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Bioactive resin-based composite with surface pre-reacted glass-ionomer filler and zwitterionic material to prevent the formation of multi-species biofilm

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ABSTRACT

Objective. This study evaluated the synergetic effect between surface pre-reacted glass-ionomer (SPRG) filler and 2-methacryloyloxyethyl phosphorylcholine (MPC), for inhibiting multi-species biofilm formation, while maintaining or even improving the original beneficial features of SPRG-filled resin-based composite (RBC).

Methods. MPC (1.5–10 wt%) was incorporated into commercial SPRG-filled RBC. Then, the inherent properties of RBC, and ion release and acid-neutralising properties associated with SPRG were investigated. Further, protein adsorptions and bacterial adhesion and viability on the SPRG-filled RBC surfaces were studied using four kinds of oral bacteria; *Streptococcus mutans*, *Actinomyces naeslundii*, *Veillonella parvula*, and *Porphyromonas gingivalis*. Finally, the thickness and biomass of the human saliva-derived biofilm model cultured on test and control samples were analysed.

Results. Addition of MPC content resulted in decreased flexural strength and wettability of SPRG-filled RBC. SPRG-filled RBC released significantly higher amounts of multiple ions as contents of MPC increased. Meanwhile, SPRG-filled RBC with 5-wt% MPC significantly improved acid-neutralising properties than those of other test and control samples ($P < 0.001$). SPRG-filled RBC with 3 wt% MPC significantly reduced the amount of adsorbed bovine serum albumin and proteins from the brain heart infusion medium as compared to the control ($P < 0.01$). A similar trend was observed in the attachment of four types of bacteria and multi-species biofilm ($P < 0.01$).

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Abbreviations: RBC, resin-based composite; SPRG, surface pre-reacted glass ionomer; MPC, 2-methacryloyloxyethyl phosphorylcholine.
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Significance. Despite limitation in terms of deteriorations of some physical properties, addition of 3% MPC to SPRG-filled RBC leads to inhibition of the attachment of multi-species bacteria on its surface, as well as inhibition of biofilm growth. Moreover, the original important bioactive features of SPRG-filled RBC such as ion release and acid neutralisations are either maintained or improved upon adding MPC.

1. Introduction

Resin-based composites (RBC) are widely used for restoring tooth cavity because of their aesthetics and direct-filling capabilities [1]. Despite numerous advantages of RBC, bacterial colonisation may still occur on RBC [2], which could consequently result in secondary caries and demineralisation owing to the production of acid from bacteria [1,3]. Hence, a RBC that would facilitate enamel remineralisation while having antibacterial effect is required.

Surface pre-reacted glass-ionomer (SPRG) filler has been developed with the pre-reacted glass-ionomer technology to impart bioactive functions to restorative materials [4]. SPRG filler consists of fluoraluminosilicate glass and a polyacrylic acid solution which have the ability to release six ions such as aluminium (Al^{3+}), borate (BO_3^{3-}), sodium (Na^{+}), silicate (SiO_3^{2-}), strontium (Sr^{2+}), and fluoride (F^{-}) [3,5]. These multiple ions have the capability to prevent demineralisation and impart acid resistivity to enamel [4,6]. Further, studies have indicated that the ions released from SPRG are associated with potential antibacterial effects, although the effects are limited in terms of inhibiting bacterial adhesion or even biofilm formation [1,5,7,8].

Zwitterionic materials possess chemical moieties with both cationic and anionic groups, and they have been used as biomaterials owing to their antifouling capacities [9–11]. 2-methacryloyloxyethyl phosphorylcholine (MPC) is one of the most popular zwitterionic materials; it has bipolar functional groups that could greatly inhibit protein adsorption and bacterial adhesion through electrostatic attraction by minimising interaction with proteins [12,13]. MPC has been previously used in dental materials such as orthodontic cements, dental composites, and dentin bonding agents [14–16]. Recently, the use of MPC has widened with investigations on the synergetic effects between the anti-biofouling property and properties such as the longevity of light-curable fluoride varnish [17] or mineralisation of calcium silicate-based cement [11]. Especially in the paper by Kwon et al. [11], the synergetic effect between bioactive components of calcium silicate based cement was evident with addition of MPC in the concentration between 1.5 wt% to 10.0 wt%. The study showed not only the addition of antibacterial properties on calcium silicate based cement, but also an increase in bioactive properties due to the zwitterionic nature of the components. Yet, there have been no reports on the synergistic effect of SPRG and MPC on the properties and functions of RBC, which may provide improved anti-bacterial/anti-biofouling functions with acid resistance/prevention of demineralisation.

Accordingly, the aim of this study was to develop composites of SPRG-filled RBC with MPC to achieve improved antibacterial properties through the anti-biofouling capacity of MPC, while maintaining or even improve the original advantageous properties of SPRG-filled RBC. The null hypothesis of this study was that the addition of MPC into SPRG-filled RBC would result in no differences of antibacterial properties and other physical/chemical properties including ion release and acid neutralization.

2. Material and methods

2.1. *Preparation of MPC-incorporated SPRG*

Commercially available MPC powder (Sigma-Aldrich, St. Louis, MO, USA) and SPRG-filled RBC (Beautifil, Shofu, Kyoto, Japan) were used in this study. MPC powder was mixed with SPRG-filled RBC at various weight percentages; 1.5, 3.0, 5.0, 7.5, and 10.0 wt%, and SPRG-filled RBC without MPC was used as a control. The compositions of the test and control materials were based on the previous studies [11,17] and are summarised in Table 1. All the samples were prepared before light polymerisation. The MPC powder were first mixed into liquid-like state of SPRG-filled RBC by through hand-mixing. The samples were then vortexed followed by through mixing using the high-speed mixer (SpeedMixer, Hauschild, Hamm, Germany), which has been shown to result in thorough dispersion of powder in many different liquid-like material including those previous studies that used of light-curable fluoride varnish [17] or calcium silicate-based cement [11]. The specimens were prepared with different shapes, as described below in Section 2.2 and 2.3. After the preparation, the samples were polymerised using a light-emitting diode (LED) light-curing unit (Elipar S10; 3M ESPE Co., Seefeld, Germany).

