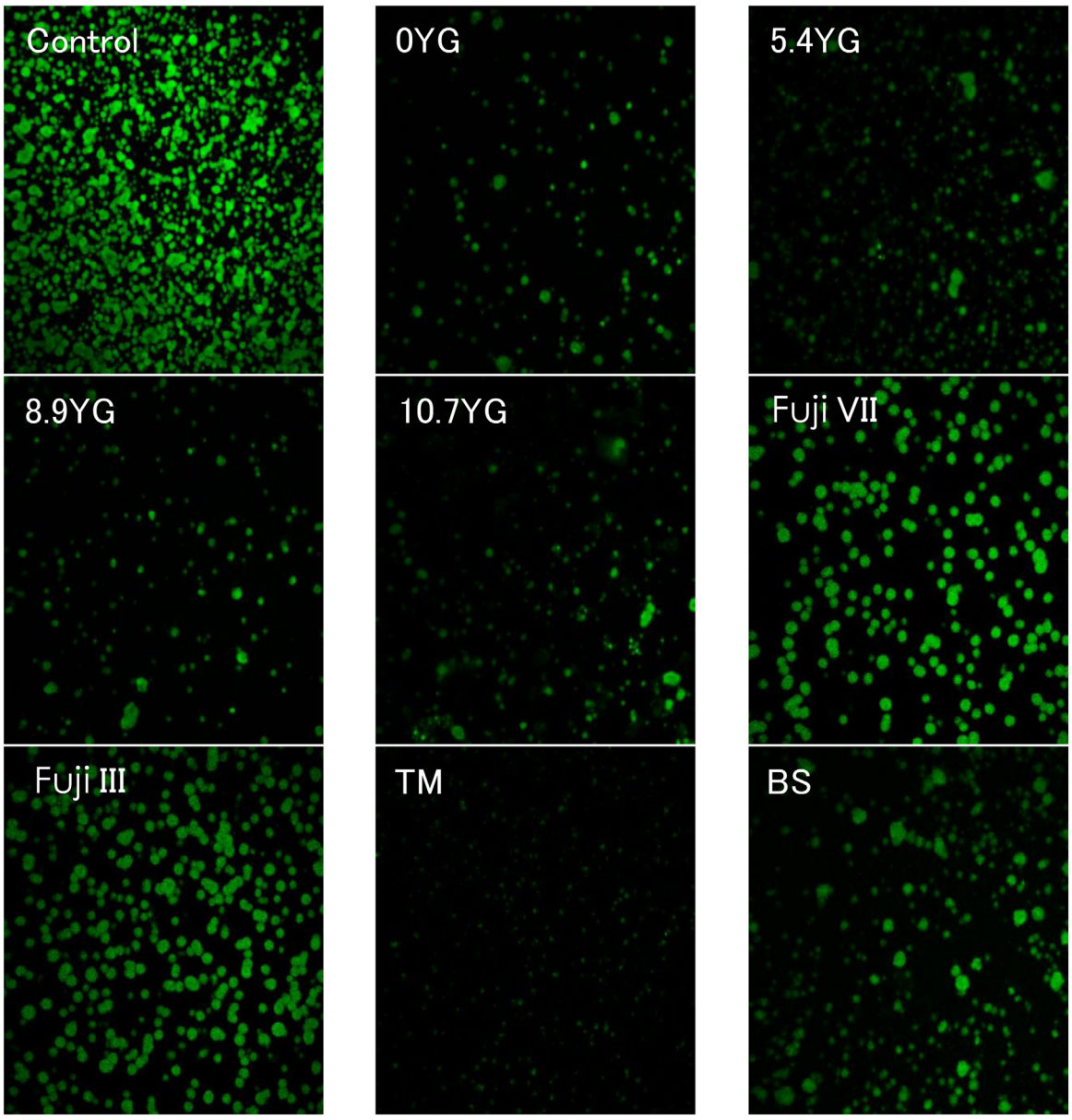

Fig. 8 – Images of calcein-AM stained experimental biofilms by confocal laser microscopy. New materials inhibited biofilm formation more than the glass that did not release fluoride ions.

but exposure to hydrochloric acid is highly unlikely. Thus, the release of various ions with exposure to hydrochloric acid, as shown in this study, may not occur. The effects of acidic environments on mechanical strength should be investigated further.

