

Fluoride varnish containing chitosan demonstrated sustained fluoride release

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Fluoride varnish is a professionally applied product that prevents dental caries. However, fluoride varnishes do not provide sustained fluoride release. The objective of this study was to prepare fluoride varnish formulations containing various amounts of chitosan that would generate sustained fluoride release. We evaluated their chemical structure, viscosity, and *in vitro* fluoride release. Furthermore, the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and direct contact test were used to determine varnish cytotoxicity. We found that all fluoride varnish formulations had the same chemical structure. Their viscosity demonstrated a chitosan concentration-dependent increase. *In vitro* fluoride release showed a sustained fluoride release. The chitosan fluoride varnishes were cytotoxic to human gingival fibroblasts. We propose the new fluoride varnish formulation as a potential material to be used as a sustained release fluoride varnish.

Keywords: Chitosan, Fluoride varnish, Sustained release

INTRODUCTION

Dental caries is a major oral health problem, with the global decayed, missing, and filled teeth scores of 12-year-old children increasing over the past 10 years¹. There are several methods for dental caries prevention, including oral hygiene instruction, pit and fissure sealants, and topical fluoride application, *i.e.* fluoride gels and fluoride varnishes. However, the most effective caries-protective results occur when fluoride is consistently maintained at a low level in the oral cavity². The salivary fluoride levels after using fluoride toothpaste, mouth rinsing, gel and varnish were analyzed and the results indicated that the fluoride level remained above the baseline for 48 h after applying fluoride varnish^{3,4}.

The advantage of fluoride varnishes over fluoride gels is that the varnish increases the contact time of fluoride on the tooth surface⁵. Therefore, fluoride concentration can be maintained in the oral environment and form calcium fluoride that deposits on the tooth surface to prevent dental caries⁶⁻⁸. There are many studies demonstrating that the professional application of fluoride varnish prevents dental caries⁹⁻¹². The amount of fluoride released from a varnish typically decreases in a few hours and continues to decline for up to 24 h, while the cumulative fluoride ion release increases in the first hour up to 24 h¹³. However, the release of fluoride ions from dental material can be controlled or sustained by encapsulating or coating sodium fluoride particles with either natural polymers or synthetic polymers, such as gelatin^{14,15}, ethylcellulose¹⁵, and polysiloxane¹⁶.

Chitosan is a linear copolymer of D-glucosamine

and N-acetyl-D-glucosamine¹⁷. This polymer is used in biomedical applications because of its biodegradability, biocompatibility, nontoxicity, and antimicrobial-antifungal properties¹⁷. Chitosan has been studied in a wide range of dental applications, including drug delivery systems¹⁸⁻²⁰, guided tissue regeneration^{21,22}, surface modification of dental implants²³⁻²⁵, qualitative modification of toothpaste^{26,27}, and dental restorative materials²⁸. Furthermore, chitosan was previously investigated for the controlled release of fluoride ions using spray drying²⁹ and emulsion dispersion techniques²⁷. These studies demonstrated sustained fluoride ion release. We hypothesized that a controlled release system using chitosan would maintain fluoride release at a low level over time. However, the influence of chitosan on fluoride release from fluoride varnish has not been investigated. The purpose of this study was to prepare new fluoride varnishes containing various amounts of chitosan and characterize the chemical structure, viscosity, *in vitro* fluoride release, and cytotoxicity of these varnishes.

MATERIALS AND METHODS

Fluoride varnish preparation

The base varnish was prepared by mixing fully hydrogenated rosin (Foral™ AX-E, Eastman Chemical, Kingsport, TN, USA) with absolute ethanol (EMSURE®, Merck, Darmstadt, Germany) at a 3:1 (w/w) ratio in a closed vessel on a stirrer at room temperature for 24 h. Sodium fluoride (NaF), particle size <45 μm, (EMPROVE®, Merck) was added to the varnish base at

a concentration of 50 mg/mL. One percent (w/v) chitosan (molecular weight 310,000–375,000 Da and $\geq 75.0\%$ degree of deacetylation) (Sigma-Aldrich®, Merck, St. Louis, MO, USA) in 1% (v/v) acetic acid (Sigma-Aldrich®, Merck) was added at concentrations of 20, 40, 60, or 80 $\mu\text{L/mL}$. The ingredients were mixed together in a closed vessel on a stirrer at room temperature for 1 h. The well-mixed fluoride varnishes containing 20, 40, 60, or 80 μL 1% w/v chitosan were labeled as FVN+20CS, FVN+40CS, FVN+60CS, and FVN+80CS, respectively. Fluoride varnish without chitosan (FVN) served as the control.

