

## IN VITRO EFFICACY OF CCP-ACP, NANO- HYDROXYAPATITE, AND PHOSPHORYLATED CHITOSAN-ACP AS ANTI-EROSIVE AGENTS ON ENAMEL

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### ABSTRACT

**Purpose:** This study assessed the anti-erosive effect of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), nano-hydroxyapatite (nano-HAP) and phosphorylated chitosan Amorphous Calcium Phosphate (Pchi-ACP) on enamel.

**Materials and methods:** A total of twenty five human incisors were ground and divided into five groups (n=5). Group1: intact enamel (negative control group) - Group 2: enamel + soft drink (positive control group). - Group3: enamel + soft drink+ CPP-ACP. Group4: enamel + soft drink+ 10% nano-HAP. Group5: enamel + soft drink+ Pchi-ACP. Four consecutive intervals of the immersion procedure were performed at 0, 8, 24 and 32 h for a total of 8 min. After each of the demineralizing immersion interval, the anti-erosive agent for groups 3, 4 & 5 was applied for 3 min. The total time for remineralization was 12 min. The root mean-square roughness ( $R_{rms}$ ) was obtained from Atomic force Microscope images and the differences in the averaged values among the groups were analyzed by ANOVA, followed by Tukey's post hoc test. The level of significance was set at  $P < 0.05$ .

**Results:**  $R_{rms}$  of different groups at baseline was not statistically significant. However, after erosive demineralization,  $R_{rms}$  of group 2 increased and was significantly higher than all other groups.  $R_{rms}$  also significantly increased in group 3 subjected to CPP-ACP treatment after erosive demineralization. On the other hand, groups 4 and 5 did not show any significant change in  $R_{rms}$ .

**Conclusions:** This study had revealed that nano-HAP and Pchi-ACP are promising remineralizing nano-biomaterials

**KEYWORDS:** Enamel erosion, phosphorylated chitosan ACP, nano-hydroxyapatite, CCP-ACP.

### INTRODUCTION

Dental erosion has been usually characterized as a chemical disintegration of the hard tissues of teeth by acids not involving bacteria.<sup>1</sup> It is common in

modern societies due to the expanded utilization of acidic beverages.<sup>2</sup> The progression of disintegration is a chemical process in which the inorganic phase of the tooth is demineralized. Enamel has no capacity to repair when it is subject to caries or erosion as it

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is an acellular structure.<sup>3</sup> Thus, the reconstruction of enamel relies on the application of remineralizing anti-erosive agents.

Recently, fluoride alternatives were proposed as anti-erosive agents; like Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), nano-hydroxyapatite (nano-HAP) and Chitosan. Milk-derived CPP-ACP was introduced and researched as a topical agent. It releases calcium and phosphate ions which increase their activity on enamel surface. This mode of action is favorable in plaque-covered enamel or within subsurface caries lesions. Since erosion occurs in the absence of plaque, thus the role of CPP-ACP as an anti-erosive agent remains controversial.<sup>4</sup> Nano-HAP is believed to be a novel biocompatible and bioactive material. It is widely utilized in medicine and dentistry as a bone substitute and for tooth remineralization.<sup>5</sup> There is evidence that nano-sized particles have similar chemical and crystal structure to that of enamel.<sup>6</sup> It was reported that nano-HAP can effectively remineralize enamel carious lesions.<sup>7</sup>

Chitosan is a linear co-polymer of glucosamine and N-acetyl glucosamine produced by N-deacetylation of chitin. Chitosan and its derivatives have been emerging as another class of novel biomaterials with characterized biological activity, biocompatibility and biodegradability.<sup>8</sup> Among these chitosan derivatives is the phosphorylated chitosan (Pchi). Pchi has the quality of being bactericidal,<sup>9</sup> biocompatible, bioabsorbable,<sup>10</sup> and has some metal chelating properties.<sup>11</sup> Due to chelating capacity of Pchi to calcium particles, immobilized Pchi atoms can bind calcium ions to form nucleating sites.<sup>12</sup> For this reason, Pchi could be utilized for remineralizing enamel subsurface lesions. On the other hand, poor mismatch between enamel apatite and ACP at the microstructural level will render it difficult for ACP to stay bound to the tooth surface and maintain the bioavailability of ions to enhance enamel remineralization.<sup>13</sup> Therefore, the nano-complexes of Pchi-ACP may transform into HAP crystals within lesions to remineralize enamel. This

assumption could be supported by the non-classical nucleation theory.<sup>14</sup>

Only few studies have tested the effect of specific polymers such as chitosan on erosion development. Therefore, this study was conducted to investigate the remineralizing effect of CPP-ACP, nano-HAP, and Pchi-ACP on enamel erosive lesions. The null hypothesis tested was that the tested materials will have similar anti-erosive remineralizing action on enamel.

