ORIGINAL RESEARCH

Carboxymethyl Chitosan—Fluoride-doped Amorphous Calcium Phosphate: A Novel Remineralizing Gel

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Abstract

Background: There is a need for innovative remineralizing gel formulations based on calcium and phosphates that can slowly release fluoride ions and enhance the formation of fluorapatite crystals that are more resistant to dissolution in an acidic environment.

Aim: The aim of the work was to formulate a remineralizing agent that remineralizes enamel through the release of Ca^{2+}, PO_4^{3-}, and F- ions for a prolonged period of time.

Materials and methods: The gel was based on carboxymethyl chitosan (CMC) as a bioinspired gelling agent and on Fluoride-doped Amorphous Calcium Phosphate (F-ACP) as a remineralizing agent. This gel was tested *in vitro* on the enamel of extracted premolars after demineralization with methacrylic acid gel.

Results: When compared to the control group and demineralized enamel group, the enamel slabs remineralized with CMC/F-ACP showed a higher calcium phosphate ratio in Energy-Dispersive X-ray (EDX) and better surface morphology under scanning electron microscope (SEM).

Conclusion: Remineralization tests performed on demineralized human permanent teeth proved that CMC/F-ACP gel has excellent efficacy, inducing a complete remineralization of the outermost layers of enamel as well as a full restoration of lost mineral content.

Keywords: Carboxymethyl chitosan, Fluoride-doped Amorphous Calcium Phosphate, Remineralizing gel.

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Introduction

Dental caries is a cyclic phenomenon involving phases of loss and gain of the mineral component of enamel and dentine (referred to respectively as demineralization and remineralization) rather than a continuous and unidirectional process of demineralization.[1] The occurrence of dental caries progresses from the initial demineralization, that is the dissolution of enamel apatite crystals, to a visible white spot lesion, followed by dentin involvement, and finally by tooth decay or cavitation. The progression through these stages is due to a constant imbalance between pathogenic and protective factors, which leads to the breakdown of apatite crystals and the net loss of calcium, phosphate, and other ions from the tooth. This process is known as demineralization.[2]

Remineralization refers to supplying calcium and phosphate ions from an external source to encourage ion deposition into crystal voids in demineralized enamel to create a net mineral gain.[3] When net mineral loss and net mineral gain are in balance, a state of health prevails in the oral cavity.[4] As the disease progresses from white spot lesions to frank caries, dentists need to take more dire measures, such as drilling and filling the tooth and surgical intervention to save the tooth, although these treatments treat the disease and not the cause.

It has taken almost a century for dentistry to go from the pioneering notion of "extension for prevention" to the more recent concept of "Minimally Invasive Dentistry (MID)."[5] MID is a concept that combines prevention, remineralization, and minimum intervention for the repair and restoration of teeth.[6,7] It strives to manage noncavitated white spot lesions noninvasively favoring remineralization to minimize disease progression and enhance aesthetics, strength, and function.[8]

The MID clearly states the necessity for clinically effective methods to remineralize early carious lesions. Fluoride-facilitated remineralization of teeth is considered the keystone of current caries control theories. Fluoride converts hydroxyapatite (HA) crystals to fluorapatite crystals that are highly resistant to acids that demineralize the enamel and dentin. Fluoride also inhibits bacterial growth by affecting the enzyme enolase in glycolysis.[9] However, it is necessary to follow safety protocols and instructions while using fluoridated toothpaste and mouthwashes, as accidental consumption of high doses of fluoride can be fatal. According to recommended daily allowances, a 0.1 mg fluoride/kg body, weight/day in children up to the age of 8 is regarded safe, creating no evident form of fluorosis in permanent teeth. However, the quantity of fluoride in drinking water, dietary supplements, and topical treatments must be considered, as they are all recognized as potential sources of fluorosis. Furthermore, the likely hazardous

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dosage of fluoride is 5 mg/kg body weight, which necessitates therapeutic intervention and hospitalization.[10] Because most dental products include a substantial amount of fluoride, there is a considerable risk of chronic ingestion by young children, surpassing the hazardous dose.[11]

Several innovative remineralization techniques such as bioactive calcium and phosphate, casein phosphopeptide (CPP) stabilized amorphous calcium phosphate (ACP), self-assembling peptide, and nano-HA have been invented and marketed to induce deep remineralization of demineralized enamel and dentin while diminishing the possible dangers associated with the use of fluoride-rich dental care products, as well as favoring caries control.[4,10] The most-used commercial remineralizing agents are listed in Table 1.