Fluoride varnish characterization

The chemical structure of the varnishes was analyzed by Fourier transform infrared spectroscopy (FTIR; Perkin Elmer Spectrum One, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) with a universal attenuated total reflectance (UATR) accessory. The resolution of the analyzing condition was set at 4.0 cm^{-1} and each fluoride varnish sample was scanned 64 times.

Varnish viscosity was measured using a viscometer (HAAKE™ Viscotester™ iQ Air, ThermoFisher Scientific, Karlsruhe, Germany). The parallel plates (35 mm diameter) were used as the measuring geometry. One milliliter of each fluoride varnish formulation was filled in the lower plate. The upper plate, which was connected to the rotor, was set in the working position. The space between the upper and lower plate was 1 mm. The shear rate was set to vary from 0.01 up to 150 1/s and the temperature was 25°C . One hundred data points were collected during the test. The viscosity of each fluoride varnish is presented as a function of shear stress and shear rate.

In vitro fluoride release

Varnish blocks were prepared in a circular acrylic mold (8 mm diameter, 1 mm deep) at room temperature until the varnish block was fully set. For each fluoride varnish formulation, 9 varnish blocks were prepared and divided into 3 groups ($n=3$) for the fluoride release measurement in triplicate. Each varnish block was placed in a capped plastic container containing 3 mL artificial saliva and kept in an incubator (Memmert®, 100-800, Memmert, Schwabach, Germany) at 37°C . The artificial saliva was composed of 3.90 mmol Na_3PO_4 , 4.29 mmol NaCl, 17.98 mmol KCl, 1.10 mmol CaCl_2 , 0.08 mmol MgCl_2 , 0.50 mmol H_2SO_4 , 3.27 mmol NaHCO_3 , and distilled water, at pH 7.2³⁰. The varnish block was changed to a new container and the fluoride ion concentration was measured after 1, 2, 3, 4, and 5 h incubation at 37°C , respectively. The measurement was also performed every day for 14 days. The varnish blocks were rinsed with de-ionized water for 30 s and blotted dry with blotting paper before being placed in a new container. Two milliliters of the test solution were placed in a new container and 200 μL of TISAB III solution (Sigma-Aldrich®, Merck) was added. This solution was stirred on a stirrer for 30 s. The fluoride ion selective electrode (Orion®, 9609BNWP, Thermo Fisher Scientific, Waltham, MA, USA) was placed in the

solution for 1 min and the amount of released fluoride was measured using an electrochemistry meter (Orion®, VERSASTAR, Thermo Fisher Scientific).

Cytotoxicity test

In this study, to evaluate varnish cytotoxicity, we used human gingival fibroblasts (hGFs), which were obtained from the gingival tissues of 3 healthy donors. The donors provided informed consent before the gingivectomy procedure. The study protocol was approved by The Human Research Ethics Committee of The Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2017-082).

The gingival tissues were cut into small pieces and placed into 35-mm cell culture dishes (SPL Life Sciences, Gyeonggi-do, Korea). Culture medium [Dulbecco's Modified Eagle's Medium (DMEM)] supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotic-antimycotic solution (Gibco™, ThermoFisher Scientific) was added to the culture dishes. The gingival tissues were cultured at 37°C in an incubator in a humidified 5% CO_2 atmosphere. The culture medium was changed every 2 days until the hGFs reached 95% confluence, and fifth passage hGFs were used in the experiments. The cytotoxicity of the fluoride varnishes were evaluated using the extract and direct contact tests described in ISO 10993-5³¹.