## MATERIALS AND METHODS

### Remineralizing agents:

Three remineralizing agents were selected for this study: CPP-ACP (Tooth Mousse, GC Corporation, Tokyo, Japan). It is a topical crème with bio-available calcium and phosphate. Second, 10% (Nano-HAP) which was prepared by wet chemical reaction of calcium nitrate, with ammonium hydroxide in the form of hydro gel with a concentration of 10 wt % Hydroxyapatite. The average grain size was  $100 \pm 5$  nm (L),  $20 \pm 3$  nm (D). The reaction was performed at room temperature. Finally, Pchi-ACP was prepared. The preparation was done by mixing 2.0 g of commercially available low molecular weight chitosan (Sigma-Aldrich, USA) (75–85 % deacetylated, molecular weight of  $2.2 \times 10^4$ ) with phosphorus pentoxide in methane sulphuric acid (20 mL). After that, the mixture was held overnight at  $-20^\circ$  C, and then precipitated with methanol and collected by centrifugation (5,000 RPM, 10 min). The product was washed with acetone and collected by three cycles of centrifugation and then dried in a vacuum desiccator. In order to prepare the nano-complexes of Pchi-ACP,  $\text{CaCl}_2$  was dissolved in solution of Pchi (0.5 % w/v) to a final concentration of 10 mM under stirring.  $\text{K}_2\text{HPO}_4$  was added into the solution to a final concentration of 6 mM. The pH was adjusted to be 7.0 using 1 M KOH.<sup>15</sup> The materials composition, manufacturers and lot numbers are presented in **table (1)**.

TABLE (1): Product names composition, lot numbers, and manufacturers of materials

Product name	Composition	# Lot	Manufacturer
<b>GC Tooth Mousse</b>	Glycerol, CPP-ACP, D-sorbitol, CMC-Na, Propylene glycol, Silicon dioxide, Titanium dioxide, Xylitol, Phosphoric acid, Flavoring, Zinc oxide, sodium saccharin, ethyl p-hydroxybenzoate, Magnesium oxide, Guar gum, Propyl-hydroxybenzoate, Butyl p-hydroxybenzoate	170118A	C Corporation, Japan
<b>10% Nano-hydroxyapatite</b>	Wet chemical reaction of calcium nitrate, with ammonium hydroxide ((NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> )		NanoTech Egypt for photo-Electronics
<b>Pchi-ACP</b>	Chitosan mixed in methane sulphonic acid and phosphorous pentoxide then CaCl <sub>2</sub> and K <sub>2</sub> HPO <sub>4</sub> were added		NanoTech Egypt for photo-Electronics

### Enamel specimen preparation:

A total of 25 human upper incisors were collected for this study. The incisors were free from cracks, caries, white spot lesions, fractures and enamel hypoplasia and they were extracted for periodontal reasons. After their extraction, teeth were cleaned from any soft tissue debris, disinfected and stored in deionized water (pH = 7.413). The roots of teeth were cut using diamond disc (**Dentorium, Germany**) with a water spray, and teeth were embedded in an acrylic resin cylinder with the enamel surface exposed and labial surfaces facing upwards. Labial surfaces were ground under running water using 320-, 600-, and 1200-grit waterproof silicon-carbide abrasive papers (**3M products**) removing about 200  $\mu\text{m}$  of the surface of the tooth.<sup>16</sup> Thereafter, the specimens were cleaned using 5% NaOCl for an hour then washed with deionized water for five minutes. The specimens were stored in deionized water during the whole experiment. The baseline root mean-square roughness,  $R_{\text{rms}}$  was measured for all the specimens before starting the erosive challenge.