4.2. *Chemical properties*

GIC is prepared by mixing glass powder and a polyacrylic acid solution, whereupon multivalent cations, such as aluminum, are released after exposing the glass surface to acid [42]. Such free ions crosslink carboxy groups of acid polymers to form a gel matrix and set the cement [39]. Calcium ions detected in this study included those used for crosslinking carboxy groups in acid polymers and free ions in the matrix. Calcium and phosphate ion levels released from sealant materials, when detected, were higher in the lactic acid and citric acid solutions than in ultrapure water (pH 6.9). Given that GIC easily releases ions in an acidic environment [43] and that the release of calcium and phosphate ions increases under acidic conditions, GIC is a material that can effectively supply ions required for remineralizing tooth substances. Under neutral conditions, the forward reaction in which calcium reacts with polyacrylic acid to produce salt is faster than the reverse reaction, where products become redissolved. Thus, at equilibrium, calcium ions are mostly used to produce insoluble calcium salt and are not easily released once the cement is set [44,45]. Calcium ion elution under acidic conditions can be explained by matrix dissolution [43,44]. The new materials, 5.4 YG, 8.9 YG, and 10.7 YG, were made from powder with a high calcium

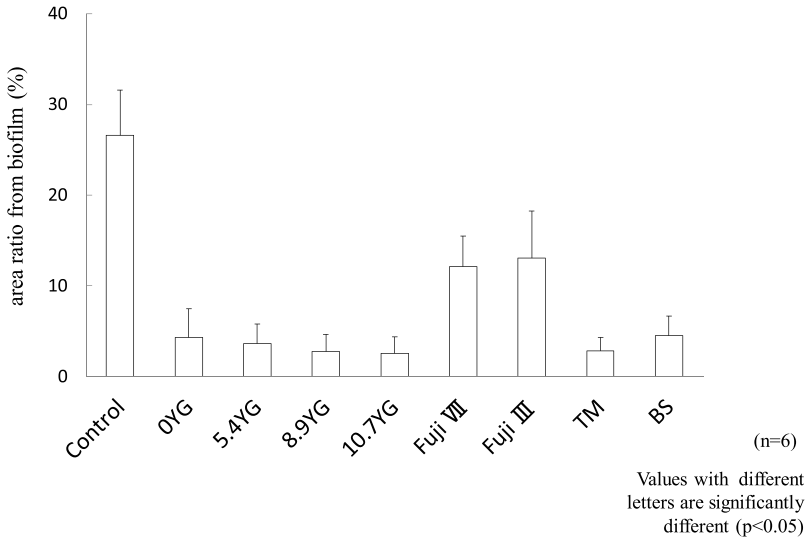

Fig. 9 – Biofilm area calculated from image analysis.
New materials reduced the biofilm formation more significantly than did the glass control, Fuji VII, and Fuji III ($p < 0.05$).

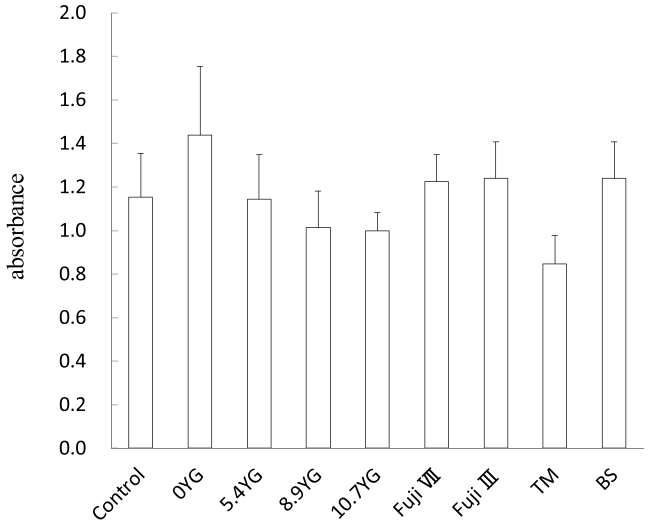

Fig. 10 – Values of the cell proliferative activity as determined using WST-1.
The resulting absorbance values suggested that adding the sealant material-exposed medium to the cell culture did not significantly change the cell proliferative activity.

content. More calcium ions were released from the glass, and consequently, more calcium bound to carboxyl groups in the matrix. This result may explain the increased calcium ion levels released upon acid exposure. Commercial GIC contains strontium to increase contrast, while Fuji VII does not contain calcium [30]. Both strontium and calcium are alkaline earth metals, and strontium is easily incorporated into hydroxyapatite as a substitute for calcium [46]. Their ionic radii are similar (Sr2 + 1.13 Å, Ca2 + 0.99 Å), and substituting strontium for calcium only negligibly changes the GIC structure. Furthermore, substituting strontium for calcium in hydroxyapatite increases acid resistance and promotes remineralization [47]. Among the new materials tested, 10.7 YG and 0 YG contained calcium only and strontium only, respectively, while 5.4 YG and 8.9 YG contained both. Materials containing both calcium and strontium are likely superior to those containing either calcium or strontium alone, based on the expected balance between crystallization performance and acid resistance.

