

# Reinforcement of calcium phosphate cement using alkaline-treated silk fibroin

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**Background:** Bone cement plays an important role in the treatment of osteoporotic vertebral compression fractures. Calcium phosphate cement (CPC) is a potential alternative to poly(methyl methacrylate), currently the gold standard of bone cements. However, the poor mechanical properties of CPCs limit their clinical applications. The objective of this study was to develop reinforced CPCs for minimally invasive orthopedic surgeries by compositing silk fibroin (SF) with  $\alpha$ -tricalcium phosphate.

**Methods:** SF solution was treated with calcium hydroxide and characterized by Zeta potential analyzer and Fourier transform infrared spectroscopy. The alkaline-treated SF (tSF) was composited with  $\alpha$ -tricalcium phosphate to obtain tSF/CPC composite, which was characterized using mechanical tests, scanning electron microscopy, handling property and biocompatibility tests, and sheep vertebral augmentation tests.

**Results:** Upon treatment with calcium hydroxide, larger SF particles and more abundant negative charge appeared in tSF solution. The tSF/CPCs exhibited a compact structure, which consisted of numerous SF-CPC clusters and needle-like hydroxyapatite (HAp) crystals. In addition, high transition rate of HAp in tSF/CPCs was achieved. As a result, the mechanical property of tSF/CPC composite cements was enhanced remarkably, with the compressive strength reaching as high as  $56.3 \pm 1.1$  MPa. Moreover, the tSF/CPC cements showed good injectability, anti-washout property, and decent biocompatibility. The tSF/CPCs could be used to augment defected sheep vertebrae to restore their mechanical strength.

**Conclusion:** tSF/CPC may be a promising composite bone cement for minimally invasive orthopedic surgeries.

**Keywords:** bone cement, silk fibroin, calcium phosphate, calcium hydroxide, reinforcement

## Introduction

Poly(methyl methacrylate) (PMMA) bone cements are commonly used for prosthesis fixation or vertebral augmentation in orthopedics surgeries, such as joint replacement, vertebroplasty (VP), and kyphoplasty (KP). However, PMMA bone cements have intrinsic limitations, including monomer toxicity, nonbiocompatibility, and excessive heat generation upon solidification.<sup>1</sup> Alternatively, calcium phosphate cements (CPCs), first invented in the 1980s, have received increasing attention as a type of biodegradable and biocompatible bone grafting and filling material.<sup>2</sup> Recently, minimally invasive surgeries, such as VP and KP, in which high-strength bone cements are needed, have been developed to relieve the morbidity of vertebral compression fractures.<sup>1,3</sup> However, due to the poor mechanical properties of CPCs, their applications in orthopedic surgeries at such high load-bearing regions are largely limited.<sup>4</sup>

To date, many approaches, including porosity reduction, fiber reinforcement, and dual setting, have been sought in order to improve the mechanical properties of CPCs.<sup>5</sup> The porosity reduction of CPCs is based on the optimization of an intrinsic cement

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property by changing the liquid/powder ratio, which may adversely affect the handling property of cement.<sup>6-9</sup> Both fiber reinforcement and dual setting require mixing of CPC with other components.<sup>10-12</sup> Some synthetic polymers, such as carbon nanotubes and poly(lactide-co-glycolide) (PLGA) and natural polymers, such as chitosan and cellulose have been shown to enhance the mechanical properties of CPCs. By controlling the growth of crystals of hydroxyapatite (HAp) in CPCs, such polymers can affect the conversion rate and HAp crystal types and as a result, strengthen CPCs.<sup>13-17</sup> However, the weak interfacial adhesion between the organic and inorganic phases still remains a major concern, which limits further enhancement of CPCs.<sup>18</sup> Hence, a number of studies have attempted to modify these fillers to ameliorate interfacial bonding between the 2 phases.<sup>19,20</sup>

Silk fibroin (SF), a popular material with good biocompatibility, is a promising biomaterial for biomedical applications.<sup>20</sup> Previously, SF has been used in CPCs by various groups, including us, to improve their mechanical properties.<sup>21-25</sup> The -COOH and -OH groups on SF can react with Ca<sup>2+</sup> from tricalcium phosphate (TCP) and form nuclear sites to grow HAp.<sup>15,19</sup> pH also affects HAp formation by regulating TCP hydration and SF structure.<sup>20,21</sup> Attempts have also been made to use SF-HAp complex, acidified SF, and *N*-acetyl cysteine to enhance the interfacial bonding between SF and CPCs.<sup>24-26</sup> However, their final mechanical performance is still insufficient for clinical needs.