1. Extract test

The extraction medium was prepared according to ISO 10993-12³². The fluoride varnishes were immersed in DMEM at ratio of 4 g/20mL and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. The hGFs (2.5×10^4 cells/well) were cultured in 24-well plates (SPL Life Sciences) at the same condition. The culture medium in each well was subsequently removed and 1 mL extraction medium was added to the hGFs culture, and the cytotoxicity was determined after incubating at 37°C for 24, 48, and 72 h. The 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell viability³³. Briefly, at the end of each culture period, the culture medium in each well was removed and 50 μL MTT reagent (5 mg/mL PBS) and 300 μL DMEM without phenol red were added. The plates were incubated at 37°C for 4 h. After the incubation period, the solution in each well was removed and 1 mL dimethyl sulfoxide (DMSO; AMRESCO LLC, Solon, Ohio, USA) solution was added to dissolve the precipitated formazan crystals. The optical density (OD) was measured using a microplate reader (EPOCH, BioTek Instruments, Winooski, VT, USA). The percentage of cell viability was calculated using the following equation:

$$\text{Percentage of cell viability} = (\text{OD of the experimental group} / \text{OD of the control group}) \times 100$$

The untreated hGFs and DMSO served as the positive control and blank group, respectively.

2. Direct contact test

Ten microliters of fluoride varnish were dropped on clean glass cover slips placed in 35-mm cell culture

dishes (3 cover slips/culture dish). The dishes were then seeded with hGFs (2.5×10^4 cells/dish) and incubated in a humidified 5% CO_2 atmosphere. The cells were observed every 24 h using an inverted phase contrast microscope (OLYMPUS CKX41, Olympus, Tokyo, Japan). The images were obtained at 24, 48, and 72 h.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistic 22 program. The data of the *in vitro* fluoride release were analyzed by the Shapiro-Wilk test to determine normality. The homogeneity of variance was tested and the group means for fluoride release were compared using one-way ANOVA. The differences between the groups were analyzed by the Dunnett and Scheffe test. The significance was determined at $p < 0.05$.

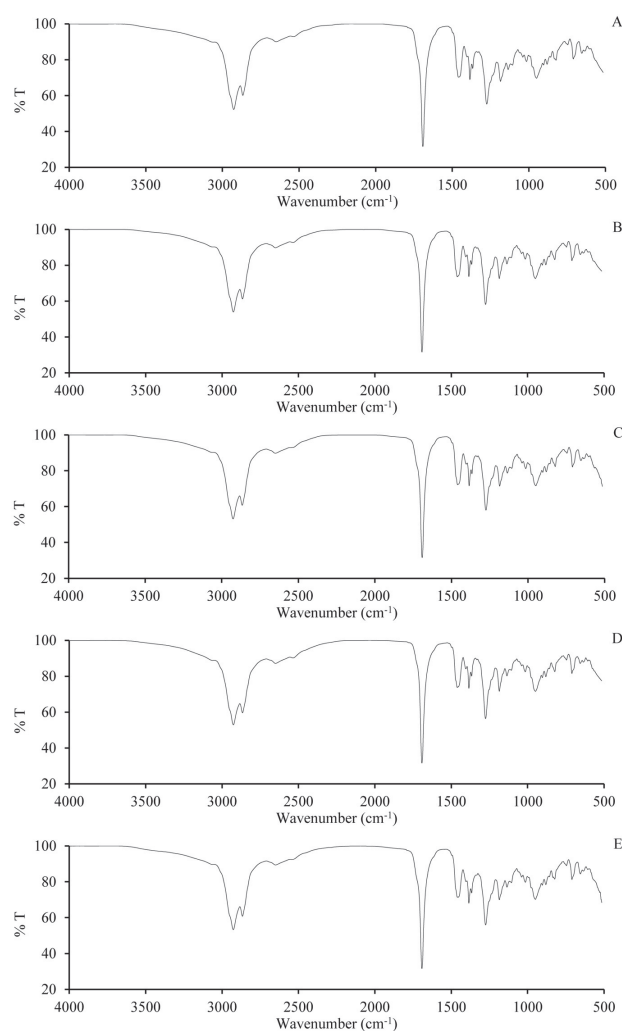


Fig. 1 FTIR spectrum of A) fluoride varnish without chitosan (FVN) and B), C), D), and E) fluoride varnishes containing 20, 40, 60, and 80 μL 1% w/v chitosan (FVN+20CS), (FVN+40CS), (FVN+60CS), and (FVN+80CS), respectively.

