The incorporation of phosphorylated chitosan/amorphous calcium phosphate nanocomplex into an experimental composite resin

Ju NIU1, Di LI1, Zeying ZHOU1, Jingyue ZHANG1, Dandan LIU1, Wendi ZHAO1, Chengji ZHAO2 and Xiaoqiu LIU1

1 Department of Prosthodontics, Hospital of Stomatology, Jilin University, Changchun, China
2 Alan G MacDiarmid Institute, College of Chemistry, Jilin University, Changchun, China

Corresponding author, Xiaoqiu LIU, E-mail: xqliu@jlu.edu.cn

This study evaluated the effect of incorporating phosphorylated chitosan/amorphous calcium phosphate nanocomplex (Pchi/ACP) into an experimental light-cure composite resin on mechanical-chemical properties and human dentin remineralization. The results showed that the mechanical strength and contact angles of the resins decreased with the increase incorporation of Pchi/ACP. Release concentrations of calcium in saline solution were measured at different time points, showing the incorporation of Pchi/ACP significantly increased calcium release within 14 days, and kept steady thereafter. Finally, the demineralized dentin slabs treated with our resins for four weeks were characterized by SEM-EDS. Various amounts of apatite were formed on the dentin slabs which were treated with the resins containing Pchi/ACP, whereas no apatite was formed without Pchi/ACP. In conclusion, the Pchi/ACP-incorporating composite resin can be a promising dental material due to its favorable mechanical and remineralization properties.

Keywords: Remineralization, Dentin, Composite resin, Amorphous calcium phosphate

INTRODUCTION

Dentin defects are common oral diseases in clinical practice, which are resulted from caries, traumas, acid corrosion, wedge-shaped defects and other causes. The traditional light-cure composite resin, using bisphenol A diglycidylether methacrylate as the monomer, has the excellent aesthetics, clinical operation and bonding performance. It has become the most commonly used dental restorative material since its introduction in the 1960s. However, the service life of composite resin is less than ten years1), which is attributed to the following two aspects: (i) because of the complexity of oral environment and the polymerization shrinkage of composite resin, the marginal adaption of the material is inevitably damaged with the time going by, resulting in secondary caries and backfilling, (ii) dentin tissue has a higher ratio of the organic matrix, lower surface energy as well as the fluid infiltration. Thus, the collagen fibers will be exposed and hydrolyzed after water absorption2). In addition, the exposed collagen fibers are further degraded by matrix metalloproteinases, which weaken the bonding strength between the adhesive and dentin3).

Therefore, finding effective methods to maintain the integrity of the hybrid layer is an important issue in restorative dentistry. In recent years, biomineralized materials have become ideal for the remineralization of defective dentin tissue, such as calcium silicate-based materials4,5), calcium phosphate materials including β-tricalcium phosphate6), amorphous calcium phosphate (ACP)7), hydroxylapatite8,9), and fluoride10). Incorporating these bioactive components into dental light-cure composite resins and applying them to dentin defects can effectively enhance the remineralization of dentin and improve the stability of the hybrid layer. ACP is a spherical mineral precursor with excellent bioactivity and biocompatibility in the remineralization process11). Moreover, because of the high specific surface area of ACP nanoparticles, the release of calcium ions is relatively high even at a low filling ratio12). Therefore, ACP is used as the mineral source in our experimental composite resins.

However, ACP is easy to spontaneously change into a more stable crystalline state in the liquid environment due to its extremely unstable thermodynamics13), thus influencing the release of mineral ions. Schweikle et al.12) produced zinc-doped ACP of high stability which showed no signs of crystallization at least 20 days. Zhang et al.14) found that modifying ACP with zirconium could significantly impede the conversion of ACP to hydroxyapatite, which was conducive to the sustained release of calcium and inorganic phosphorus ions in composite resin materials. Polycarboxylate macromolecules, such as polyacrylic acid15) and polyaspartic acid16), have also been verified to stabilize ACP at nanoscale due to the electrostatic interaction between carboxyl groups and calcium ions. Niu et al.17) found that the cationic polymer also played the same role by attracting phosphate ions in the biomineralization solution. Moreover, some metal ions such as magnesium18) and zinc12,19) could stabilize ACP by slowing down the nucleation rate of hydroxyapatite, and the physical properties of ACP remained unchanged. Chitosan and its derivatives are novel biomaterials with extensive sources, desirable biocompatibility, and complete biodegradability10). Phosphorylated chitosan
(Pchi) has been widely studied in the field of medical biomaterials due to its bioactivity, cytocompatibility, and anti-inflammatory properties, especially the ability to encapsulate Ca\(^{2+}\) and PO\(_4\)\(^{3-}\) to form metastable ACP nanoparticles\(^{19}\).