2.2. *Water sorption and solubility*

The water sorption test was adapted from a previous study [18]. Each material was placed in a mould of 20 mm diameter and 1.5 mm height to form a disc. The sorption and solubility of the RBC were determined according to the International Standard, ISO 4049 [19]. The mean diameters of the samples were calculated by measuring two diameters, and the mean thicknesses of the samples were calculated by measuring the thickness of three equally spaced points on the circumference. These values were then used to calculate the volume (V) (in 0.01 mm^3) of the samples Then, the samples were weighted on an analytical balance (accurate to 0.01 mg) (XS105, Mettler Toledo AG, Greifensee, Switzerland) with a reproducibility

Table 1 – Composition of the control and experimental groups.

Groups	Resin-based composite (RBC) with surface pre-reacted glass-ionomer (SPRG) filler (Beautifil), wt%	2-Methacryloyloxyethyl Phosphorylcholine (MPC), wt%
Control	100	0
1.5% MPC	98.5	1.5
3% MPC	97.0	3.0
5% MPC	95.0	5.0
7.5% MPC	92.5	7.5
10% MPC	90	10.0

of 0.1 mg until a constant mass (m_1) was obtained. Then, all the samples were immersed in distilled water and placed in a water bath maintained at 37 °C for 7 days. After that, the samples were blotted until they were visibly free from moisture, and dried by waving in air for 15 s, and then, they were weighted to determine the final mass (m_2). Finally, the samples were placed in a desiccator and weighted daily until a constant dry mass (m_3) was obtained. Water sorption (W_{sp}) in g/mm^3 was calculated using the Eq. (1) and water solubility (W_{sl}) was calculated using Eq. (2).

$$W_{sp} = \frac{m_2 - 3}{V} \quad (1)$$

$$W_{sl} = \frac{m_1 - m_3}{V} \quad (2)$$

2.3. Flexural strength

For the preparation of the samples, each material was placed in a mould (25 mm × 25 mm × 2 mm) without air bubbles or voids. Then, all the samples were polymerised following the method described above using an LED light-curing unit (Elipar S10; 3 M ESPE Co., Seefeld, Germany). Then, the samples were stored in distilled water at (37 ± 1) °C for 24 h. Maximum loads were measured with a universal testing machine (Instron 5942, Instron, Norwood, MA, USA) according to ISO 4049 [19]. Flexural strength was calculated as,

$$S = 3P_{max}L/(2bh^2) \quad (3)$$

where, P_{max} is the fracture load, L is the span, and b and h are the width and thickness of the specimen.

2.4. Wettability

The wettability was determined in accordance with previous studies [20,21], where the distilled water was chosen as the reference liquid [20,22]. Each material was placed in a mould with a diameter of 15 mm and thickness of 1 mm to form a disc. The static contact angle following 10 s after a 2 μL droplet of distilled water (Sigma-Aldrich, St. Louis, MO, USA) was placed on the sample surface was measured using a video contact angle goniometer (SmartDrop, Femtobiomed Inc, Gyeonggido, Korea).

2.5. Ion release

To fabricate the samples, each material was moulded into a disc using a mould 15 mm in diameter and 1 mm in height. Then, each sample was stored in 5 mL of distilled water at 37 °C. After 24 h, samples containing eluted ions were collected, and the concentration of each ion was determined. Elemental analysis of the Na^+, SiO_3^{2-}, Sr^{2+}, BO_3^{3-}, and PO_4^{3-} and F^- ions released from discs was performed using an inductively coupled plasma optical emission spectrometer (Optima 8300, PerkinElmer, MA, USA) and fluoride ion electrodes (920A, Orion, Boston, USA).

2.6. Acid neutralisation

The samples were prepared *via* the same method described above for the flexural strength. To characterise the acid neutralisation ability, a solution of lactic acid (Sigma-Aldrich, Steinheim, Germany) (pH 4.0) was prepared. Three specimens with 25 mm × 25 mm × 2 mm dimensions were immersed in 2.14 mL of the lactic acid solution, yielding a specimen volume to lactic acid solution volume ratio of 0.14 to 1 [23,24]. After immersing the samples in the lactic acid solution, the change in the pH of the acid solution was monitored using a digital pH-meter (Orion 4 Star, Thermo Fisher Scientific Inc., Singapore), which was calibrated at pH 4.01 and pH 7.00 immediately before use. The pH was measured as soon as the specimens were submerged in the lactic acid solution. The pH change of the solution and the time required for the solution pH to increase from 4.0 to 5.5 were analysed.

2.7. Protein adsorption

Protein adsorption was tested out according to a previously established method [15]. Each material was placed in a mould with a diameter of 15 mm and thickness of 2 mm to form a disc. All samples were immersed in a fresh phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) for 1 h at room temperature and immediately immersed in a protein solution of either bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL, USA) or brain heart infusion (BHI; Difco, Sparks, MD, USA) broth (both at a concentration of 2 mg of protein per mL of PBS and a volume of 100 μL). After incubation at 37 °C for 1 h, the samples were gently rinsed twice with fresh PBS. After 4 h of incubation under sterile humid conditions at 37 °C, any protein that was not adhered to the surface was removed by washing twice with PBS. The amount of protein adhered to samples was measured using 200 μL of micro-bicinchoninic acid (Micro BCA™ Protein Assay Kit; Pierce Biotechnology) followed by incubation at 37 °C for 30 min. Quantitative analysis of the proteins adsorbed on the surfaces was performed following the measurement of the absorbance at 562 nm using a micro-plate reader (Epoch, BioTek Instruments, VT, USA).

2.8. Bacterial attachment, colony forming units, and viability

Bacterial analyses were carried out using four different bacterial species. The selected species represent initial colonisers: *S. mutans* (ATCC 25175) and *Actinomyces naeslundii* (KCOM 1942), early coloniser: *Veillonella parvula* (KCOM 1301), and late coloniser: *Porphyromonas gingivalis* (KCOM 2799). *S. mutans* was cultured in BHI broth (Difco, Sparks, MD, USA) under aerobic conditions in an incubator at 37 °C. *A. naeslundii*, *V. parvula*, and *P. gingivalis* were obtained from Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea). They were all cultured in 3.8% BHI broth supplemented with 0.5% yeast extract (Becton Dickinson and Co. Sparks, MD, USA), 4 μg/mL resazurin (Sigma-Aldrich, St. Louis, MO, USA), 5 μg/mL hemin (Sigma-Aldrich, St. Louis, MO, USA), 0.05% L-cysteine HCl·H_2O (Sigma-Aldrich, St. Louis, MO, USA), and 2 μg/mL vitamin K (Sigma-Aldrich, St. Louis, MO, USA), under anaerobic condition. Anaerobic atmosphere was generated using an Anaeropack system (Mitsubishi Gas Chemical, Tokyo, Japan).