It has been reported that these remineralizing agents show promising potential in preventing dental caries *in vitro* studies and animal studies. The drawbacks of these substances have also been reported. A significant disadvantage of tricalcium phosphate (TCP) is the development of calcium phosphate complexes. If fluorides are present, calcium fluoride formation inhibits remineralization by reducing the quantities of bioavailable calcium and fluoride.[12] The disadvantage of ACP-based remineralization systems is the formation and precipitation of insoluble calcium and phosphate ions complexes on teeth that can't be available for remineralization.[13] Although CPP-ACP and CPP/Fluoride-doped Amorphous Calcium Phosphate (F-ACP) have proved to be excellent remineralizing agents; they cannot be used in patients with lactose intolerance due to the presence of milk protein casein.[14,15] However, there is satisfactory evidence from *in vitro* and animal studies that advocate that this technology is promising in the prevention of enamel caries.[7] Nevertheless, in humans, the only class I clinical trial in a population with high-risk was unsuccessful in showing a variance in enamel caries reduction among control and experimental groups.[16]

Thus, considering all the advantages and disadvantages of the various remineralizing agents, we formulated a novel remineralizing gel using carboxymethyl chitosan (CMC) and biomimetic ACP doped with fluoride ions (F-ACP). Chitosan is a biopolymer derived from 70% deacetylation of chitin in a basic solution. Chitosan and its derivatives have developed as novel biomaterials due to their low toxicity, biodegradability, biocompatibility, and biological activity. Chitosan is a polycationic mucoadhesive polymer that allows a sustained release of bioactive substances over a period of time. Active ingredients like fluoride incorporated with chitosan can act as potent remineralizing agents for teeth.[17] F-ACP is a promising remineralizing agent that contains citrate which maintains ACP with fluoride in a dry and nonreactive state up to a span of 1 year.[18] Therefore a combination of CMC and F-ACP would give rise to a remineralizing agent that can overcome the drawbacks of other remineralizing agents.

Materials and Methods

Ethical approval was obtained for the present study from the Institutional Review Board at the Texilla Americal University with the number 2020/BC/0271.

Materials Used

Carboxymethyl chitosan (CMC) was procured from ChemCruz™ Biochemicals Private Limited, United States of America (Lot No-G1720). F-ACP was prepared at the King Saud University by following the formulation instructions mentioned by Iafisco et al. in Science Reports.[18]

Preparation of the F-ACP Gel

The CMC/F-ACP gel was prepared by mixing 50 gm of CMC/F-ACP with 45 gm of ultrapure water in a transparent container (50 gm of CMC/F-ACP consisting of 2.5 gm of CMC with 2.5 gm of F-ACP containing 750 ppm fluoride). CMC/F-ACP powder is added to the water while stirring with a spatula until any clump is dissolved. Clumps are detected by passing strong light through the container. The final gel is white and opaque with strong consistency, yet able to slowly flow when the container is tilted. The gel is stored at 4°C until used and is allowed to warm up to room temperature before use.

Preparation of Artificial Caries

Human premolars without visible evidence of defects were obtained from the dental clinic. The teeth were subjected to mechanical cleaning immersed in 0.1% thymol and stored at 4° until used. Roots were separated and cut into mesial and distal surfaces using a diamond saw as shown in Figure 1. The enamel slabs obtained were ultrasonically cleaned and rinsed in 75% ethanol. A demineralization zone was created with a 3×3 mm^2 outline, and the rest of the tooth was covered with water-resistant nail varnish (Fig. 1).

Demineralization–Remineralization Cycle

Demineralization

The enamel slabs were immersed in 30 mL acidic gel of 1% gelatin with 0.2 M aqueous solution of methacrylic acid prepared as described by Simeonov et al.[19] The gel was renewed every 24 hours for 4 days.