In the early stage of this study, 3,3’,5,5’-tetramethyl biphenyl (TMBP) was synthesized with higher stability, heat-resistance and strength than bisphenol A epoxy resin\(^{20}\). In this work, TMBP was reacted with acrylic acid to generate 3,3’,5,5’-tetramethyl biphenyl epoxy acrylate (TMBPEA). TMBPEA monomer is better in low polymerization shrinkage and high mechanical properties due to the presence of biphenyl structure. Therefore, TMBPEA was selected as the matrix monomer in our composite resin for better properties.

The purposes of this study were to synthesize the novel bioactive composite resin containing Pchi/ACP and investigate its mechanical-chemical properties. Moreover, the release concentration of calcium and human dentin remineralization ability were analyzed by ion chromatograph and SEM-EDS, respectively.

**MATERIALS AND METHODS**

*Synthesis of Pchi/ACP*

Pchi was synthesized according to the technique reported by Nishi et al.\(^{18}\). 2.0 g chitosan (Degree of deacetylation ≥90%, BBI, Shanghai, China) was dissolved in 14 mL methanesulfonic acid (Aladdin, Shanghai, China), followed by adding an appropriate amount of P2O5 (Aladdin). The mixture was stirred at 10–5°C for 1.5 h. Thereafter, the product was kept at −20°C overnight, then precipitated and washed with methanol and acetone. The final product Pchi was collected by a vacuum filter and dried under vacuum condition. In order to detect whether phosphate group was successfully grafted on chitosan, Pchi was characterized using Fourier Transform Infrared Spectroscopy (FT-IR, VECTOR22, Bruker, Ettlingen, Germany) in a KBr matrix.

In the preparation of Pchi/ACP complex, Pchi, CaCl\(_2\)•2H\(_2\)O and Na\(_2\)HPO\(_4\) were sequentially added to deionized water. It was noted that Pchi should be added slowly and dissolved under continuous stirring. The final solution contained 50 g/L Pchi, 100 mmol/L CaCl\(_2\)•2H\(_2\)O, 60 mmol/L Na\(_2\)HPO\(_4\), and the ratio of calcium to phosphorus in the solution was 5:3. After freezing the solution at −80°C for 24 h, Pchi/ACP complex was obtained by lyophilization technique. The morphology and particle size of Pchi/ACP were observed under TEM (JEM-1200EX, JEOL, Tokyo, Japan). XRD (Empyrean, PANalytical, The Netherlands) was used to evaluate the amorphous state of Pchi/ACP.

*Synthesis of Pchi/ACP contained composite resin*

TMBPEA monomer was obtained by the ring-opening esterification of TMBP with acrylic acid (Aladdin). Purified TMBP powder, a small amount of triphenylphosphine (Aladdin) and hydroquinone (Sinopharm, Shanghai, China) were added into a three neck flask. The reactants were stirred and heated to 90°C under the protection of nitrogen, then a certain amount of acrylic acid was added. The reaction temperature was controlled at 105°C and continued for 4 h.

The biomimetic composite resin was prepared by incorporating the lyophilized Pchi/ACP powder and some other essential ingredients into TMBPEA monomer. Our experimental resin co-monomer blend was formulated using TMBPEA monomer and 2-hydroxyethyl methacrylate (HEMA, Aladdin), and they were made light-cure by adding 0.5 wt% of camphorquinone (Aladdin) and 0.5 wt% of dimethylaminomethyl methacrylate (Sigma-Aldrich, St. Louis, MO, USA). The hydrophilic monomer HEMA was chosen as a viscosity diluent and calcium release adjuvant. In order to enhance the mechanical and chemical properties, different sizes of SiO\(_2\) were added into the compound. The weight ratio of micron silica (2 μm, Aladdin) to nano silica (30 nm) was 9:1. Four composite resin systems were subsequently created with different ratios of Pchi/ACP and SiO\(_2\) (Table 1). All processes were carried out in dark conditions.

*Properties of the experimental composite resin*

1. **Scanning electron microscopy (SEM) measurement**

The fracture micro-morphologies of each group material were analyzed by SEM. Specimens with a length of 25 mm, a width of 2 mm and a height of 2 mm of each group were brittlely broken in liquid nitrogen. Then these fractured specimens were adhered to the sample stage with conductive adhesive and sprayed with gold before being examined.