### Erosive challenge and remineralization of the specimens

A soft drink Coca-Cola, (Coca-Cola Company, Egypt) was chosen for the demineralization process. The pH of soft drink was determined using a digital pH meter<sup>17</sup> (**U-Tech, Singapore**). The beverage was

kept refrigerated at constant temperature of 20°C. The specimens were randomly assigned to five groups each made of five samples.

- **Group 1:** intact enamel (negative control group) not subjected to erosion or remineralization.
- **Group 2:** enamel + soft drink (positive control group).
- **Group 3:** enamel + soft drink+ CPP-ACP.
- **Group 4:** enamel + soft drink+ 10% nano-HAP.
- **Group 5:** enamel + soft drink+Pchi-ACP.

The specimens in group 1 were stored during the whole experimentation and they did not receive any treatment. The specimens in the rest of the groups were immersed in 6mL of the soft drink for 2min at 20° C, and then they were rinsed with deionized water. Four consecutive intervals of the immersion procedure were performed at 0, 8, 24 and 36 h for a total of 8 min using freshly opened bottles of Coca Cola for each immersion.<sup>18</sup> After each of the demineralizing immersion interval, the anti-erosive agent assigned for each group was applied for 3 min by using micro applicators with fine tips of 2.0 mm. The remineralizing agents were not brushed to the surfaces of the specimens in groups 3, 4 and 5. The total time for remineralization was 12 min then the agent was wiped off and washed with distilled water.

### Testing methods: Atomic Force Microscope (AFM) observations

After 24 h from the last procedure of demineralization and remineralization,<sup>19</sup> specimens were observed with AFM for the second time. AFM was operated in contact mode using nonconductive silicon nitride probe, manufactured by (**Bruker Nano Inc., Camarillo, CA, USA**) using scan rate 1 Hz. Images were 25  $\mu\text{m}$  x 25  $\mu\text{m}$  with resolution of 256 x 256 pixels. Pro-scan 1.8 software was used for controlling the scan parameters and IP 2.1 software for image analysis.

### Statistical analysis

Statistical analysis was then performed using a commercially available software program (**SPSS 18; SPSS, Chicago, IL, USA**) to compare the mean root mean square roughness ( $R_{\text{rms}}$ ) at baseline and at the end of the study. As data was parametric, significance of the difference between groups was evaluated using one-way analysis of variance (ANOVA test), followed by Tukey's post hoc test. The level of significance was set at  $P < 0.05$ .

## RESULTS

Table (2) shows that  $R_{\text{rms}}$  of different groups at baseline was not statistically significant. After

erosive demineralization,  $R_{\text{rms}}$  of group 2 increased and was significantly higher than all other groups according to ANOVA test ( $p=0.001$ ). For group 3, the  $R_{\text{rms}}$  also significantly increased after erosive demineralization followed by CPP-ACP treatment ( $p=0.38$ ). On the other hand, groups 4 and 5 did not show any significant change in  $R_{\text{rms}}$  compared to baseline.

### AFM images results:

AFM images are clear and explanatory which is reflected on the scores of different groups. This enables the comparison between the patterns of the outermost layer of enamel. Figure (1) shows that the intact enamel surface is smooth except for minor elevations. Figure (2) shows obvious cavities and increase in enamel surface elevations after erosive demineralization. Figure (3) reveals enamel surface with globular non-homogeneous deposits and adherent irregularities after CPP-ACP treatment. Figure (4) shows homogeneous deposits so that the enamel was hidden by a homogeneous layer after nano-HAP treatment. Figure (5) also shows formation of a thick homogenous layer after Pchi-ACP treatment.

**Table (2):** Descriptive statistics and test of significance of  $R_{\text{rms}}$  in tested groups at baseline and after erosive demineralization and treatment

Groups	Baseline		After erosive demineralization & remineralization		t-value	P- value
	Mean	SD	Mean	SD		
1) Intact enamel	67.35	21.11	67.35 <sup>a</sup>	21.11	0	1 <sup>ns</sup>
2) Enamel+ soft drink	43.74	12.52	103.22 <sup>b</sup>	30.89	-7.587-	0.001*
3) Enamel+ soft drink + CPP-ACP	47.53	14.29	63.85 <sup>a</sup>	18.37	-2.801-	0.038*
4) Enamel+ soft drink + Nano-HAP	66.29	22.12	57.11 <sup>a</sup>	19.13	1.379	0.227 <sup>ns</sup>
5) Enamel+ soft drink + Pchi-ACP	68.51	24.34	59.64 <sup>a</sup>	20.25	1.413	0.232 <sup>ns</sup>

Significance level  $p < 0.05$ ,

\*significant

ns= non-significant

Different superscript letters in the same column are significantly different according to ANOVA test.