In this study, we proposed to use SF treated with calcium hydroxide, referred to here as tSF, as curing liquid for  $\alpha$ -TCP to prepare tSF/CPC composite bone cements. We hypothesized that after treatment with calcium hydroxide, the increased number of negative charges on tSF chains could provide more nuclear sites and facilitate HAp formation after mixing with  $\alpha$ -TCP. The handling property, setting time, mechanical properties, and biocompatibility of tSF/CPC composite bone cements were determined.

## Materials and methods

### Preparation of tSF solution

*Bombyx mori* (silkworm) SF solution was prepared according to a published protocol.<sup>27</sup> The SF solution was condensed to 20 wt% using polyethylene glycol (>99%, Sinopharm Chemical Reagent, Shanghai, China) solution. Then the condensed SF solution was treated with saturated calcium hydroxide (99%, Sinopharm Chemical Reagent) solution. In brief, a certain amount of saturated clarification limewater was added to 5 mL condensed SF solution to reach a final pH of 8.0, 8.5, 9.0, and 9.5. After being placed in the oven for 4 hours, the treated SF solutions were named as 8tSF, 8.5tSF, 9tSF,

and 9.5tSF, respectively. An untreated SF solution (pH=7.5) named SF was used as a control group. For CPCs without SF, PBS solution or saturated limewater-modified PBS with different pH (8.0, 8.5, and 9.0) was also prepared.

### Preparation of CPCs

The  $\alpha$ -TCP (99%, Alfa Aesar, Ward Hill, MA, USA) powder was mixed with curing liquid for 2 minutes until a paste was formed. CPC was prepared using PBS as curing liquid. Alkaline (ALK)/CPCs prepared using PBS at pH values of 8.0, 8.5, and 9.0 as curing liquids, were named 8ALK/CPC, 8.5ALK/CPC, and 9ALK/CPC, respectively. SF/CPC was obtained with SF solution, and 8tSF/CPC, 8.5tSF/CPC, and 9tSF/CPC were separately prepared using 8tSF, 8.5tSF, and 9tSF solutions. The compositions of various CPCs are shown in Table 1.

### Characterizations

The particle size and zeta potential of SF and tSF solutions were measured by Zetasizer Nano ZS90. SF and 8.5tSF solutions were freeze-dried by Freeze Drier (Christ Alpha1-2) for Fourier transform infrared spectroscopy (FTIR) (Nicolet 6700, Thermo Scientific, Chelmsford, MA, USA) test. CPC, 8.5ALK/CPC, SF/CPC, and 8.5tSF/CPC cements were ground into powders to be characterized by X-ray diffraction (XRD) (X'Pert-Pro MPD, PANalytical, Almelo, the Netherlands).

### Mechanical tests

To evaluate the compression strength of CPCs, the mechanical properties of CPCs, ALK/CPCs, SF/CPCs, and tSF/CPCs were tested using Universal mechanical testing machine (E10000, Instron, Norwood, MA, USA) with 5 repetitions for each group. The cements were hardened and filled in a mold for 1 hour and then ejected into pillar mold with diameter of 6 mm and height of 12 mm. After that, the

**Table 1** The compositions of different CPCs

Sample	Composition	
	Curing liquid	Powder
CPC	PBS (pH =7.4)	$\alpha$ -TCP
8ALK/CPC	Treated PBS (pH =8.0)	
8.5ALK/CPC	Treated PBS (pH =8.5)	
9ALK/CPC	Treated PBS (pH =9.0)	
SF/CPC	Untreated SF solution at pH of 7.4	
8tSF/CPC	Treated SF solution at pH of 8.0	
8.5tSF/CPC	Treated SF solution at pH of 8.5	
9tSF/CPC	Treated SF solution at pH of 9.0	