2. **Contact angle measurement**

The as-prepared four groups of mixtures were separately

Table 1 Division of experiment groups

| Groups | Components |
|--------|------------|
| A      | 40 wt% TMBPEA/HEMA+35 wt% Pchi/ACP+25 wt% SiO\(_2\) |
| B      | 40 wt% TMBPEA/HEMA+25 wt% Pchi/ACP+35 wt% SiO\(_2\) |
| C      | 40 wt% TMBPEA/HEMA+15 wt% Pchi/ACP+45 wt% SiO\(_2\) |
| D      | 40 wt% TMBPEA/HEMA+0 wt% Pchi/ACP+60 wt% SiO\(_2\) |

TMBPEA, 3,3’,5,5’-tetramethyl biphenyl epoxy acrylate; HEMA, 2-Hydroxyethyl methacrylate; Pchi/ACP, phosphorylated chitosan and amorphous calcium phosphate nano-complex
filled into a round stainless steel mold (15 mm in diameter and 1 mm in thickness) without bubbles, and the surfaces were covered with polyethylene films and glass slides. Both sides of each sample were cured by nine-points surrounding method (Fig. 1) using a LED unit (1,200 mW/cm², bluephase N, Ivoclar Vivadent, Austria), and each point continued for 20 s.

The 15×1 mm resin discs were polished and placed on a contact angle analyzer (JC2000D1, POWERACH, Shanghai, China) to obtain the contact angles. Each group contained three discs, and three places were measured for each disc. The results of each group were the arithmetic mean of nine results.

3. Flexural strength and elastic module measurements
A 25×2×2 mm elongated stainless steel mold was used to prepare specimens. Each curing position overlapped with the previous along the radius. Six points were selected for each side and cured for 120 s in all. All the specimens were polished on each side and stored in deionized water bath for 24 h at 37°C. The exact thickness (mm) and width (mm) were measured with a micrometer before subjecting them to the three-point bending tests at a universal test machine (AG-10TA, Shimadzu, Kyoto, Japan), operated at a 20 mm span with a loading speed of 1 mm/min. Six specimens of each group were subjected to the test. Afterwards, the flexural strength (FS) and elastic modulus (EM) were calculated using the following two formulas.

\[
FS = \frac{3FL}{2wh^2} \quad (1)
\]

\[
EM = \frac{kL^3}{4wh^4} \quad (2)
\]

where F is the maximum load (force), L is the length of the support span, w and h are the actual width and thickness of each specimen, respectively, and k is the slope of the straight line segment of the load displacement curve.

4. Vickers hardness measurement
Specimens were prepared using a 6×3 mm stainless steel mold and cured for 60 s on each side. The hardness of each specimen were measured on a Vickers indenter (HMV-G 20ST, Shimadzu) with a crosshead loading force of 100 g for 10 s. Each group contained three specimens and each specimen was measured for three times. The results of each group were the arithmetic mean of nine results.

5. Calcium ion release measurement
Calcium ion release concentrations were measured only for the Pchi/ACP-incorporating groups. A total number of 15×1 mm round specimens were made, five specimens for each group. All the specimens were respectively immersed in 50 mL saline solution (pH 7.4) after which all the specimens were kept in an incubator at 37°C. After 1, 4, 7, 14, 21 and 28 days, calcium ion release concentrations of the materials were quantitatively measured by ion chromatograph (ICS-1000, DIONEX, CA, USA).

6. Remineralization properties
Simulated body fluid (SBF) was regarded as the remineralized medium because it has very similar concentration and pH to human blood plasma. SBF was prepared by dissolving 142 mM NaCl, 4.2 mM NaHCO₃, 5 mM KCl, 1 mM K₂HPO₄·3H₂O, 1.5 mM MgCl₂·6H₂O, 2.5 mM CaCl₂, 0.5 mM Na₂SO₄ in deionized water, 1 M HCl and tris(hydroxymethyl)-aminomethane were used to adjust pH to 7.4.

Five intact human third molars were extracted and stored in 1% chloramine solution at 4°C. The teeth were collected after the patients’ informed consents were obtained under a protocol approved by the Ethics Committee of the Faculty of Dentistry at Jilin University, China. All teeth were used within three months after extraction. After removing the surface enamel, a 1 mm thick dentin slab was prepared by making two parallel cuts perpendicular to the longitudinal axis of each tooth through a low-speed linear cutting machine (STX-202A, Kejing, Shenyang, China) under water cooling. The shape of these slabs was trimmed to a diameter of 1 cm and a thickness of 1 mm. The slabs were wet polished with 800-, 1200-, 1500-, and 2000-grit silicon carbide papers and ultrasonically cleaned with deionized water.