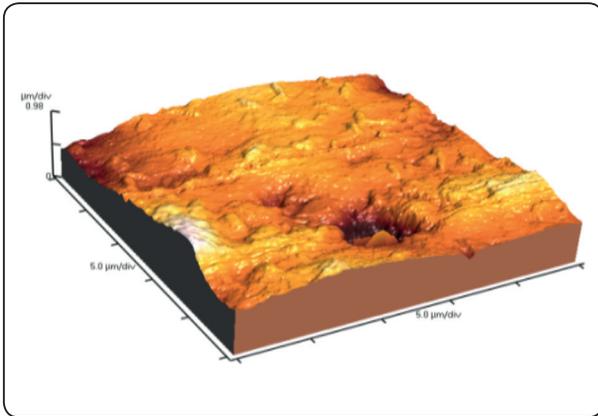


Fig. (1) 3D AFM image for group 1

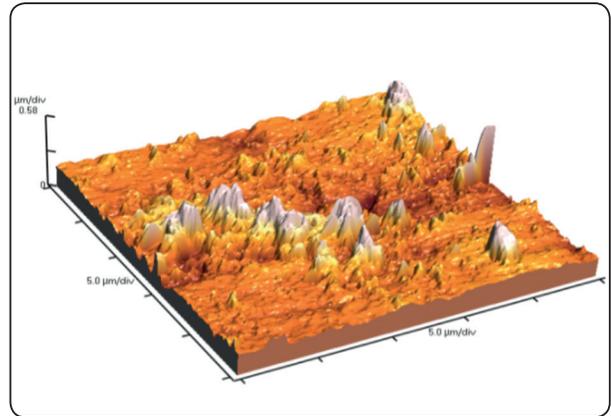


Fig. (2) 3D AFM image for group 2 after erosive demineralization

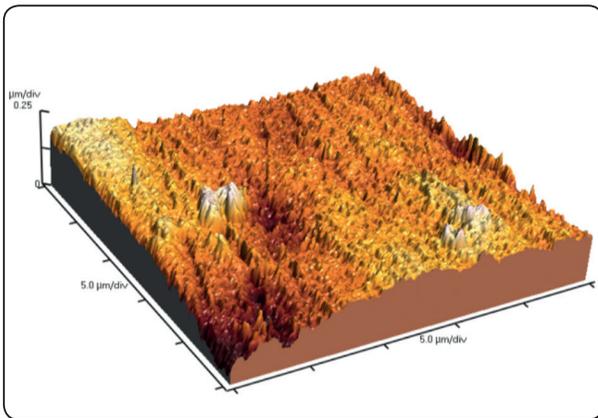


Fig. (3) 3D AFM image for group 3 after erosive demineralization and CPP-ACP

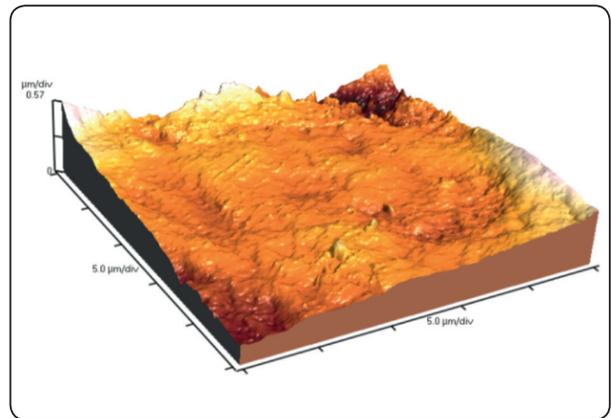


Fig. (4) 3D AFM image for group 4 after erosive demineralization and nano-HAP

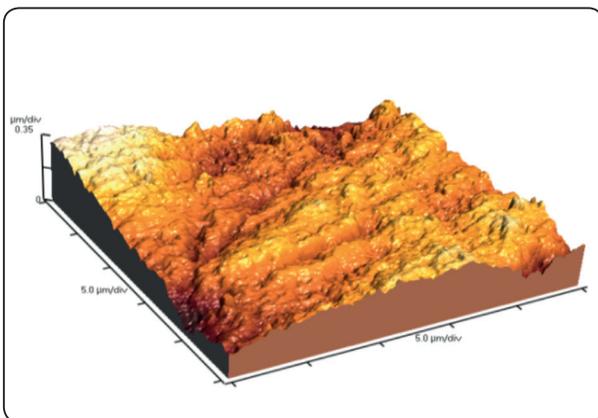


Fig. (5) 3D AFM image for group 5 after erosive demineralization and Pchi-ACP

## DISCUSSION

Tooth material loss has been vastly discussed. However, little attention has been made to investigate the tooth surface change during erosion.<sup>19</sup> The development of erosion involves a chemical process in which the inorganic phase of the tooth is demineralized.<sup>20</sup> Biomineralization of enamel is a regulated process which involves protein-protein and protein-mineral interactions.<sup>21</sup> The knowledge about the remineralizing potential of biomaterials based on nanotechnology should be widened to develop preventive strategies for patients subject to high erosive challenges.

The current invitro study was designed to assess the effectiveness of different remineralizing anti-erosive agents on eroded enamel surface using AFM which is not focused on in literature till now. The uniqueness of this study is that it compares three fluoride alternative anti-erosive agents. The Pchi-ACP is a relatively newer remineralizing agent so its comparative studies are few. The materials were carefully selected to serve the aim of the study.

The CPP are produced from the tryptic digest of casein then aggregated with calcium phosphate and purified with ultrafiltration.<sup>22</sup> CPP keeps calcium and phosphate in an amorphous, non-crystalline state that helps them enter enamel. It was demonstrated that the remineralizing effect of CPP-ACP on enamel is similar to that of saliva.<sup>23</sup> In 2009, Ranjitkar et al,<sup>24</sup> suggested that the erosion inhibiting potential of CPP-ACP probably involves remineralization action. This was the reason for selecting CPP-ACP in the current research.

Nano-HAP is chemically homogenous with enamel.<sup>25</sup> It was proven that nano-HAP was more efficient to prevent erosion compared to micro-HA.<sup>25</sup> Huang et al, 2009,<sup>26</sup> revealed that different concentrations of nano-HAP (1, 5, 10, and 15%) were effective compared with placebo on enamel remineralization, and 10% nano-HAP was the best concentration. Haghgoo et al, 2011,<sup>27</sup> showed that enamel microhardness (decreased due to erosive lesion) was enhanced significantly after exposure to nano-HAP solution. Thus, it was of value to stress on the effectiveness of 10% nano-HAP.