**Abbreviations:** ALK, alkaline; CPC, calcium phosphate cement; PBS, phosphate buffer saline; SF, silk fibroin; tSF, SF treated with calcium hydroxide.

cements were placed at 37°C, 100% relative humidity for 3 days. After the mechanical tests, the fractured sections of CPCs were characterized by scanning electron microscopy (SEM) (Quanta 250FEG, Thermo Fisher Scientific Electron Microscopy, Hillsboro, OR) and energy dispersive spectrometer (GENESIS, EDAX, Mahwah, NJ).

## Handling properties of CPCs

The handling properties of CPC, 8.5ALK/CPC, SF/CPC, and 8.5tSF/CPC were tested. Both the initial setting time (I-ST) and final setting time (F-ST) were determined at 37°C using a Gilmore apparatus, as described previously.<sup>28</sup> To determine the washout resistance of the cements, they were molded into pallet and then immersed in PBS solution immediately. After that, the appearance of CPCs immersed in PBS at 37°C for 20 minutes was recorded by photographing. For injectability test, the CPC was prepared and inserted into a 2 mL syringe after mixing for 3 minutes, then the paste was ejected from a 11–13G needle and recorded by photographing.

## Cell culture

Extracts from SF/CPC and 8.5tSF/CPC cements were prepared following the international standard procedure (ISO 10993) for cytotoxicity tests. Briefly, the cements were soaked in alpha-minimum essential medium ( $\alpha$ -MEM) solutions (Hyclone, Novato, CA, USA) (3 cm<sup>2</sup>/mL) at 37°C for 24 hours, then the extracts were collected and sterilized before use. MC3T3-E1 cells (Cell Bank, Shanghai Institutes for Biological Sciences, CAS, Shanghai, China) were seeded at a density of 10,000 cells/well in a 96-well plate. After 4 hours of incubation, the medium was changed into the obtained extracts containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin–streptomycin (Gibco, USA).  $\alpha$ -MEM medium with no cements immersed was used as control group. The medium was refreshed every 3 days. Cell Counting Kit-8 (CCK-8) tests were performed and the OD at 450 nm was measured at 1, 3, 5, and 7 days of culture. For cell attachment test, MC3T3-E1 cells were seeded on 8.5tSF/CPC plate at a density of 5,000 cells/cm<sup>2</sup>. After overnight culturing, the cements were dehydrated in graded ethanol and dried in a critical point dryer. The cell morphology on the specimens was imaged by SEM.

## Sheep vertebral augmentation tests with CPCs

The sheep vertebral augmentation test was approved by the Institutional Animal Care and Use Committee of Soochow University. All the experiments were performed following the National Institutes of Health Guide for the Care and Use

of Laboratory Animals. In brief, spine specimens (L2–L6) were obtained from fresh female adult small-tail Han sheep with weight range from 40 to 50 kg provided by the Central Animal Facility of Soochow University. After removing other tissue, a hole ( $\Phi=6$  mm) was drilled across the middle of the specimen. To make the vertebra upright (slant angle  $<1^\circ$ ), the top and bottom of vertebrae was covered with PMMA. The drilled vertebrae were considered the defect group, which simulated the osteoporotic vertebrae. In the experimental groups, the drilled vertebrae (in 6 duplicates) were injected with cements to mimic cement-enhanced osteoporotic vertebrae. Six intact specimens and 6 drilled specimens served as blank and control group for mechanical tests. These samples were soaked in PBS for 1 day at 37°C before mechanical test (E10000, Instron). In order to eliminate the effects of rheological properties, such as creep and relaxation of the vertebral bodies, it was applied the preload (200N) for 30 seconds. The loading rate was 5 mm/min during the biomechanical test.