The prepared dentin slabs were thoroughly demineralized in order to eliminate false positive results of remineralization. All the dentin slabs were incubated in ethylene diamine tetraacetic acid solution (pH=8.0) by stirring at room temperature for five days. After that, the slabs were rinsed with deionized water for five times, and then cleaned ultrasonically for 10 min.

The previously demineralized dentin slabs were used to evaluate the remineralization capability of our composite resin. Each group of resin disc (15×1 mm) was kept in close contact with a demineralized dentin slab.
by the clamps. Then they were soaked in 50 mL SBF at 37°C for four weeks. At last, the dentin slabs were taken out, rinsed with deionized water, and analyzed after drying with SEM-EDS (XL30 ESEM-FEG, FEI, Columbia, MD, USA). EDS spectrums referred to the whole image and the elemental proportions were an average over the whole image. The elemental analysis (wt% and at%) of each sample were performed applying the ZAF correction method. By applying the quantitative approach, Ca/P could be calculated.

Statistical analysis
The data reported in the present study were mean values. Significant statistical differences between groups of data were determined using one-way and two-way analyses of variance (SPSS, version 23.0). Tukey’s multiple comparison tests were used to compare the data at a $p$-value of 0.05.

RESULTS
Characterization of phosphorylated chitosan and Pchi/ACP
As shown in Fig. 2, the spectra of chitosan showed characteristic -OH frequency band at 3,422 cm$^{-1}$. However, in Pchi spectra, the peak at 3,422 cm$^{-1}$ became narrower and weaker, which might result from the participation of -OH in the esterification reaction. Meanwhile, the amide bending vibration peak at 1,655 cm$^{-1}$ and the amino absorption peak at 1,599 cm$^{-1}$ disappeared in Pchi. Two new peaks, formed by the chitosan phosphorylated reaction, appeared at 1,660 cm$^{-1}$ and 1,540 cm$^{-1}$. Moreover, the P=O and P-O bands at 1,220 cm$^{-1}$ and 1,041 cm$^{-1}$, respectively, which were the characteristic peaks of Pchi were observed. In Pchi/ACP spectrum, there was the displacement and decrease of the peaks in the range of 1,300–1,600 cm$^{-1}$, and no sharp peaks of calcium phosphate crystals appeared. It could be inferred that there were some intermolecular interactions between Pchi and ACP particles.

The amorphous character of Pchi/ACP was confirmed by XRD (two diffraction broad bands in the region of $2\theta=3°$–$50°$) in Fig. 3. Compared with the chitosan before modification, the position of the characteristic peak in Pchi at $2\theta=20°$ was basically unchanged. However, the height of the peak in Pchi was lower than that in chitosan, which demonstrated that phosphate group could decrease the crystallinity of chitosan.

The morphology of Pchi/ACP was observed under TEM (Fig. 4). The result showed that the ultrastructure of Pchi/ACP was spherical nanoparticle with a diameter of 50–100 nm, rather than the faceted angular shape of crystalline calcium phosphates.

Properties characterization of the experimental composite resin SEM analysis
The section morphologies of each group material were showed in Fig. 5. In the resin system, the matrix monomers of each group were uniformly mixed with SiO$_2$ particles and Pchi/ACP. From group A–C, with the increase of Pchi/ACP content, the surfaces of the composite resin were getting more and more smoother, which could not see any raised structures. Instead, the surface of group D was rough and uneven on which agglomerated SiO$_2$ nanoparticles can be seen. Moreover, SiO$_2$ nanoparticles were dispersed among micron particles to form a firm mechanical interlocking structure.
1. Contact angle
The contact angles between different groups were significantly different, and decreased with the increase content of Pchi/ACP (Fig. 6). The contact angle of the composite resin containing 35 wt% Pchi/ACP in group A was the lowest in the four groups, which was 37% lower than that of the resin without Pchi/ACP in group D.

2. Mechanical properties
The mechanical strength decreased with the increase content of Pchi/ACP, as shown in Fig. 7. The flexural
strength increased from group A to group D (Fig. 7A). The incorporation of 35 wt% Pchi/ACP in group A material resulted in the significant decrease in the flexural strength compared with group D material without Pchi/ACP. However, there was no difference between group B (25 wt% Pchi/ACP) and group C (15 wt% Pchi/ACP), nor group C and group D (0 wt% Pchi/ACP) (Fig. 7A). What's more, there was a statistically significant decrease trend in the elasticity modulus of four groups materials, especially for group A, which was 64% lower than group D (Fig. 7B). In addition, the Vickers hardness of each group material decreased significantly with the increase content of Pchi/ACP (Fig. 7C).