Chitosan is a naturally occurring deacetylated product of chitin from crustacean's exoskeleton.<sup>28</sup> Due to its positive charge and amino groups, chitosan can bind to negatively charged teeth surfaces.<sup>29</sup> It was speculated that chitosan can form a layer-by-layer build-up on enamel, providing better protection against erosion.<sup>30</sup> Nano-complexes of Pchi-ACP could be a good method to provide stable ACP to be adsorbed on enamel surface.

For this reason, Pchi-ACP was selected.

In this study, grinding of enamel surfaces was done for standardization. This would eliminate natural variations on enamel surface, moreover, variations between different tooth sites and types.<sup>1</sup> It is important to mention that natural tooth surfaces erode more slowly than polished surfaces.<sup>24</sup> After grinding, cleaning of specimens was performed using 5% NaOCl for an hour, which would not alter enamel surface.<sup>20</sup> According to the recommendation by Shellis et al,<sup>31</sup> the specimens are better stored moist between cycles. Enamel specimens were washed and stored in deionized water which is in accordance with the technical report of ISO (1991).<sup>32</sup>

In order to mimic soft drinks consumption habits and their actual *in vivo* erosive potential, the protocol followed in the present study was four consecutive intervals of 2 minutes, at 0, 8, 24 and 36 hours. This was considered to simulate, as closely as possible, the natural consumption of Cola drink during the main daily meals.<sup>33</sup> The soft drink is commonly used to produce erosive enamel lesions. The time that the tooth surface is exposed to acid should be minimal, so that the surface change produced is initial erosion, and not an initial carious lesion.<sup>34</sup> The Cola drink has the highest erosive potential<sup>35</sup> compared to other soft drinks. Its pH level is below 4, thus it is expected to be effective because erosion is correlated to pH.<sup>1</sup> In this study, the new Cola drink was used in each new cycle to ensure that it was carbonated and to reduce the buffering effect from ions dissolved from enamel.<sup>36</sup> The Cola was kept at a constant temperature of 20° C as the effect of temperature can be significant.<sup>37</sup> Artificial saliva was not used during the erosion challenge because its use would buffer the acidity of the Cola drink and limit the erosion process.<sup>36</sup>