Table 1: Commercially available remineralizing agents

Sl. no	*Remineralizing agent*	*Trade name*	*Active ingredients*
1	TCP	Clinpro™	Calcium, fluoride
2	ACP	Enamelon™	Calcium, phosphate, fluoride
3	Calcium carbonate carrier	SensiStat™	Calcium, arginine complex
4	Calcium hydroxide	Dycal™	Calcium
5	CPP-ACP	Recaldent™, MI Paste™	Calcium, phosphate
6	CPP/F-ACP	MI Paste Plus™	Calcium, phosphate, fluoride
7	Nano-HA	Reminpro™	HA
8	Sodium calcium phosphosilicate–bioactive glass powder	Novamin™	Bioactive glass, HA
9	Bioactive HA	Curasept™	Fluoride ion-doped HA
10	Zinc HA	Biorepair™	HA

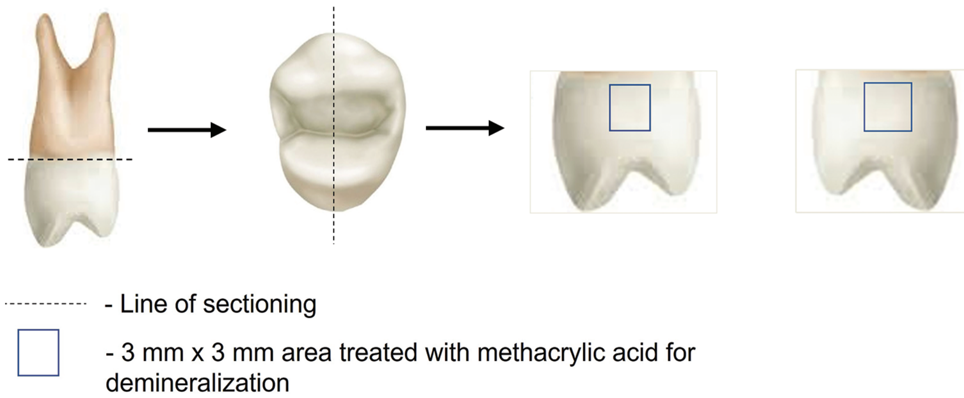
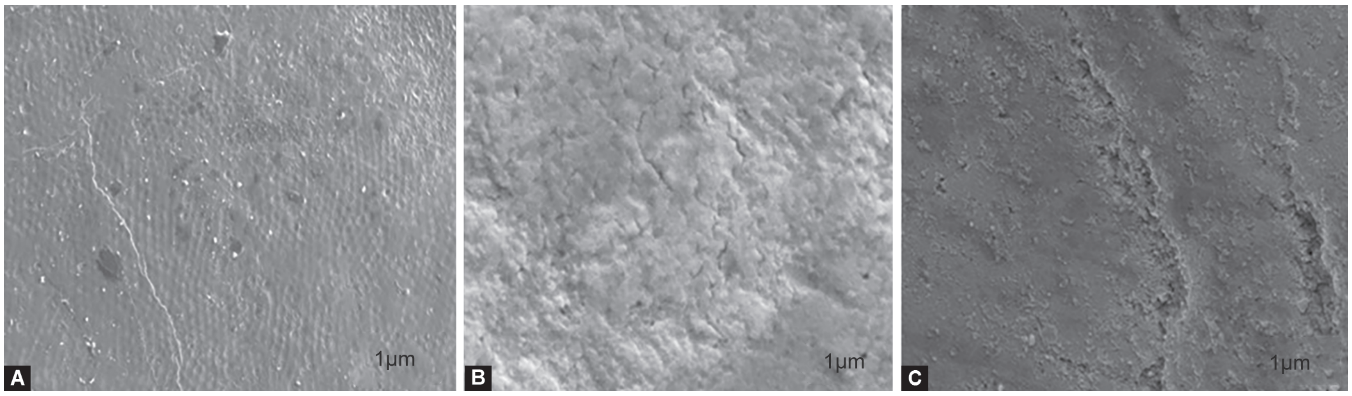


Fig. 1: Sectioning of teeth



Figs 2A to C: SEM micrographs of (A) Control, sound enamel; (B) Demineralized enamel; (C) Demineralized enamel treated with CMC/ F-ACP gel

After demineralization the solutions were immersed in distilled water for 1 minute, sonicated for 5 minutes, and stored at 4°. Both symmetrical slabs from each tooth were used.

- The first slab was categorized as a demineralized group.
- The second slab was subjected to remineralization.

Remineralization

The test samples were immersed in CMC/F-ACP gel for 6 hours for 7 days. After 6 hours, the samples were removed from the remineralization media and cleaned and dried at room temperature for 7 days (Fig. 2).