Although the phosphorus contents were the same among the four new materials, the level of phosphate ions released increased in a calcium content-dependent manner, likely because negatively charged phosphate ions were dissolved with positively charged calcium ions in accordance with the principle of electroneutrality. Both ions were released in greater amounts into the citric acid solution (pH 5.0) than into the lactic acid solution (pH 5.0). Zalizniak et al. [31] reported that selective chelation of calcium and aluminum by citric acid degraded cement. Similar chelation likely occurred in this study, thus degrading a large volume of cement. Fluoride ion levels were high in the matrix made from the new materials, resulting in high fluoride content in the matrix. Thus, the levels of fluoride ions released from the new materials were higher than those released from Fuji VII and Fuji III.

Sealants are suitable for treating subsurface demineralization in healthy dentin and enamel. The enamel surface is constantly demineralized and remineralized and is covered by a hypercalcified layer formed by calcium and phosphate ions released from teeth via subsurface demineralization or by these ions from saliva. Tooth remineralization is induced by calcium and phosphate ions from saliva and demineralized teeth [48], and fluoride ions are taken up and converted into fluorapatites in nearby teeth, improving the teeth's acid resistance and strengthening the dentin. Compared with GIC-based Fuji VII and Fuji III and resin-based BS, the new materials had significantly high levels of sustained fluoride release. Therefore, the new materials are expected to improve anticariogenic effects and acid resistance and strengthen tooth substances. TM also had significantly higher levels of sustained fluoride release than did the other materi-

als. Due to hydrolysis, fluoride ions are released slowly and irreversibly from TM, which is a polymer of methyl methacrylate and methacrylic fluoride and is therefore a fluoride-ion sustained-release polymer [49]. This sustained-release property distinguishes TM from the other materials.

GIC, which contains high fluoride ion levels in the matrix, releases the ions in large quantities immediately after polymerization, followed by a gradual decrease. However, GIC has been shown to incorporate and recharge itself with fluoride ions when immersed in a solution containing a high fluoride concentration [50–52]. In the present study, the newly prepared GIC-based materials released the most fluoride ions 1 h after placing them in a lactic acid solution, followed by a gradual decrease (Fig. 5). These results are consistent with those of previous studies showing that after releasing fluoride ions from the cement surface layer, a state of equilibrium is established over time, and eventually, a slow and sustained release of fluoride begins as the ions inside the cement spread toward the surface [42,53]. In other words, before fluoride recharges, a gentle and constant slope forms after the initially large fluoride release, but after recharging, the graph shows little sustained fluoride release after the initial large fluoride release (Fig. 5). This finding suggests that biphasic release consisting of early fast release and late slow and sustained release occurs before recharge, while monophasic release consisting of early fast release occurs after recharge. Furthermore, significantly higher levels of sustained fluoride release were observed after immersion in the APF solution than were observed immediately after sample preparation. A previous study also showed that due to matrix deterioration and recharged fluoride ion release, the number of fluoride ions released surpassed that observed in a newly made sample, thus increasing the amount of fluoride released [54]. Being acidic, APF increases the level of sustained fluoride release by dissolving glass in addition to recharging fluoride [54]. Despite the highest amount of sustained fluoride release before recharging, TM did not release fluoride ions after recharging, revealing that after releasing many fluoride ions in the early phase, TM does not take up fluorine into its hardened body even when recharged (Figs. 5 and 6). Accordingly, the properties of GIC-based materials underscore the ability of the new materials and Fuji VII and III to recharge fluoride, highlighting the difference between the GIC-based materials and resin-based TM, which cannot recharge fluoride.

BS is a bioactive material that slowly releases various ions from the glass-ionomer phase of the S-PRG filler. Quantitatively, the glass-ionomer phase of the BS used in this study is only part of the material, which also contains resin. This factor likely contributed to the smaller amount of sustained fluoride release from the BS than from the new materials. BS also releases strontium ions, not calcium ions, suggesting that its glass-ionomer phase does not contain calcium ions.