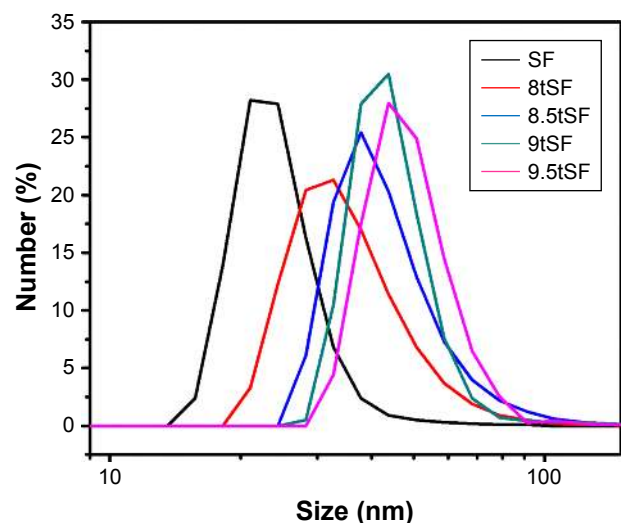
## Statistical analysis

All statistical analyses were performed using SPSS software. Kruskal–Wallis one-way ANOVA tests followed by Tukey post hoc tests were used. Unpaired Student's *t*-tests were also used where appropriate. Difference is considered statistically significant when *P* is  $<0.05$ .

## Results

### ALK treatment of SF

SF was treated with saturated ALK, ie, calcium hydroxide, to prepare tSF solutions with various pH. Figure 1 shows



**Figure 1** DLS analysis of 8tSF, 8.5tSF, 9tSF, and 9.5tSF.

**Abbreviations:** SF, silk fibroin; DLS, dynamic light scattering; tSF, silk fibroin treated with calcium hydroxide.

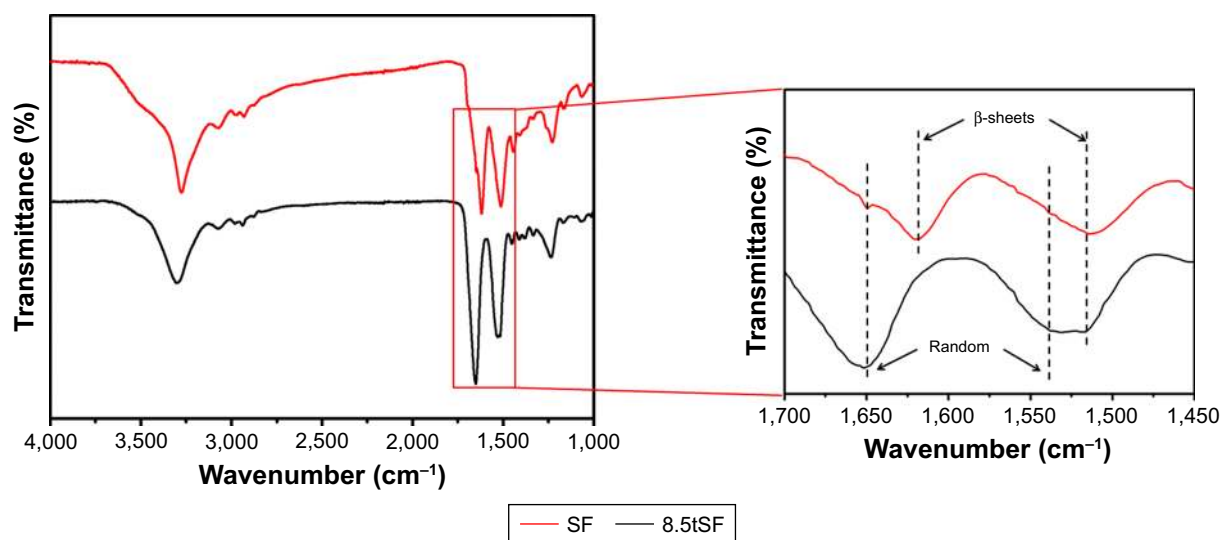
**Table 2** The zeta potential of different solutions

Sample	tSF	8tSF	8.5tSF	9tSF	9.5tSF
Zeta potential (mV)	-1.23	-7.58	-15.60	-10.37	-5.13