3. Calcium ion release
From the overall trend in Fig. 8, there was a significant positive correlation between calcium ion release concentration and time within 14 days for groups A and B, while group C exhibited no significant correlation. After 14 days, the calcium concentration of each experimental group reached almost constant. And calcium ion release concentration of group A was the highest at each time point, followed by groups B and C.

4. Remineralization properties
SEM and corresponding EDS analyzes of remineralized dentin slabs were shown in Fig. 9. The dentin slabs treated with our experimental resins displayed diffuse calcium phosphate deposits and the presence of phosphorus and calcium peaks. It was evident that the number of apatite deposited on the dentin surface varied with the contents of Pchi/ACP in resins. The incorporation of Pchi/ACP significantly increased the apatite deposits and weight fractions of calcium and phosphorus elements on dentin surface. The dentin slab, which was treated with the composite resin containing 35 wt% Pchi/ACP, was deposited the most apatite and covered the most orifices of dentinal tubules, followed by groups B and C (Fig. 9A–C). EDS analyses of their respective constituent elements showed the same results, too. The weight ratio of calcium-to-phosphorus (Ca/P) on the dentin slab which was remineralized by group B material (25 wt% Pchi/ACP) was 1.76, showing the newly formed apatite was most likely hydroxyapatite (Ca/P=1.67). However, the weight ratios of Ca/P in group A (35 wt% Pchi/ACP) and group C (15 wt% Pchi/ACP) were less than 1.67, indicating that hydroxyapatite had not been formed or other calcium phosphorus complexes were formed. On the contrary, the dentin slab treated with the composite resin without Pchi/ACP of group D presented no apatite deposition in Fig. 9D. As a comparison, the thoroughly demineralized dentin slab with no treat.
without any treat showed collapsed dentin collagen, on which the orifices of dentinal tubules shrank. Moreover, there was almost no element of calcium and phosphorus as shown in EDS results (Fig. 9E).

**DISCUSSION**

For the first time, Pchi/ACP was incorporated into the dental light-cure composite resin, which could release high concentration of calcium and make the apatite-defected dentin remineralized in aqueous media. Among the four groups, the material containing 35 wt% Pchi/ACP was not recommended because of its lowest mechanical properties and highest hydrophilicity. However, the material containing 15 wt% Pchi/ACP had poorest ability of calcium ion release and remineralization of dentin in liquid environment. Compared with the above two groups, the material containing 25 wt% Pchi/ACP not only showed relatively ideal mechanical properties and hydrophilicity, but also showed higher calcium ion release concentration and more apatite deposits on the surface of dentin. Therefore, within the scope of this study, the results demonstrated that it was feasible to add 25 wt% Pchi/ACP into the hydrophilic TMBPEA composite resin system.

Traditional treatment of dental caries supports that the softened dentin tissue should be completely removed before filling and repairing. However, in order to achieve the maximum retention of tooth tissues and the preservation of pulp activity, many dentists believe that the softened dentin near the pulp cavity is not necessary to be removed. Nowadays, the compacted filling of bioactive ions release materials has achieved the goal of remineralization on softened dentin to restore its hardness and morphology. *In vivo* and *in vitro* studies have shown that the remineralization materials could both increase the mineral content and improve the mechanical strength of hard tissues. In addition, the biomimetic mineralization of dentin plays an active role in preserving the integrity of the hybrid layer and improving the durability of dentin adhesion. Moreover, it is also significant in preventing the acidic substances produced by bacteria.

ACP is a precursor to hydroxyapatite, which can slowly release calcium ions into aqueous media and form hydroxyapatite on hard tissues. However, the application of ACP in remineralization area is limited due to its instability. Through the exploration of the biomimetic mineralization of natural dentin, researchers have found that non-collagen proteins, such as dentin matrix protein 1 (DMP-1) and dentin sialophosphoprotein (DSPP), can bind with Ca$^{2+}$ to stabilize ACP at nano scale and slow down the formation of large crystals. The phosphate groups in DMP-1 and DSPP can induce the metal chelation reaction with Ca$^{2+}$, thus inducing the separation of liquid phase and preventing the phase transition of ACP. In addition, the carboxyl group (-COOH), sulfonyl group (-SO$_2$H) and phosphate group (-PO$_3$H$_2$) have also been verified to follow the same mechanism for the stabilization of ACP. However, the extraction and purification of natural non-collagen proteins are indeed challenging and costly. As a popular bioactive material with rich sources, chitosan is obtained by the deacetylation of chitin and widely used in the fields of biomedicine. In general, chitosan is usually chemically modified with various functional groups in order to play different roles in different fields. Our studies have proved that Pchi can induce the nucleation of hydroxyapatite and remineralization of hard tissues. Our study further demonstrated that Pchi could be used as a stabilizer for ACP, and the incorporation of Pchi/ACP complex into the dental composite resin could lead to the remineralization of defected dentin.