In this study, atomic absorption spectrometry, absorption spectroscopy, and a fluoride ion-selective electrode method were used to measure the sustained calcium, phosphorus, and fluoride releases, respectively. Using the different methods may have caused the measurement accuracy to differ. Therefore, future studies should measure ions under the same conditions by using, for example, inductively coupled plasma optical emission spectroscopy.

4.3. *Experimental measurement of antibacterial effects and suppression of biofilm formation*

Measurement of total viable bacterial counts to evaluate antibacterial effects and the suppression of biofilm formation revealed that the new materials and controls suppressed *S. mutans* growth and biofilm formation better than the glass materials with no sustained fluoride release.

Measurement of sustained ion release showed that all materials used in this study released fluoride ions, and measuring the total viable bacterial counts revealed that TM, with the highest sustained fluoride release, had the lowest total viable bacterial counts. Together, these findings strongly suggest that fluoride ions are associated with antibacterial effects and the ability to suppress biofilm formation. A previous study showed that fluoride ions released from dental materials suppressed *S. mutans* growth [55], and in another study, fluoride suppressed *S. mutans* growth and inhibited enolase (phosphopyruvate hydratase) in carbohydrate metabolism [56]. Fluorine is thought to suppress acid production by inhibiting the activity of enolase, an enzyme that converts the glycolytic intermediate, 2-phosphoglycerate, into phosphoenolpyruvate (PEP), thereby downregulating PEP production and in turn decreasing phosphotransferase activity, which requires PEP (the PEP-PTS system) [57]. Ono et al. [58] reported that composite resins containing fluoride ions suppress biofilm formation. These mechanisms are believed to have also contributed to suppressing *S. mutans* growth and biofilm formation by fluoride ions in the present study.

In a study investigating the antibacterial activity of GIC-based materials with bioactive properties [59], Vogel et al. demonstrated that various elements are present in a state of high valency, and positive ions bind to the surface of negatively charged biofilms, generating a net positive charge, which in turn attracts negative ions. In another study [60], Domon-Tawaraya et al. reported that divalent metal ions facilitate binding between fluorine and bacteria, and during glucose metabolism, fluoride ions are released gradually from the bacteria, suppressing acid production. This finding suggests that the new materials used in the present study have antibacterial effects and suppress biofilm formation by promoting fluoride ions to bind to bacteria via sustained release of various ions.

4.4. *Measurement of cell proliferative activity*

GIC releases various ions such as silicate, strontium, and aluminum [61]. The present study revealed that the new materials slowly released silicate and aluminum ions, in addition to calcium, phosphate, and fluoride ions, while 0 YG, 5.4 YG, and 8.9 YG released strontium ions. Therefore, these ions are expected to exert various effects in humans. However, in recent years, more emphasis has been placed on the cellular toxicity of these ions. In the present study, WST-1 was used to measure cell proliferative activity. In the WST-1 method, cell proliferation is measured by colorimetrically measuring dehydrogenation by dehydrogenases such as lactate dehydrogenase [62]. Here, colorimetric assay results showed no significant differences in cytotoxicity between the samples

and controls, suggesting that all materials tested, including the new ones, are essentially not cytotoxic.

5. Conclusions

1 Compressive strength did not differ significantly between the new materials and the conventional GIC-based sealants Fuji VII and Fuji III (which lack calcium), suggesting that mechanical strength is unaffected by calcium content.
2 The sustained levels of calcium and phosphate ion release were significantly higher among the new sealants 5.4 YG, containing 5.4 mol % calcium; 8.9 YG, containing 8.9 mol % calcium; and 10.7 YG, containing 10.7 mol % calcium than among the conventional sealants Fuji VII and Fuji III and the resin-based sealants TM and BS. Therefore, the new materials are expected to have superior reinforcement and remineralization effects on the tooth substance.
3 The sustained level of fluoride ion release was increased in the new materials compared with the conventional sealants Fuji VII and Fuji III and with BS, suggesting that the new materials have greatly superior anticariogenic effects. In addition, cell proliferation assay data suggest that the new materials are not cytotoxic.
4 The new materials can recharge fluoride ions.

Based on these findings, we produced cement for new sealants by reinforcing the material properties of conventional GIC-based sealants. Among the new sealants, 5.4 YG, 8.9 YG, and 10.7 YG were superior because they showed mechanical strength comparable to that of commercial products, excellent antibacterial effects, the ability to suppress biofilm formation, and high levels of sustained calcium ion release.

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