Evaluation of Enamel Remineralization

After remineralization, the control group enamel slabs, demineralized enamel slabs, and the enamel slabs remineralized with CMC/F-ACP were examined under a scanning electron microscope (SEM) to evaluate the surface changes in the enamel after remineralization. The mineral content in enamel slabs, before and after remineralization was evaluated by Energy-Dispersive X-ray (EDX) spectrometer.

All samples from the three groups were examined with SEM to evaluate the morphological changes in enamel surface before and after remineralization by using a field-emission gun SEM microscope (FEG-SEM ΣIGMA, ZEISS NTS Gmbh, Oberkochen, Germany). In addition, enamel mineral content in the samples was evaluated by EDX spectrometry through a microanalysis system integrated into an SEM microscope (INCA Energy 300, Oxford Instruments, Abingdon-on-Thames, United Kingdom).

Slabs were mounted on SEM aluminum stubs by using carbon adhesive tape. The samples were then coated with 10 nm of gold with a Polaron E5100 sputter-coater (Polaron Equipment, Watford, Hertfordshire, United Kingdom). Micrographs for morphology analysis were acquired in secondary electrons acquisition mode using an acceleration voltage of 5 kV in six random regions of the samples with magnification between 50,000× and 5,000×. EDX microanalysis was performed in six random regions of the samples on squared areas of 2 × 2 µm at an acceleration voltage of 15 keV and was carried out at 5,000× magnification.

The outcome of the remineralization was evaluated by comparing the enamel slabs that originated from one tooth.

Statistical Analysis

Statistical data testing was carried out in the software Statistical Package for the Social Sciences-22.0, IBM product of Chicago (United States of America). All the continuous response observations of mineral concentrations were presented in terms of mean (standard deviation). These data were explored for the test of normality by using the Kolmogorov–Smirnov test, which revealed normal distribution. Analysis of variance was performed to compare the mean responses of mineral concentrations among study groups. *post hoc* Tukey's test was applied to compare mean mineral concentrations between the groups. A p-value ≤ 0.05 was considered a statistically significant result.

Results

SEM Characterization of Enamel

Figure 3 depicts the SEM analysis of enamel in the control group that has not been demineralized or remineralized. The surface appears smooth with few irregularities. The scratched

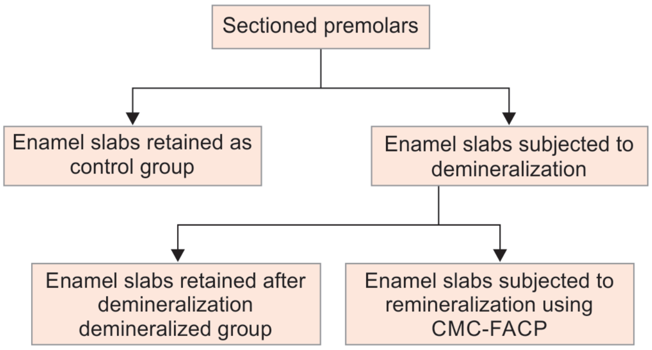


Fig. 3: Methodology

appearance of clinically sound enamel can be correlated to abrasions due to brushing. The usual perikymata pattern on the tooth surface at the time of eruption progressively fades away to be replaced by a scratched pattern.

SEM Characterization of Demineralized Enamel

Figure 3 depicts SEM analysis of demineralized enamel which shows a deeply eroded, pitted enamel surface. The surface appeared disorganized, irregular, and rough indicating that demineralization of the superficial layer of enamel had occurred.

SEM Characterization of Enamel Remineralized with CMC/F-ACP

Figure 3 depicts SEM analysis of remineralized enamel which shows that there are deposits around the lacunae created due to demineralization. Interprismatic porosities can be seen that appear to be occluded due to the deposition of a fine granular layer over the demineralized enamel surface.

The mineral content of the enamel samples has been measured by SEM-EDX analysis (Table 2). It can be observed that the calcium content is statistically different between the three groups as the demineralized group presents a lower calcium content while the enamel treated with CMC/F-ACP gel has a significantly higher calcium content that is different both from the control to demineralized group ($p < 0.001$). These differences confirm on one hand that the demineralization treatment effectively induced a mineral loss, while the treatment with CMC/F-ACP restored the lost mineral content. On the contrary, the mean phosphorous content was nonsignificant in all groups compared to the control group. The Ca/P molar ratio reflects the changes in calcium content in the three groups as demineralized enamel has a significantly lower Ca/P ratio due to the loss of calcium while the treated group has a higher Ca/P due to the additional deposition of calcium.