In this study, the mechanical strength gradually decreased with the increase content of Pchi/ACP in the resin system. The possible reasons are as follows: (i) although Pchi/ACP has ideal biological activity, it has no functional groups bound with the matrix monomer which hinders the interface interaction between the monomer and Pchi/ACP; (ii) the agglomeration and uneven distribution of ACP nanoparticles in the resin system; (iii) Pchi is a hydrophilia biological product which increases the internal pores of materials when wet. Although the composite resin of group A containing 35 wt% Pchi/ACP had the lowest flexural strength, elastic modulus, and Vickers hardness, it still conformed to ISO 4049. According to the contact angles results, the values decreased with the increase content of Pchi/ACP which corresponded to the hydrophilic compounds of Pchi and HEMA. Nonetheless, these hydrophilic compounds also increase the mineral saturation inside the material and provide a hydrophilic environment for ACP to release mineral ions.

In the dental composite resin system, inorganic filler has an important influence on the mechanical and chemical properties, especially the content, particle size and shape of the filler. Wang et al. added micron SiO$_2$ and nano SiO$_2$ to the resin system, and found that the overall performances of the mixed SiO$_2$ resin system were more excellent compared with the system containing only micron SiO$_2$ or nano SiO$_2$. Nanoparticles were evenly dispersed among large particles in the matrix SiO$_2$ system, which could improve the mechanical-chemical properties and have no effect on the release of mineral ions.

The results of calcium release concentrations confirmed that Pchi/ACP is the source of calcium in our experimental composite resin. The concentration of calcium rose with the increase content of Pchi/ACP (Fig. 8). Since the main mechanism for ion release involved water-assisted diffusion, the release of calcium from our composite resin under the relatively neutral condition may be attributed to the high water solubility and hydrophilicity of Pchi/ACP complex. Interestingly, the release concentration of calcium showed a almost flat trend after 14 days. It is speculated that excessive calcium spontaneously precipitated into hydroxyapatite in the saline solution.

It is well known that the hard tissues of vertebrate contain many acidic proteins, such as osteopontin,
bone sialoprotein, dentin matrix protein 1 and dentin sialophosphoprotein\(^2\). These proteins, including multiple aspartic acids and glutamic acids repetitive sequences, are able to generate electrostatic effects with Ca\(^{2+}\) in the liquid environment and serve as nucleation sites of apatite. By this way, apatite crystals are formed and deposited on the surface of hard tissues. In this work, the release of calcium from our composite resin was another indispensable basis for the formation of apatite and the remineralization of dentin tissue. Calcium ions were released into the aqueous media and ingested by the phosphorylated proteins of demineralized dentin, which are the possible mechanisms for the growth and precipitation of crystals induced by Pchi/ACP. The analyzes of SEM and corresponding EDS confirmed that many of the dentinal tubules in the Pchi/ACP-incorporating groups were found to be closed with various amounts of deposits accumulated on the dentin surfaces. The proportions of calcium and phosphorus elements on the dentin surface also increased with the increase contents of Pchi/ACP in the materials accordingly.

The results of present study showed that compared with the composite resin without Pchi/ACP, the mechanical and chemical properties decreased with the increase content of Pchi/ACP in the resin system. However, calcium ion release concentration and remineralization capacity should be considered comprehensively. In conclusion, our TMBPEA composite resin containing 25 wt% Pchi/ACP is still acceptable as a novel remineralization dental material within the limits of this study. Moreover, adhesives between the experimental composite resin and dentin tissue should be considered in further study, and the effect of adhesives on the calcium ion release and apatite formation should be explored.

ACKNOWLEDGMENTS
This study was financially supported by the National Natural Science Foundation of China (Grant No. 81371184) and Department of Science and Technology of Jilin Province (Grant No. 20160519017JH).