For microscopic examination of the attached bacteria, bacteria on the samples were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M PBS for at least 30 min at room temperature. Then, the samples were post-fixed with 1% OsO_4 dissolved in 0.1 M PBS for 2 h, dehydrated in an ascending gradual series of ethanol, treated with isoamyl acetate, and subjected to critical point drying (LEICA EM CPD300; Leica, Wien, Austria). Then, the discs were coated with Pt (5 nm) using an ion coater (ACE600; Leica) and examined by scanning electron microscopy (FE-SEM; Merin, Carl Zeiss, Oberkochen, Germany) at 2 kV.

To evaluate bacterial colony forming units (CFU), the adherent bacteria were harvested in 1 mL BHI broth or modified BHI broth through sonication (SH-2100; Saehan Ultrasonic, Seoul, Korea) for 5 min. Then, 100 μL of this bacterial suspension was spread onto a BHI agar plate and incubated at 37 °C for 24 h. Then, the total numbers of colonies were counted.

Finally, the viability of the adherent bacteria was examined by staining with a live/dead bacterial viability kit (Molecular Probes, Eugene, OR, USA), according to the manufacturer's protocols. Equal volumes of Syto 9 dye and propidium iodide, which stain live and dead bacteria, respectively, from the kit were mixed thoroughly. Of the mixture, 3 μL was added to 1 mL of the bacterial suspension prepared as described above. After 15 min incubation at room temperature in dark, the stained samples were observed under a confocal laser microscope (CLSM, LSM700, Carl Zeiss, Thornwood, NY, USA). Live bacteria appeared green while the dead bacteria appeared red.

2.9. Saliva-derived biofilm model and biomass measurement

Human saliva has been shown to be ideal for growing plaque microcosm biofilms *in vitro*, with the advantage of maintaining much of the complexity and heterogeneity of the dental plaque *in vivo* [25]. A protocol from a previous study was adapted [26], where human saliva was collected from healthy adult donors who had no active caries or periodontal disease, and had not taken antibiotics within the previous 3 months. Participants did not brush teeth for 24 h and abstained from food/drink intake for at least 2 h prior to donating saliva. Saliva was collected from six individuals and mixed in equal proportions. The mixed saliva was then diluted in sterile glycerol to a concentration of 30% and stored at −80 °C to be used as a biofilm model [27]. The biofilm model was cultured in McBain medium supplemented with mucin (type II, porcine, gastric) (2.5 g/L), bacteriological peptone (2.0 g/L), tryptone (2.0 g/L), yeast extract (1.0 g/L), NaCl (0.35 g/L), KCl (0.2 g/L), $CaCl_2$ (0.2 g/L), cysteine hydrochloride (0.1 g/L), haemin (0.001 g/L), and vitamin K1 (0.0002 g/L) at 37 °C for 24 h [25,28]. From the cultured medium, 1.5 mL of the bacterial solution was placed on the specimen. Following 8 h, 16 h, and 24 h of incubation, additional 1.5 mL of the bacterial solution was placed on the specimen, after each period. Biofilms were allowed to grow for a total of 48 h. Specimens were stained with live/dead bacterial viability kit (Molecular Probes, Eugene, OR, USA) using the same method described above for bacterial staining. The biofilm was then visualised at five randomly chosen positions using CLSM (LSM700, Carl Zeiss, Thornwood, NY, USA). Axially stacked biofilm images were captured and thicknesses of the biofilm were calculated using the software (Zen, Carl Zeiss, Thornwood, NY, USA). Additionally, the COMSTAT plug-in (Technical University of Denmark, Kongens Lyngby, Denmark) along with the ImageJ (NIH, Bathesda, MA, USA) software was used to determine the average biomass.

2.10. Statistical analysis

All statistical analyses were performed using IBM SPSS software, version 23.0 (IBM Korea Inc., Seoul, Korea) for Windows, with data from at least three independent experiments. The results obtained from the control and experimental groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test. Comparison between two groups was used as the independent *t* test. $P < 0.05$ is considered statistically significant.

3. Results

3.1. Physical and mechanical properties

Increasing trend of water solubility was noted with increasing amount of MPC added to SPRG-filled RBC (Fig. 1A). Samples with 7.5 and 10% MPC showed significantly higher level of water solubility than other test and control samples. Yet, all the samples met the requirement of ISO 4049, which specifies a water solubility of less than 7.5 μg/mm^3.

As for the flexural strength, the results reveal a decreasing trend of strength with increasing amount of MPC added. In particular, samples with 7.5% MPC (40.64 ± 5.86 MPa) and 10% MPC (43.74 ± 3.01 MPa) showed significantly lower values of flexural strength, with the values being four times lower than that of the control (200.96 ± 5.17 MPa) ($P < 0.001$, Fig. 1B). Additionally, only 1.5, 3, and 5% MPC groups along with the control fulfilled the minimum requirements (80 MPa) specified in ISO 4049.