RESULTS

FTIR analysis

The interaction between fully hydrogenated rosin and chitosan was characterized using FTIR spectroscopy, and representative spectrograms are presented in Fig. 1. The FTIR spectra of all fluoride varnishes showed alkane C-H bond stretching at $2,926$ and $2,866$ cm^{-1} . The sharp absorption band at $1,691$ cm^{-1} represented carboxyl group C=O bond stretching. The absorption band at $1,460$ cm^{-1} resulted from alkane C-H bond scissoring. The absorption peaks at $1,366$ and $1,384$ cm^{-1} were due to methyl group C-H bond rocking. The absorption bands at $1,275$ and 949 cm^{-1} represented C-O bond stretching and carboxyl group O-H bond bending, respectively.

Viscosity

We analyzed shear stress as a function of shear rate for the fluoride varnishes (Fig. 2). The FVN and FVN+20CS groups had slightly increased shear stress with an increasing shear rate. In contrast, the FVN+40CS, FVN+60CS, and FVN+80CS groups demonstrated a rapid increase in shear stress that then gradually increased. The FVN+80CS group showed the highest shear stress, followed by the FVN+60CS, FVN+40CS, and FVN+20CS groups, while the FVN group produced the lowest shear stress. The viscosity of the fluoride varnishes was determined (Fig. 3). The viscosity of all groups decreased as the shear rate increased. The FVN group demonstrated the lowest viscosity, and chitosan fluoride varnish viscosity increased in a chitosan concentration dependent-manner.

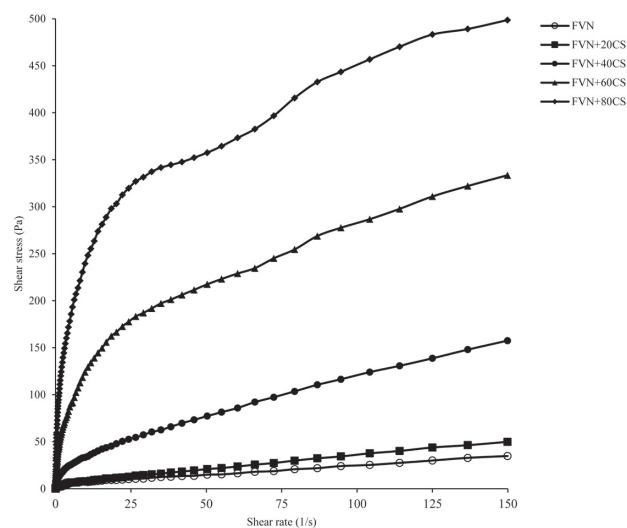


Fig. 2 Shear stress as a function of shear rate for the fluoride varnish (FVN) and fluoride varnishes containing 20, 40, 60, and 80 μL 1% w/v chitosan (FVN+20CS), (FVN+40CS), (FVN+60CS), and (FVN+80CS), respectively, at 25°C .

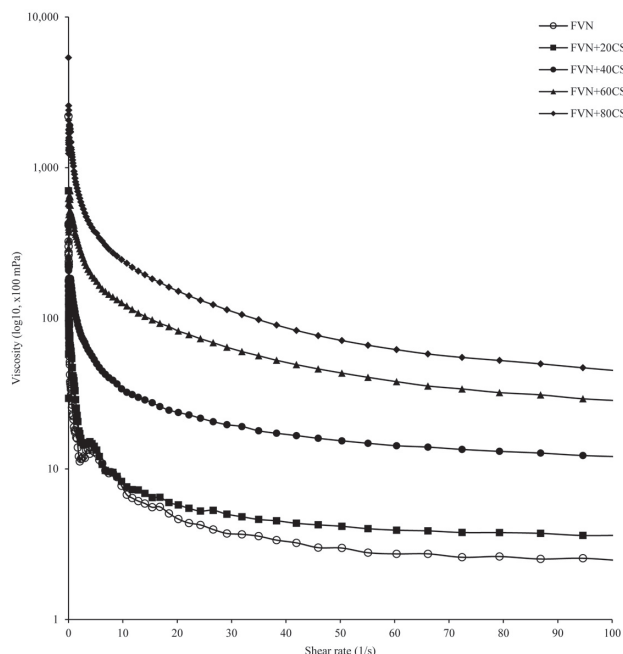


Fig. 3 Viscosity of the fluoride varnish (FVN) and fluoride varnishes containing 20, 40, 60, and 80 μL 1% w/v chitosan (FVN+20CS), (FVN+40CS), (FVN+60CS), and (FVN+80CS), respectively, at 25°C.