Discussion

The demineralization–remineralization cycle concept has opened up the possibility of remineralizing a carious lesion in its early stages.[20] As a result, the focus on caries prevention moved to develop early detection tools. The use of remineralization as a noninvasive treatment for incipient carious lesions has been hailed as a promising step in the clinical management of the condition. It eliminates the usual divide between preventative and rehabilitative care.[21] A range of fluoride or bioavailable calcium and phosphate medications like CPP-ACP that can remineralize early carious lesions are available.[4] However, thorough reviews by Chen et al. and Li et al. found a lack of valid scientific evidence to support CPP-ACP's remineralization impact.[22,23]

It is also considered that there is currently a lack of research concerning the remineralizing efficacy of nanotechnology-based biomaterials, which could be an essential preventive treatment for patients with high caries risk.[24]

Various kinds of biomimetic calcium phosphates (CaPs) have been proposed, such as hydroxyapatite (HA), fluoro-HA (FHA), and ACP.[25,26] These substances can be mixed into restorative materials or administered directly to the tooth surface. Because of their biomimetic property, larger surface area and reactivity, and improved capacity to adhere, and penetrate the enamel and dentinal lesions, nanostructured CaPs are more effective for remineralization therapies than their macrosized counterparts.[27] To the best of our knowledge, most of these materials are unavailable in the market because of the snags in the scale-up process, production costs, and concerns regarding the biocompatibility of stabilizing agents.

Iafisco et al. created a new biomimetic ACP doped with fluoride ions (F-ACP) to produce materials with improved anticaries and remineralizing characteristics. The doping of fluoride with citrate was significant, which didn't affect the physicochemical properties of ACP. However, it did result in a quicker conversion to the crystalline apatite phase in water.[18] Citrate played a significant role in stabilizing the precursor ACP phase.[28] The advantage of the topical delivery of fluoride interceded by ACP nanoparticles is that it is delivered directly into the carious lesion, which enhances its efficacy of fluoride and reduces its side effects.[18]

In the present study, to improve the efficacy of F-ACP, a novel remineralizing gel based on a CMC hydrogel containing F-ACP particles was formulated, defined as CMC/F-ACP. Chitosan and its derivatives are promising biomaterials due to their low toxicity, biodegradability, biocompatibility, and biological activity.[29] CMC is a carboxylated derivative of high interest for MID application as it mimics the dentin matrix protein 1, which is involved in stabilizing ACP during amelogenesis before it converts into enamel HA.[30] Therefore, CMC is a promising gelling agent and has been used together with F-ACP to produce a remineralizing gel that could act as a Tooth Mousse.

The remineralizing action of CMC/F-ACP gel has been tested on demineralized human enamel and assessed by SEM microscopy (Fig. 3). The enamel treated with CMC/F-ACP gel has been compared to sound enamel (control group, Fig. 3) and to demineralized enamel (Fig. 3). The surface of sound enamel appears pitted with a few irregularities, which could be abrasion due to brushing or mastication. The pitted appearance is due to shallow depressions marking the sites of the short Tomes processes of ameloblasts.[31] Sometimes the pits are deeper, and the surface has a honeycomb appearance.

In the present study, the demineralizing agent used was 1% methacrylic acid. Methacrylic acid is known tobe a minimally invasive organic acid that imitates the chemical environment of the oral cavity.[19,32] The demineralized enamel shows a deeply eroded enamel surface. Indeed, the surface is irregular and rough, presenting erosion pits which indicates that demineralization of the enamel superficial layer has occurred. This finding was similar to the findings of Degli Esposti et al., wherein they analyzed the remineralizing capacity of a commercial toothpaste (Biosmalto Caries Abrasion and Erosion, Curasept S.p.A., Saronno, Italy).[33] The tooth samples were sectioned and demineralized with 37 with respect to phosphoric acid gel and later remineralized with the toothpaste under examination. The tooth samples demineralized with 37% phosphoric acid gel appeared eroded and rough, similar