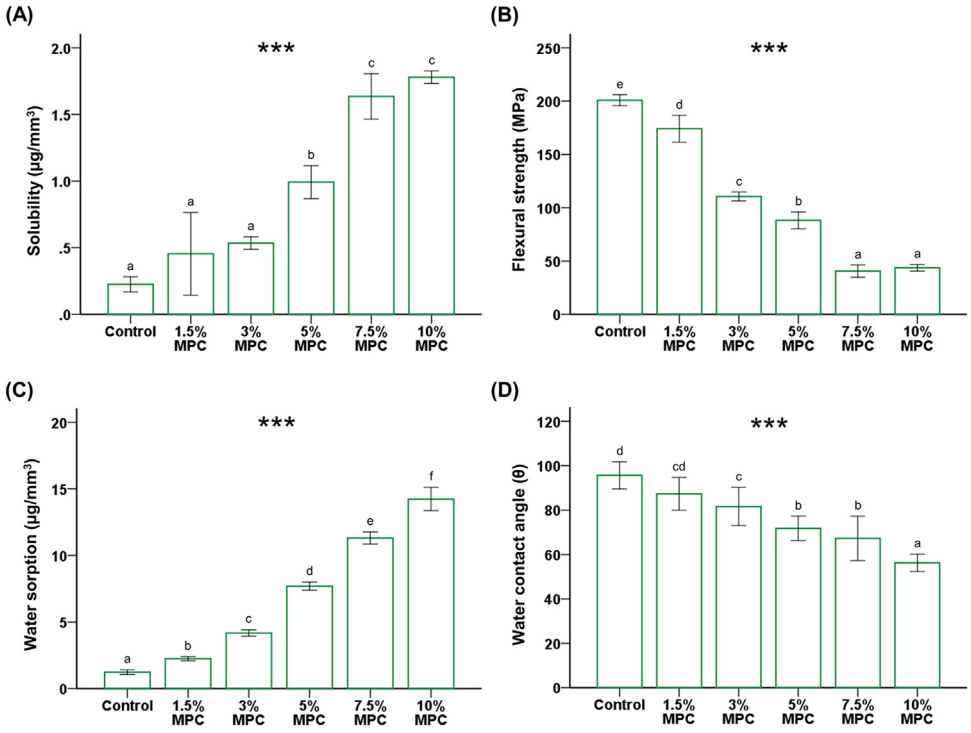

Fig. 1 – Comparison of the water solubility (A), flexural strength (B), water sorption (C), and water contact angle (D) between different groups of samples. Different letters above bars indicate significant differences. ***$P < 0.001$ for comparison between SPRG-filled RBC with different amounts of MPC.

The water sorption showed a similar trend as the water solubility; significant differences were observed among the test and control samples, while the sample with 10% MPC showed the highest water sorption ($14.24 \pm 0.86\ \mu g/mm^3$) ($P < 0.001$, Fig. 1C). All groups satisfied the ISO requirements for water sorption, with the water sorption requirement stated as $40\ \mu g/mm^3$.

Finally, the wettability of the samples was evaluated by measuring the water contact angle on their surfaces (Fig. 1D). The average contact angle of the control sample was found to be close to 100° ($95.62 \pm 6.13°$). No significant differences between the water contact angles of the control and 1.5% MPC was observed (Fig. 1D). The sample with 10% MPC showed a dramatically decreased contact angle ($56.25 \pm 3.91°$), which is about half that of the control.

Overall, it was evident that samples with 7.5 and 10% MPC showed significantly degraded physical and chemical properties compared to those of the control.

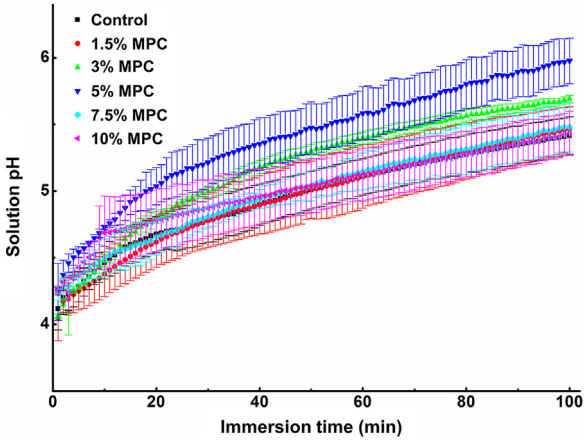

Fig. 2 – pH of the acid solution according to the immersion of different SPRG-filled RBC samples: SPRG-filled RBC with no MPC (Control) and SPRG-filled RBC with 1.5, 3, 5, 7.5 and 10% MPC.

3.2. *Ion-releasing and acid-neutralising properties*

The ions released from the control and the test samples are listed in Table 2. The results indicate an increasing trend of ion release for Na^+, SiO_3^{2-}, BO_3^{3-}, PO_4^{3-}, and F^- with an increase in the amount of MPC added. The 1.5% MPC samples showed significantly higher release of Sr^{2+} and F^- ions than the control ($P < 0.001$). As for all of ions measured, there were no significant differences between the 1.5 and 3% MPC samples; however, the release from samples with 5% or higher contents of MPC were found to be significantly higher than those from the rest ($P < 0.001$). Especially, the amount of PO_4^{3-} ions released from sample with 7.5% MPC was more than double compare to the 5% MPC sample (22.87 ± 2.33 ppm *vs* 9.71 ± 2.86 ppm). Fig. 2 shows the time-dependent changes in the pH of the lactic acid solution in which the samples were immersed. The control took 100 ± 0.14 min to reach pH 5.5 from pH 4, which was significantly reduced to 54 ± 0.18 min for the sample with 5% MPC ($P < 0.001$). However, there are no significant differences in the time taken to increase the pH from 4 to 5.5 amongst the rest of the test samples, even those with higher contents of MPC.

Table 2 – Concentrations of Na, Si, Sr, B, F, and P based on the release concentration from each group.

	Concentration (ppm) released												
	Control		1.5% MPC		3% MPC		5% MPC		7.5% MPC		10% MPC		P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Na^+	0.60^a	0.09	0.92^{ab}	0.06	1.35^b	0.20	3.42^c	0.45	6.90^d	0.22	14.05^e	0.47	<0.001
SiO_3^{2-}	0.31^a	0.04	0.53^{ab}	0.09	0.70^b	0.06	1.11^c	0.13	1.91^d	0.05	3.86^e	0.31	<0.001
Sr^{2+}	1.11^a	0.2	1.82^b	0.31	2.16^b	0.52	2.09^b	0.49	2.08^b	0.24	3.59^c	0.18	<0.001
BO_3^{3-}	1.22^a	0.11	2.26^{ab}	0.38	3.57^b	0.27	7.69^c	0.97	14.47^d	0.25	29.87^e	1.21	<0.001
F^-	0.47^a	0.05	0.73^b	0.13	0.77^b	0.16	1.06^c	0.12	2.48^d	0.12	5.92^e	0.09	<0.001
PO_4^{3-}	0.00^a	0.00	3.97^{ab}	2.81	6.78^{bc}	2.16	9.71^c	2.86	22.87^d	2.33	34.95^e	2.92	<0.001

Different superscript letters indicate statistically significant differences between groups.