REFERENCES

1) Simecek JW, Diefenderfer KE, Cohen ME. An evaluation of replacement rates for posterior resin-based composite and amalgam restorations in US Navy and Marine Corps recruits. J Am Dent Assoc 2009; 140: 200-209.
2) Hashimoto M, Ohno H, Sano H, Kaga M, Oguchi H. In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. Biomaterials 2003; 24: 3795-3803.
3) Chauvaasen-Miller C, Fioretti F, Goldberg M, Menashi S. The role of matrix metalloproteinases (MMPs) in human caries. J Dent Res 2006; 85: 22-32.
4) Sauro S, Osorio R, Watson TF, Toledano M. Therapeutic effects of novel resin bonding systems containing bioactive glasses on mineral-depleted areas within the bonded-dentine interface. J Mater Sci Mater Med 2012; 23: 1521-1532.
5) Hinata G, Yoshiha K, Han L, Edanami N, Yoshiha N, Okiji T. Bioactivity and biomimeralization ability of calcium silicate-based pulp-capping materials after subcutaneous implantation. Int Endod J 2017; 50 Suppl 2: e40-e51.
6) Sauro S, Osorio R, Osorio E, Watson TF, Toledano M. Novel light-curable materials containing experimental bioactive micro-fillers remineralise mineral-depleted bonded-dentine interfaces. J Biomater Sci Polym Ed 2013; 24: 940-956.
7) Allegrini S Jr, da Silva AC, Tsujita M, Sales MB, Gehrke SA, Braga FJC. Amorphous calcium phosphate (ACP) in tissue repair process. Micros Res Tech 2018; 81: 579-589.
8) Sujana A, Venugopal JR, Velmurugan B, Gora A, Salla M, Ramakrishna S, Hydroxyapatite-interwinned hybrid nanofibres for the remineralization of osteoablasts. J Tissue Eng Regen Med 2017; 11: 1853-1864.
9) Guillaume O, Geven MA, Sprecher CM, Stadelmann VA, Grijpma DW, Tang TT, et al. Surface-enrichment with hydroxyapatite nanoparticles in stereolithography-fabricated composite polymer scaffolds promotes bone repair. Acta Biomater 2017; 54: 386-398.
10) Saxena N, Cremer MA, Dolling E, Nurohoman H, Habelitz S, Marshall GW, et al. Influence of fluoride on the mineralization of collagen via the polymer-induced liquid-precursor (PILP) process. Dent Mater 2018; 34: 1378-1390.
11) Dickens SH, Flaim GM, Takagi S. Mechanical properties and biochemical activity of remineralizing resin-based Ca-PO\(_4\) cements. Dent Mater 2003; 19: 558-566.
12) Schweikle M, Bjornay SH, van Helvoort AT, Haugen HJ, Sikorski P, Tainten H. Stabilisation of amorphous calcium phosphate in polyethylene glycol hydorgels. Acta Biomater 2019; 90: 132-145.
13) Zhang F, Allen AJ, Levine KJ, Vaudin MD, Skrctie D, Antonucci JM, et al. Structural and dynamical studies of acid-mediated conversion in amorphous-calcium-phosphate based dental composites. Dent Mater 2014; 30: 1113-1125.
14) Qi Y, Ye Z, Fok A, Holmes BN, Espanol M, Ginebra MP, et al. Effects of molecular weight and concentration of poly(acrylic acid) on biomimetic mineralization of collagen. ACS Biomater Sci Eng 2018; 4: 2758-2766.
15) Kim D, Lee B, Thomopoulos S, Jun YS. In situ evaluation of calcium phosphate nucleation kinetics and pathways during intra- and extrabrillar mineralization of collagen matrices. Cryst Growth Des 2016; 16: 5359-5366.
16) Niu LN, Jee SE, Jiao K, Tonggu L, Li M, Wang L, et al. Collagen intrabrilral mineralization as a result of the balance between osmotic equilibrium and electroneutrality. Nat Mater 2017; 16: 370-378.
17) Yang X, Xie B, Wang L, Qin Y, Henneman ZJ, Nancollas GH. Influence of magnesium ions and amino acids on the nucleation and growth of hydroxyapatite. Cryst Eng Comp 2011; 13: 1153-1158.
18) Nishi N, Ebina A, Nishimura S, Tsutsuwa H, Hasegawa O, Tokura S. Highly phosphorylated derivitives of chitin, partially deacetylated chitin and chitosan as new functional polymers: preparation and characterization. Int J Biol Macromol 1986; 8: 311-317.
19) Sauro S, Osorio R, Watson TF, Toledano M. Influence of phosphoproteins’ biomimetic analogs on remineralization of mineral-depleted resin-dentin interfaces created with ion-releasing resin-based systems. Dent Mater 2015; 31: 759-777.
20) Zhang CL, Na H, Mu JX, Yu WZ, Fu TZ, Zhang XG, et al. Regen Med 2017; 11: 1853-1864.
21) Schwendicke F, Frencken J, Bjornay SH, Maltz M, Manton D. Bioactivity and biomineralization ability of calcium silicate-based pulp-capping materials after subcutaneous implantation. Int Endod J 2017; 50 Suppl 2: e40-e51.
22) Innes N, Frencken J, Bjornay SH, Maltz M, Manton D, Ricketts D, et al. Managing carious lesions: consensus recommendations on carious tissue removal. Adv Dent Res 2016; 28: 58-67.
23) Innes N, Frencken J, Bjornay SH, Maltz M, Manton D, Ricketts D, et al. Managing carious lesions: consensus recommendations on terminology. Adv Dent Res 2016; 28: 49-
23) Profeta AC. Preparation and properties of calcium-silicate filled resins for dental restoration. Part II: Micro-mechanical behaviour to primed mineral-depleted dentine. Acta Odontol Scand 2014; 72: 607-617.