Table 2: EDX analysis of enamel samples

Variable	*Control*	*Demineralized*	*CMC/F-ACP*	*Σ*
Ca (with respect to)	30.02 (1.55)	21.27 (1.81)	57.15 (5.88)[a]	<0.001
P (with respect to)	17.31 (3.91)	21.34 (2.46)	16.57 (2.42)	0.035
Ca/P (mol)	1.81 (0.40)	1.00 (0.03)	3.49 (0.49)[a]	<0.001

[a] Significant mean difference of specific group vs control and Demin groups at $p \leq 0.05$; Ca, calcium; P, phosphorus; Ca/P, calcium phosphate ratio

to our demineralized enamel samples.[33] In another study conducted by Gjorgievska et al. remineralizing the potential of various toothpaste formulations such as toothpaste containing bioactive glass, HA, and strontium acetate was examined. This was an *in vitro* study and tooth samples were sectioned and demineralized using acidic artificial caries gel and later remineralized with various toothpastes. The demineralized enamel when observed under SEM appeared porous and rough analog to our demineralized enamel samples. This determines that the samples in our study were well-demineralized.

The enamel is treated with CMC/F-ACP gel (Fig. 3). On the contrary, it shows the presence of new mineral deposits on the rough surface created by demineralization. It can be observed that interprismatic porosities were occluded due to a deposition of a fine granular layer over the demineralized enamel surface. Therefore, demineralized enamel was remineralized by the gel.

The EDX is a microanalytical technique used for elemental analysis at an ultrastructural level. It is used in combination with SEM where SEM does the structural analysis of the sample and EDX does the elemental analysis. The principle of EDX is based on the energy discharged in the form of X-ray photons when electrons from an external source hit the atoms in a material, thus producing peculiar X-rays of that element. When the sample under examination is bombarded by a beam of electrons, electrons are emitted from the atoms present on the surface of the specimen (secondary electrons). An electron from the higher shell fills in the electron vacancy, and an X-ray is emitted (characteristic X-rays) in order to balance the energy variance between the two electrons. The EDX detector assesses the number of emitted X-rays vs the energy released. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy vs relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements present in the specimen using a computer-based program.[34]

The EDX reports in the present study showed that calcium content in samples remineralized with CMC/F-ACP was higher and significant compared to the calcium content in demineralized samples and samples of the control group (Table 2). The calcium phosphate ratio in CMC/F-ACP treated samples was also significantly higher than the demineralized and control groups. This indicates that there was CMC/F-ACP acted as a strong remineralizing agent. High calcium content in enamel post remineralization was also reported by Gjorgievska et al.[35] where remineraling agents used were Mirasensitive hap+, Mirawhite®tc, and Sensodyne®Rapid Relief. The calcium contents after examination by EDX were reported to be 54.48 for Mirasensitive hap+, 58.6 for Mirawhite®tc, and 52 for Sensodyne®Rapid Relief. The calcium content in CMC/F-ACP samples reported in our study was 57.15.[35]

The mechanism of action of ACP is based on two philosophies:

- The slow and consistent release of calcium and phosphate ions from ACP creates a supersaturated environment that induces remineralization.[18]
- The calcium and phosphate ions attach to the hard tissue surface such as enamel and convert to HA crystals.[18]

In the case of F-ACP, it has been observed that F-ACP converted to F-HA crystals and the release of calcium and phosphate ions from F-ACP has been gradual and persistent in artificial saliva. The newly formed F-HA crystals form an enamel-like crystalline layer that is similar to the native crystals. Thus, it can be said that F-ACP successfully restores the enamel structure in its native form.[18]

Conclusion

In the present study, a novel hydrogel formulation was devised for the treatment of dental caries. The hydrogel contained CMC as a bioinspired gelling agent and F-ACP, which was already proven to be an excellent material for dental remineralization. Together, the two components allow for easy formulation of a gel that is suitable to be used as a remineralizing agent and provides all the ions necessary to stimulate enamel remineralization. In addition, the slowed interaction between water and F-ACP given by the gel formulation allows for to extension of the remineralization effect of F-ACP as well as increasing the storability of the material. Remineralization tests performed on demineralized human permanent teeth proved that CMC/F-ACP gel has excellent efficacy, inducing a complete remineralization of the outermost layers of enamel as well as a full restoration of lost mineral content.

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