3.3. *Protein adsorption*

There was general decrease in BSA adsorption with addition of MPC up to 3%, but the amount of adsorbed BSA increased after that point (Fig. 3A). The amount of adsorbed BSA was significantly lower on sample with 3% MPC compare to that on the control ($P < 0.01$), while there were no statistical differences in adsorbed BSA among the control, 1.5% MPC, 5% MPC, 7.5% MPC and 10% MPC ($P > 0.05$). Fig. 3B shows the amount of proteins adsorbed from BHI medium, with the results being similar to those with BSA adsorption. Again, the 3% MPC sample showed significantly lower adsorption compared to those of other samples, while there were no statistical differences among rest of samples ($P > 0.05$, Fig. 3B).

3.4. *Bacterial attachment, colony forming units, and viability*

The FE-SEM images clearly show that less *S. mutans* are attached to the surfaces of test samples than on the control (Fig. 4A). Still, higher number of *S. mutans* are evident with 10% MPC samples compare to other test samples (Fig. 4A). The result was further confirmed by quantitatively analysis of CFU counts. Samples with 1.5, 3, 5, 7.5 and 10% MPC showed significantly lower CFU counts than that of the control ($P < 0.001$, Fig. 4B). Further, the CFU counts for samples with 1.5, 3, 5 and 7.5% MPC were significantly lower than that for 10% MPC sample. There are no statistically significant differences between the results for 1.5, 3, 5 and 7.5% MPC samples, but the sample with 3% MPC showed the lowest average count. The results of viability staining are consistent with the above findings. The number of viable bacteria attached to the samples, which was stained with a green fluorescent stain, was found to be greater for the control group than for the test groups (Fig. 4C). Overall, the sample with 3% MPC showed significant bacterial inhibition while showing significant lower protein adsorption compared to that of the control. Therefore, only 3% MPC sample was used for further experiments. Additional bacterial analyses were carried out using three more bacterial species: *A. naeslundii*, *P. gingivalis*, and *V. parvula*, and the results showed trends similar to that with *S. mutans* (Fig. 5). Fewer bacteria attached to 3% MPC sample than to the control, as observed in FE-SEM (Fig. 5A, D, and G). The CFU results are consistent with the FE-SEM results, with the CFU counts of 3% MPC sample being significantly lower than that of the control for all bacterial species (Fig. 5C, F, and I, $P < 0.01$). The viability results indicated that less live bacteria (visible as green) were attached to 3% MPC sample than to the control in case of all bacterial species (Fig. 5B, E, and H).

3.5. *Biofilm thickness and biomass*

The results show distinctive differences between the control and 3% MPC groups, which is consistent with those observed with a single bacterium (Fig. 6A). The images indicate a relatively thin biofilm on 3% MPC sample compared to that on the control, which was confirmed using the thickness calculated by the software, which indicated significantly thinner biofilm on 3% MPC sample than on the control ($P < 0.01$, Fig. 6B). Finally, the biomass of the biofilm was found to be significantly reduced in 3% MPC sample than in control ($P < 0.01$, Fig. 6C).

4. Discussion

In this study, a new RBC containing SPRG and zwitterionic material was investigated to study the synergetic effect of bioactivity from SPRG and antibacterial activity of zwitterionic material. We successfully incorporated MPC into SPRG-filled RBC, which not only imparted additional anti-biofouling property to the composite but also led to the maintenance or even improvement of the original advantages of SPRG-filled RBC.

Commercially available SPRG-filled RBC was used in the present study along with MPC that was previously investigated in our laboratory [11,17]. Physical properties, including the water solubility, flexural strength, water sorption, and water contact angle were evaluated in accordance with the relevant international standards or previous literature, which provided references of the basic properties of the material in terms of its use as an RBC. The results of this study indicated that high concentrations of MPC such as 7.5 or 10% would result in dramatic deteriorations of such physical properties. Also, even with low concentration of MPC, some of physical properties such as flexural strength and water sorption, deteriorated with addition of even low amount of MPC. As some of commercially available product of RBC showed low clinical success in long term period [29], these experimental groups may be limited in terms of direct application in clinic, and further studies may be required to overcome these problems.

The bioactive properties of SPRG-filled RBC have been demonstrated in previous studies [1,30]. Additionally, our previous study [11] indicated that the addition of MPC into

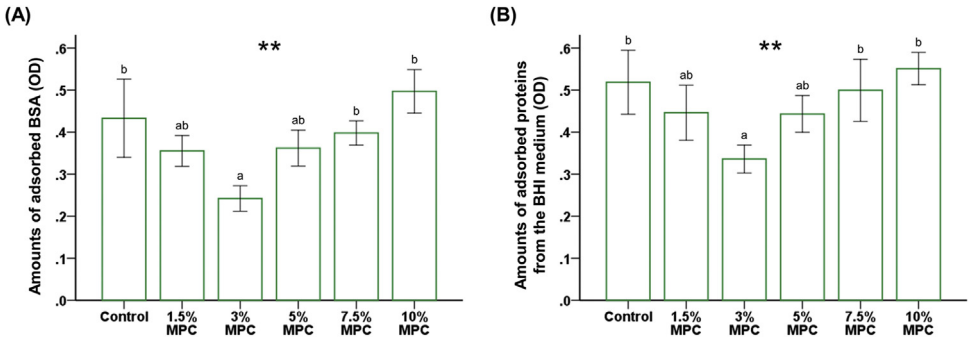

Fig. 3 – Comparison of the optical density (OD) of adsorbed bovine serum albumin (BSA) (A) and protein adsorbed form brain heart infusion (BHI) medium (B) among SPRG-filled RBC with different concentrations of MPC. Different letters above bars indicate significant differences. **P < 0.01 for comparison between SPRG-filled RBC with different concentrations of MPC.