24) Li F, Wang P, Weir MD, Fouad AF, Xu HH. Evaluation of antibacterial and remineralizing nanocomposite and adhesive in rat tooth cavity model. Acta Biomater 2014; 10: 2804-2813.

25) Profeta AC, Mannocci F, Foxton RM, Thompson I, Watson TF, Sauro S. Bioactive effects of a calcium/sodium phosphosilicate on the resin-dentine interface: a microtensile bond strength, scanning electron microscopy, and confocal microscopy study. Eur J Oral Sci 2012; 120: 353-362.

26) Moreau JL, Sun L, Chow LC, Xu HH. Mechanical and acid neutralizing properties and bacteria inhibition of amorphous calcium phosphate dental nanocomposite. J Biomed Mater Res B Appl Biomater 2011; 98: 80-88.

27) Tsuji T, Onuma K, Yamamoto A, Iijima M, Shiba K. Direct transformation from amorphous to crystalline calcium phosphate facilitated by motif-programmed artificial proteins. Proc Nat Aca Sci 2008; 105: 16866-16870.

28) Hamai R, Shirosaki Y, Miyazaki T. Apatite formation on a hydrogel containing sulfinic acid group under physiological conditions. J Biomed Mater Res B Appl Biomater 2017; 105: 1924-1929.

29) Boskey AL, Villarreal-Ramirez E. Intrinsically disordered proteins and biomineralization. Matrix Biol 2016; 52-54: 43-59.

30) Huang Z, Qi Y, Zhang K, Gu L, Guo J, Wang R, et al. Use of experimental-resin-based materials doped with carboxymethyl chitosan and calcium phosphate microfillers to induce biomimetic remineralization of caries-affected dentin. J Mech Behav Biomed Mater 2019; 89: 81-88.

31) Zhu L, Peng L, Zhang YQ. The processing of chitosan and its derivatives and their application for postoperative anti-adhesion. Mini Rev Med Chem 2015; 15: 330-337.

32) Kerch G. The potential of chitosan and its derivatives in prevention and treatment of age-related diseases. Mar Drugs 2015; 13: 2158-2182.

33) Zhang X, Li Y, Sun X, Kishen A, Deng X, Yang X, et al. Biomimetic remineralization of demineralized enamel with nano-complexes of phosphorylated chitosan and amorphous calcium phosphate. J Mater Sci Mater Med 2014; 25: 2619-2628.

34) Zheng Z, Wei Y, Wang G, Gong Y, Zhang X. Surface characterization and cytocompatibility of three chitosan/polyacrylamide composite membranes for guided bone regeneration. J Biomat Appl 2009; 24: 209-229.

35) Okulus Z, Buchwald T, Voelkel A. Calcium release from experimental dental materials. Mater Sci Eng C Mater Biol Appl 2016; 68: 213-220.

36) Wang X, Cai Q, Zhang X, Wei Y, Xu M, Yang X, et al. Improved performance of Bis-GMA/TEGDMA dental composites by net-like structures formed from SiO2 nanofiber fillers. Mater Sci Eng C 2016; 59: 464-470.

37) Marovic D, Tarle Z, Hiller KA, Muller R, Rosentritt M, Skrtic D, et al. Reinforcement of experimental composite materials based on amorphous calcium phosphate with inert fillers. Dent Mater 2014; 30: 1052-1060.

38) Marovic D, Tarle Z, Hiller KA, Muller R, Ristic M, Rosentritt M, et al. Effect of silanized nanosilica addition on remineralizing and mechanical properties of experimental composite materials with amorphous calcium phosphate. Clin Oral Investig 2014; 18: 783-792.

39) O'Donnell JN, Antonucci JM, Skrtic D. Illuminating the role of agglomerates on critical physicochemical properties of amorphous calcium phosphate composites. J Compos Mater 2008; 42: 2231-2246.