In vitro fluoride release

We investigated the amount of F^- released from the fluoride varnishes (Fig. 4). The result showed that the F^- released from each varnish formulation was the highest after 1 h and rapidly decreased after 2 h. At 1 h, the observed F^- release increased corresponding to the increase in chitosan concentration. The FVN+80CS group showed the significantly highest F^- release (29.79 ± 6.10 ppm, $p < 0.05$) and there were significant differences between the FVN (6.08 ± 0.99 ppm), FVN+20CS (7.33 ± 0.36 ppm), and FVN+40CS (8.91 ± 1.12 ppm) groups' F^- release ($p < 0.05$). From 2–5 h, the FVN+40CS and FVN+80CS groups' F^- release gradually decreased, while those of the FVN, FVN+20CS, and FVN+60CS groups demonstrated a relatively continuous release. The FVN group had the significantly lowest F^- release at each time point ($p < 0.05$).

We also determined the cumulative F^- release from the different varnishes over 14 days (Fig. 5). The FVN+20CS and FVN+60CS groups showed increased cumulative F^- concentration for up to 7 days and the concentration then relatively plateaued until 14 days. Similarly, the FVN+40CS and FVN+80CS groups demonstrated an increase in F^- concentration until 9 days, which then plateaued until 14 days. In contrast, the cumulative F^- release of the FVN group presented a time-dependent increase from day 1 until day 14. The FVN+20CS group presented the highest mean cumulative F^- release, which was significantly higher compared with the FVN group at each time point and

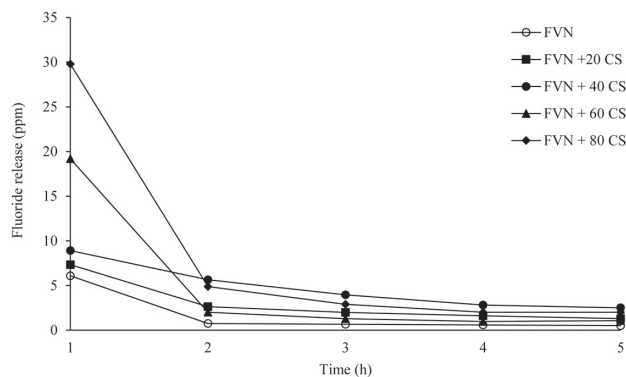


Fig. 4 Fluoride release (ppm) of the fluoride varnish (FVN) and fluoride varnishes containing 20, 40, 60, and 80 μL 1% w/v chitosan (FVN+20CS), (FVN+40CS), (FVN+60CS), and (FVN+80CS), respectively, in artificial saliva pH 7.2 at 37°C for 5 h ($n=9$).

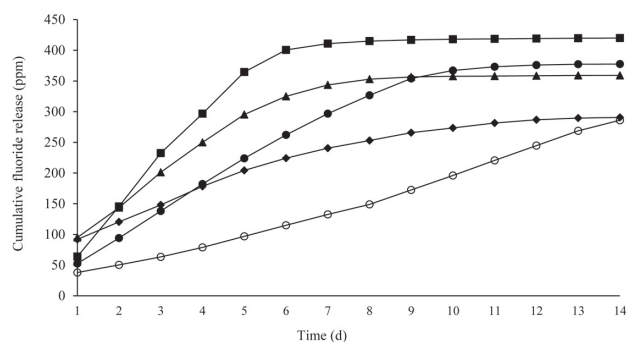


Fig. 5 Cumulative fluoride release (ppm) of the fluoride varnish (FVN) and fluoride varnishes containing 20, 40, 60, and 80 μL 1% w/v chitosan (FVN+20CS), (FVN+40CS), (FVN+60CS), and (FVN+80CS), respectively, in artificial saliva pH 7.2 at 37°C for 14 days ($n=9$).

the FVN+80CS group at day 1 and day 5–14 ($p < 0.05$). Moreover, the FVN+20CS group F^- concentration was significantly higher than that of the FVN+60CS group at day 1 and the FVN+40CS group at day 2 and 7 ($p < 0.05$). In contrast, the FVN group demonstrated the significantly lowest mean cumulative F^- compared with the other groups for the first 6 days. However, from day 7–12 the FVN group F^- concentration was not significantly different from that of the FVN+80CS group and at 13 and 14 days its cumulative F^- release was significantly different from the FVN+20CS group ($p < 0.05$).