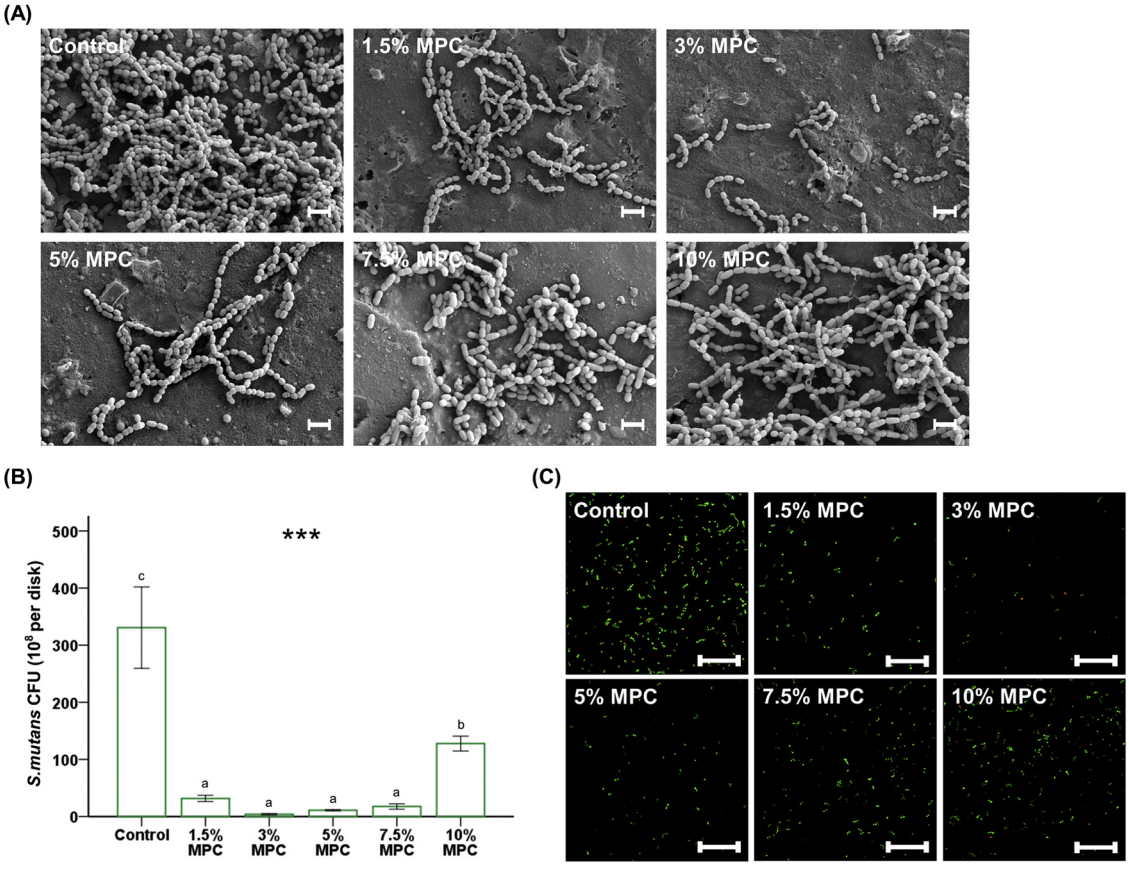

Fig. 4 – Qualitative scanning electron images of bacteria attached to the surfaces of SPRG-filled RBC without MPC (Control), and SPRG-filled RBC with 1.5, 3, 5, 7.5 and 10% MPC at a magnification of 5000× (A). Scale bar is 2 μm. Colony-forming unit (CFU) counts derived from bacteria attached on the surfaces of each SPRG-filled RBC (B). Different letters above bars indicate significant differences. ***P < 0.001 for comparison between SPRG-filled RBC with different concentrations of MPC. Representative live/dead staining images of bacteria attached on the surfaces of control and experimental groups (C). Scale bar is 500 μm.

calcium silicate based cement result in synergetic effect by increase in bioactive properties due to the zwitterionic nature of the components. Hence, the possibilities of synergetic effect of adding MPC into SPRG-filled RBC were considered by two properties: ion-releasing properties and acid neutralisation. To inhibit secondary caries, it would be ideal for the SPRG-filled RBC to increase the cariogenic pH from 4.0 to a relatively safe pH of 5.5 or above, and quickly neutralise the local acids [31]. This study demonstrates that when SPRG-filled RBC containing MPC is immersed in a lactic acid solution with pH 4.0, it could increase the pH to the critical pH of 5.5. According to the Stephan curve, it would be desirable for a specimen to quickly