AFM is a nanoindentation technique that is capable of obtaining images with atomic resolution with minimal sample preparation. Recently it was used to characterize enamel erosion. Minute specimen

preparation is one of the main advantages of AFM over other techniques.<sup>38</sup> AFM was used in the present study to verify the protective anti-erosive remineralizing effect of three biomaterials on enamel surface with images of high contrast and resolution.  $R_{rms}$  was the topographical parameter determined to quantitatively evaluate enamel surface.<sup>20</sup>

The baseline  $R_{rms}$  in this study was nearly similar to a previous study by Murakami et al,<sup>39</sup> with a range of 43-68 nm but was different from the research by Quartarone et al,<sup>40</sup> with a range of 50-120 nm. This variation could be due to variation in geographical location, age and oral environment. Coca-Cola showed high erosive potential on human enamel indicated by the highly significant increase in surface roughness in the second group. However, in the oral environment, many factors can enhance or prevent demineralization such as mineral concentration, pellicle and plaque formation, salivary flow rate and its buffering capacity.<sup>41</sup>

Specimens in the third group also showed significant increase in  $R_{rms}$ , which was in accordance with the study by Carvalho et al, 2013,<sup>42</sup> who found a non-homogeneous layer with globular irregularities in samples treated with CPP-ACP in AFM images. These globular irregularities were also cited by Poggio et al, 2009,<sup>41</sup> A possible explanation could be that erosion occurred in absence of dental plaque and that enamel was exposed to four subsequent demineralization intervals, it is highly likely that if CPP-ACP succeeded in remineralization it will not be able to advance by the time of the next interval. This indicates that the presence of calcium and phosphate is short-lived, and enamel is deprived of clinically relevant remineralization time.

On the other hand, this came in contradiction to a previous study by Willershausen et al, 2009,<sup>43</sup> who evaluated the effect of CPP-ACP paste on untreated enamel surfaces exposed to an erosive challenge. They found that there was a slight gain in the mineral contents after the application of a CPP-ACP paste, mainly in the upper enamel layer.

Interestingly, both groups 4 and 5 had  $R_{rms}$  values nearly similar to group 1. This implies that nano-HAP and Pchi-ACP can stop the progression of erosion and provide a sort of protection for enamel surface. Therefore, the null hypothesis should be rejected because CCP-ACP did not show a similar action. This may be explained by that nano-HAP forms a homogenous layer completely hiding the prismatic and interprismatic enamel structure, proved by the AFM image (Fig. 4). Another possible mechanism of action of nano-HAP would be a mechanical imbrication of the nanocrystals into the porous enamel surface. Najibfard et al, 2011,<sup>44</sup> also suggested that nano-HAP is capable of producing a thin but tightly bound layer on the tooth surface due to its hydrophilic and wetting characteristics. This was in agreement with previous studies that showed a positive effect of nano-HAP on enamel.<sup>5,7,26</sup>

ACP may act as a transient phase during enamel repair.<sup>45</sup> This process is regulated by the phosphorylated proteins that can stabilize ACP by sequestering calcium ions through the electrostatic attraction between positive charged calcium ions and negative charged phosphate groups.<sup>46</sup> Since chitosan is a biopolymer, it is likely that the reactive molecule chitosan did react with the enamel surface in the current study and formed thin, ubiquitous chitosan layer coverage.<sup>29</sup> Such chitosan-based layers have a thickness of few nanometers,<sup>47</sup> which was apparent in fig. 5. In view of the results of this study, the need for further in situ/in vivo research is recommended. This will help to verify the effectiveness of nano-biomaterials in preventing enamel erosion.

## CONCLUSIONS

Within the limitations of this in-vitro study, it can be concluded that both 10% nano-HAP and Pchi-ACP are promising remineralizing nano-biomaterials. They may provide adequate protection for enamel surface under erosive conditions.

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