Cytotoxicity test

The MTT assay results indicated that the percentage of hGF cell viability was less than 50% for all evaluated groups (data not shown). Moreover, the direct contact test exhibited many necrotic hGFs around the fluoride varnish droplets (data not shown).

DISCUSSION

The aim of this study was to investigate the effect of chitosan added to fluoride varnish in different amounts. The findings of this study indicate that the addition of chitosan had a significant effect on the fluoride varnish viscosity and *in vitro* F⁻ release behavior.

Fluoride varnish has a high concentration of fluoride (5% NaF) in a rosin base. Rosin, or colophony, is a solid form of resin that is obtained from pine trees. Rosin has been used in pharmaceutical and medical fields for coating granules, tablets, and pellets for sustained drug release³⁴. Rosin is also used in dentistry as cavity varnishes³⁵ and a component of commercial fluoride varnishes. We used fully hydrogenated rosin, which is a modified form of rosin. Fully hydrogenated rosin is tetrahydroabietic acid that has tricyclic diterpene monocarboxylic acid as a chemical structure³⁴. The FTIR analysis indicated that the chemical structure of the chitosan added fluoride varnishes was composed of alkanes (at 1,460, 2,926, and 2,866 cm⁻¹), methyl groups (at 1,366 and 1,384 cm⁻¹) and carboxyl groups (at 949, 1,275, and 1,691 cm⁻¹), which coincided with the chemical structure of fully hydrogenated rosin. However, the FTIR spectrum analysis did not demonstrate the chitosan functional groups. The FTIR spectrum absorption bands of chitosan showed stretching of the –OH and –NH groups at 3,440 cm⁻¹, bending of the N–H group and the amide of the acetyl groups at 1,623 and 1,657 cm⁻¹, and stretching of the –CN group at 1,318 cm⁻¹³⁶. These results indicated that the fully hydrogenated rosin did not chemically interact with chitosan because a new functional group was not present. Therefore, the addition of chitosan in different concentrations did not have any effect on the chemical structure of the fluoride varnish. In a previous study, a graft copolymer of chitosan and rosin was successfully prepared using microwave irradiation with potassium persulfate as the initiator³⁷. However, this copolymer was not used in dental varnish. Although the fluoride varnishes in our study did not show an interaction between rosin and chitosan, they demonstrated sustained release of fluoride ions in artificial saliva.

In our viscosity study, the results indicated that when adding the chitosan to the fluoride varnishes, the viscosity increased in a concentration-dependent manner. This may have resulted from increasing entanglement of the chitosan chains. According to the rheological behavior of chitosan solutions, the viscosity increased with the chitosan concentration^{38,39}. Furthermore, chitosan acts as a viscosity enhancing agent. Our results imply that the application of fluoride varnish containing 60, or 80 µL/mL chitosan on a tooth surface would be very difficult. This is because these were more viscous and a high shear rate or high velocity rate must be used to spread the fluoride varnish. Therefore, they cannot flow easily into the proximal surface of the tooth.

The *in vitro* F⁻ release results of our study demonstrated a burst phase in the first hour and a continued release at a slow rate through 5 h, while the

mean cumulative F⁻ release profile showed a sustained release. From these results, we propose a mechanism of modifying F⁻ release behavior by adding chitosan. Chitosan may mechanically trap NaF particles with a relatively weak interaction, while rosin may trap it strongly. Therefore, chitosan containing varnish released F⁻ at a higher rate (or at the early stage) compared with the varnish without chitosan. After a burst release of F⁻, the varnishes with and without chitosan released F⁻ at a slow rate mainly from the rosin-based matrix. Milburn *et al.*¹³ presented that the greatest rate of F⁻ release from four commercial fluoride varnishes occurred in the first four hours and began to plateau after 8–12 h. Comar *et al.*⁴⁰ analyzed the amount of F⁻ released from NaF varnishes. The authors found that the highest amount of F⁻ release was exhibited in the first 3 h and the release was decreased until 12 h. The F⁻ release profile in our study was in the same as that of Milburn *et al.*¹³ but different from the finding by Comar *et al.*⁴⁰. Although in our study the FVN+80CS and FVN+60CS groups showed a high level of F⁻ release after 1 h, they are unsuitable for application on a tooth surface due to their high viscosity. In contrast, the F⁻ release after 1 h of the other chitosan-added fluoride varnish groups were lower than 10 ppm. These varnishes are appropriate to be applied on a tooth surface due to their low viscosity.