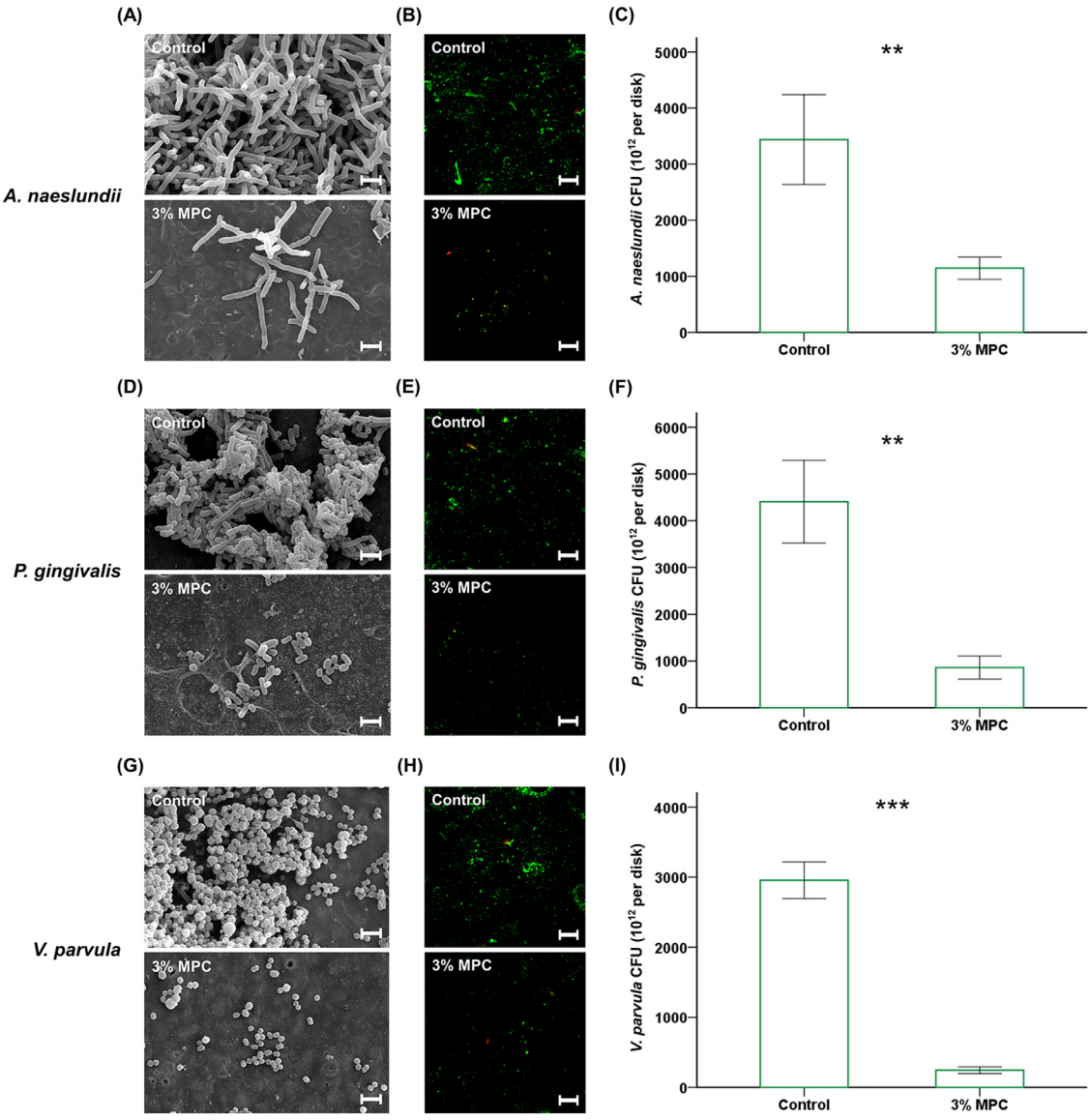

Fig. 5 – Qualitative scanning electron images of bacteria attached to the surfaces of SPRG-filled RBC without MPC (Control) and SPRG-filled RBC with 3% MPC at a magnification of 5000×, for *A. naeslundii* (A), *P. gingivalis* (D), and *V. parvula* (G). Scale bar is 2 μm. Representative live/dead staining images of bacteria attached on the surfaces of the control and 3% MPC (B, E, H) samples. Scale bar is 100 μm. Colony-forming unit (CFU) counts derived from bacteria attached on the surfaces of the control and 3% MPC (C, F, I) samples. Different letters above bars indicate significant differences. $^{**}P < 0.01$, $^{***}P < 0.001$ for comparison between SPRG-filled RBC with different concentrations of MPC.

increase the cariogenic pH from 4.0 to 5.5 in order to help resist caries [31]. This implies that quickly increasing the pH of local plaque from the cariogenic level to the safety level plays an important role in inhibiting the demineralisation of the tooth. The SPRG filler has been reported to demonstrate remarkable acid-neutralising ability by releasing ions such as Sr^{2+} and Na^{+} [8]. Ions released from the SPRG filler rapidly neutralise acids produced by the lactic acid solution, and thus would prevent enamel demineralisation. In this study, it was evident that MPC incorporated in SPRG-filled RBC at a low concentration (1.5–3%) or high concentration (7.5–10%) hardly affected the acid-neutralising properties of SPRG, while significantly improved acid-neutralising property was noted at a MPC concentration of 5% MPC. Such a result seems to arise from the release of ions from the sample.

Analysis of the ions released from SPRG-filled RBC indicated that an increased amounts of ions were released from the samples with an increased amount of MPC. Further, the sample with 5% MPC showed a significantly higher level of release of most of the ions compared to those of 1.5% MPC, 3% MPC and control samples; this trend seems similar to that of the acid-neutralisation properties. However, even with higher amount of ion released from 7.5% and 10% MPC compare to 5% MPC, there was also burst release of PO_4^{3-} ions which seems to played a role in acidity of the surrounding solutions. It is unclear how the addition of MPC results in such an increase,