The prolonged release property of F⁻ at a lower level concentration (ppm or sub ppm) is superior to the burst release because this can maintain a lower level of F⁻ longer in oral cavity². Generally, the F⁻ release profile of a fluoride varnish has two phases that are the burst release phase and the prolonged release phase. In this study, the F⁻ release was evaluated for 5 h and the cumulative F⁻ release was assessed for 14 days. The burst release phase occurred in the first hour and the first day (if focused on the cumulative F⁻ release) and the prolonged release phase was presented after that time. At the burst release phase, the fluoride varnishes containing chitosan especially the FVN+80CS demonstrated the higher F⁻ release than the FVN group. While in the prolonged release phase, the FVN+40CS group showed the better F⁻ release than the others. Even though the FVN+20CS group exhibited the highest cumulative F⁻ release, the release profile was stable at day 7.

There has been no study about the length of time that fluoride varnish is retained on enamel surfaces when patients with fluoride varnish brush their teeth. However, prolonged retention time has been clinically recommended to maximize the F⁻ reaction with enamel. Fluoride varnish can remain on the tooth surface for 1–3 days if the patient brushes gently⁴¹. In contrast, one study found that the retention time of fluoride varnish ranged from 4 h to the next morning after application⁴². However, these authors also detected that microscopic remnants of fluoride varnish were retained on enamel surfaces for a long period of time, which might function as a limited local effect of slow-release F⁻ reservoirs. Another study presented that F⁻ uptake and distribution were increased by prolonging the contact time between the varnishes and enamel from 1 to 24 h⁴³.

There are many procedures to encapsulate NaF particles with chitosan for sustained F⁻ release. Keegan *et al.*²⁹⁾ produced chitosan/fluoride microparticles that were prepared by spray drying using glutaraldehyde as the crosslinker. The authors found that the microparticles demonstrated a burst release of F⁻ in the first 2 h and slightly increased F⁻ release through 6 h. Another study prepared chitosan nanoparticles that entrapped NaF and used tripolyphosphate as the crosslinker by ionic gelation⁴⁴⁾. They showed that the loading capacity of F⁻ was 33–113 ppm and the cumulative F⁻ release steadily increased over 4 h and remained at this level for at least 24 h. Furthermore, Liu *et al.*²⁷⁾ demonstrated that the chitosan/NaF nanoparticles prepared from water-in-oil emulsion with glutaraldehyde as the crosslinker showed a burst release in the first 2 h and sustained release for at least 10 h. Compared with the previous studies, our results showed a longer time of sustained release than those of these studies. Moreover, our preparation method was simpler and less expensive compared with those studies.

In our *in vitro* study using hGFs, the results indicated that our fluoride varnishes were toxic to hGFs. These results imply that, although the released F⁻ from our fluoride varnishes was less than the certainly lethal dose (32–64 mg F/kg of body weight) and the probably toxic dose (5 mg F/kg of body weight)⁴⁵⁾, care must be used when applying these types of fluoride varnishes to the tooth surface to ensure that the fluoride varnish does not contact oral soft tissues.

Future studies will evaluate the anti-demineralization/remineralization properties on artificial caries, the amount of fluoride uptake into enamel specimens, formation of calcium fluoride, the adhesiveness on tooth surface, and *in vivo* studies will be performed to determine the anti-caries effect of our fluoride varnish. Moreover, a comparison with a commercial fluoride varnish should be conducted.

CONCLUSION

Here, we have examined a new chitosan added fluoride varnish that demonstrated a sustained F⁻ release in artificial saliva. We propose that fluoride varnish containing 40 µL/mL chitosan is a potential material for sustained release fluoride varnish. Further studies will be conducted to compare our novel varnish with a commercial fluoride varnish.

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