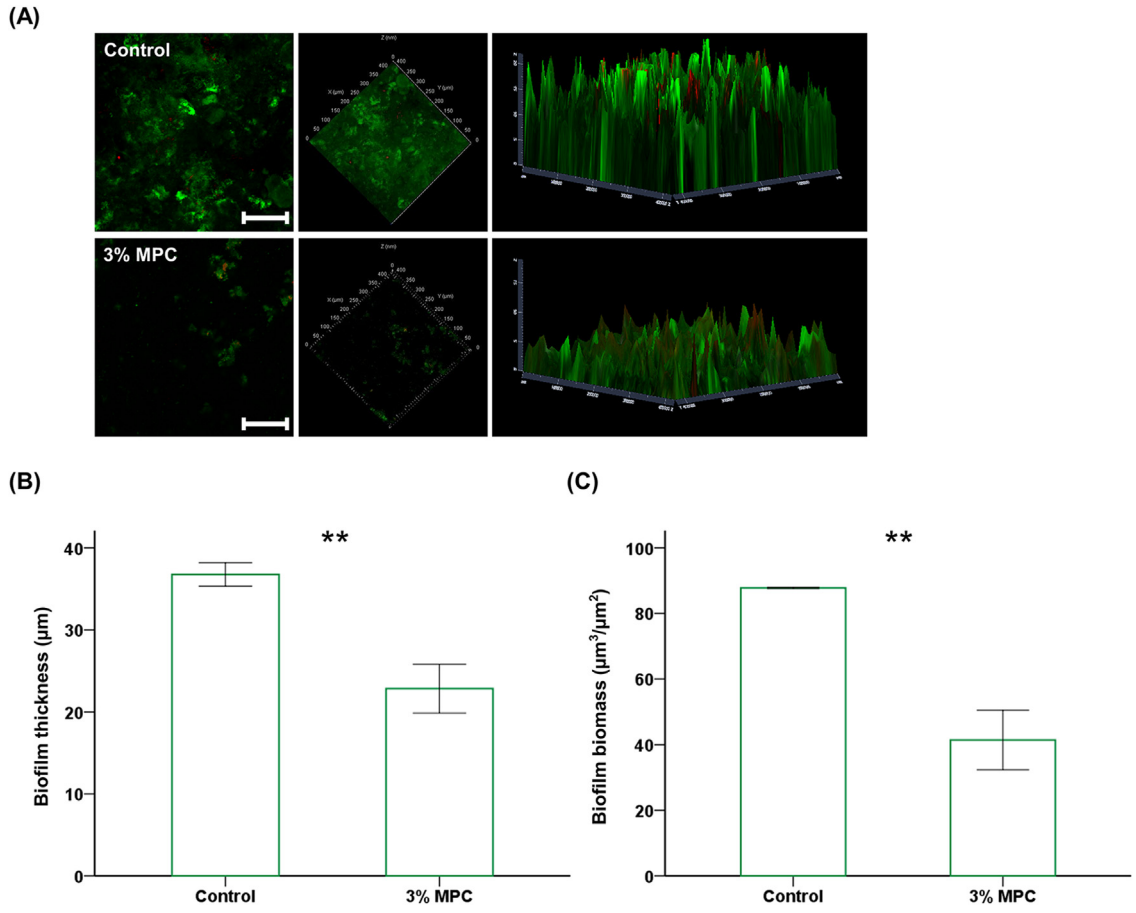

Fig. 6 – Representative live/dead staining images of biofilms attached on the surfaces of SPRG-filled RBC without MPC (Control) and SPRG-filled RBC with 3% MPC (A). Scale bar is 100 μm. Quantitative analysis of the thickness (B) and biomass (C) of the biofilms. Different letters above bars indicate significant differences. $^{**}P < 0.01$ for comparison between SPRG-filled RBC with different concentrations of MPC.

but it may be because the negatively charged polar groups of MPC have a catalytic effect on the surface, as suggested previously [11]. It may be possible that the structure of MPC, with a polar phospholipid side chain orientated such that the polar heads face outward may interact with the liquid while the non-polar tail region faces inward in the bilayer [11].

The attachment of oral bacteria to the RBC surface is initiated by the adsorbed protein, and protein attachment on the surface is essential for the bacterial attachment. Previous studies have demonstrated that the addition of MPC results in a hydrophilic surface, while most proteins are known to adsorb more on hydrophobic surfaces than hydrophilic surfaces [32]. In this study, we assessed the protein adsorption using the BSA protein, which is considered to be a protein with a quite general structure, and a mix of proteins that are present in bacterial BHI culture medium. First, it was noted that the amount of adsorbed BSA was significantly lower for 3% MPC than for the control. A similar trend was observed for the amount of proteins adsorbed from BHI medium. MPC is a part of chemical group known as a zwitterionic material. Zwitterionic materials are a family of materials that possess both cationic and anionic moieties, which are therefore characterized by highly charged groups and high dipole moment, but are still neutral in terms of overall charge [10,33]. MPC is a methacrylate with a phospholipid polar group in the side chain [32]. Phospholipid molecules generally consist of hydrophilic heads that are attracted to water and hydrophobic tails that are repelled by water [34]. Thus, in water, the phospholipids of MPC orient themselves into a bilayer in which the non-polar tails face the inner area of the bilayer and the polar heads face outward to interact with water, resulting in highly hydrophilic properties of the surface [32]. When MPC is exposed to a protein solution, the unique structure of MPC would allow a large amount of free water to be present around the phosphorylcholine group, whereas there would be no bound water in hydrated MPC [12]. The presence of bound water would cause protein adsorption, whereas the presence of free water would repel protein adsorption. Therefore, the addition of MPC to SPRG-filled RBC results in protein-repellent properties [35]. Previous studies have demonstrated that copolymerisation of MPC with resin-like polymers results in MPC immobilisation, causing long-lasting and durable prevention of protein attachment [36,37].

A critical pathogenic event in the process of biofilm formation is bacterial attachment, since this process represents a

turning point for planktonic bacteria that lead to biofilm formation [38]. Moreover, the initial step of bacterial attachment is the adsorption of salivary-derived proteins as a salivary pellicle that can mediate bacterial attachment and biofilm formation [39]. Hence, the protein-repellent properties of MPC-incorporated SPRG-filled RBC would result in resistance to bacterial attachment, which was analysed with four different bacteria: *S. mutans*, *A. naeslundii*, *P. gingivalis*, and *V. parvula*. The microorganisms tested in this study are the key bacteria that are frequently associated with oral diseases. It is well known that *S. mutans* is the initial carious bacteria. Bacteria involved in the initial colonisation of tooth surface contribute to the formation of a biofilm. Additionally, *A. naeslundii* and *V. parvula* are involved in early stage of oral biofilm formation, while *P. gingivalis* is associated with late colonisation [40,41]. Indeed, the results confirmed a significant reduction in bacterial attachment on the surfaces of all SPRG-filled RBC with MPC than on the control, as indicated by microscopy and CFU counting for all four bacteria.

Finally, we fabricated saliva-derived biofilm model and evaluated the thickness and biomass of the formed multi-species biofilm by analysing the CLSM images. Real saliva is a complex system, and McBain medium was used to mimic the saliva and enable the maintenance of stable salivary microcosms [25,27,28]. Mucin will provide a similar environment as that of natural saliva, while bacteriological peptone, tryptone, and yeast extract provide nutrition for bacterial growth [26]. To the best of our knowledge, this is the first study examining multi-species biofilm formation on SPRG-filled RBC containing MPC. The results revealed that both the thickness and biomass of the biofilm formed on 3% MPC was significantly lower than on the control. The most effective way to prevent and manage dental caries is to maintain a healthy oral cavity environment by inhibiting the biofilm formation, rather than inhibiting a single specific species of bacterium [42]. From this perspective, our evaluation confirms the anti-biofouling effect on a single bacterium as well as multi-species biofilm in SPRG-filled RBC containing 3% MPC.

5. Conclusion

Despite the limitation of the *in vitro* experiments in terms of complications in the oral environment, including salivary flow and complex interactions between materials and surrounding tissues, as well as evident deteriorations of physical properties by adding MPC, the present study clearly indicates that the addition of an appropriate amount of MPC along with SPRG to RBC results in synergetic effects; not only provide anti-bacterial properties to SPRG, but enhanced original features of SPRG such as ion release and acid neutralization.

The RBC containing SPRG filler along with 3% MPC inhibited multi-species bacterial attachment and biofilm formation. This inhibition is due to the protein-repellent properties of the hydrophilic surface with MPC, while both the release of ions and acid neutralisation properties related to SPRG were maintained or even improved with the addition of MPC.

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