**Abbreviation:** tSF, silk fibroin treated with calcium hydroxide.

that the size of tSF particles increased with pH, being 21, 30, 40, and 45 nm for 8tSF, 8.5tSF, 9tSF, and 9.5tSF, respectively. The zeta potential of SF, 8tSF, 8.5tSF, 9tSF, and 9.5tSF is shown in Table 2. The zeta potential is -1.23, -7.58, -15.60, -10.37, and -5.13 mV for SF, 8tSF, 8.5tSF, 9tSF, and 9.5tSF, respectively. After treatment with calcium hydroxide, more -COO- and -O- in SF solution was exposed and more negative ions formed. The repulsive force between these negative groups could stretch the SF chain, resulting in a larger particle size. The zeta potential of these particles becomes more negative due to the formation of negative groups with the addition of Ca<sup>2+</sup>, but negative groups can then bind to Ca<sup>2+</sup> ions, contributing to the later decrease in zeta potential.<sup>29</sup>

The FTIR spectra show apparent difference between SF and 8.5tSF (Figure 2). The major difference exists at the 2 strong absorption bands near 1,500 cm<sup>-1</sup> and 1,650 cm<sup>-1</sup>. The bands at 1,515 cm<sup>-1</sup> indicate the amide C=O bond and the band at 1,625 cm<sup>-1</sup> indicates the amide C-N bond in the SF group. These 2 bands indicate the formation of  $\beta$ -sheets in the secondary structure of SF. However, the amide C=O bond and amide C-N bond shift to 1,537 cm<sup>-1</sup> and 1,648 cm<sup>-1</sup>, respectively, for tSF, implying a random form of SF chains.<sup>30</sup> These results indicate that the secondary structure of SF changes after treatment with the calcium hydroxide solution. Compared with SF, the tSF appears to have more random chains rather than  $\beta$ -sheets.

**Figure 2** FTIR spectra of SF and 8.5tSF.

**Abbreviations:** FITR, Fourier transform infrared spectroscopy; tSF, silk fibroin treated with calcium hydroxide; SF, silk fibroin.

## XRD analysis of CPCs

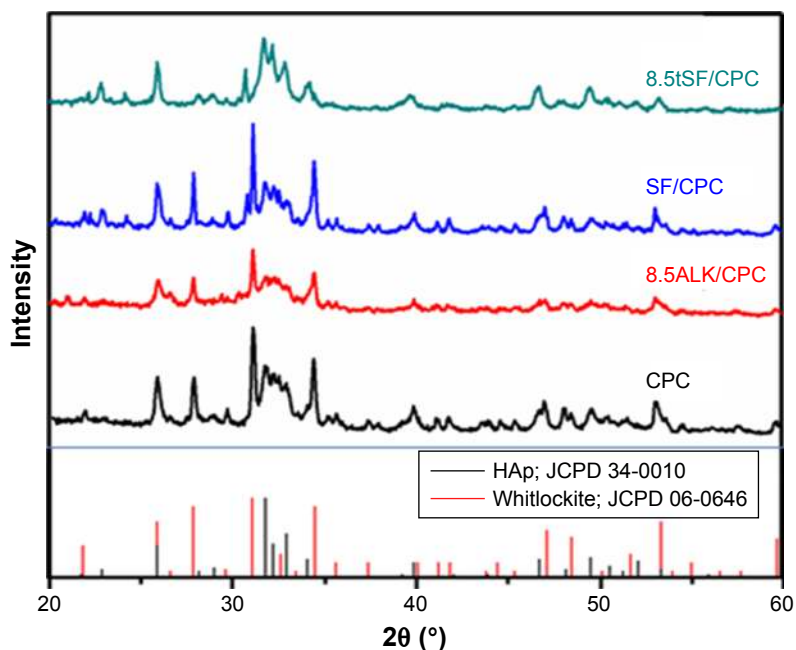
The XRD curves of CPC, 8.5ALK/CPC, SF/CPC, and 8.5tSF/CPC cements after 7 days of hardening are shown in Figure 3. The 8.5tSF/CPC exhibits obvious HAp peaks at (002), (211, 300, 202, 310, and 222), and (213).<sup>23</sup> However, peaks of whitlockite (Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>) at 2 $\theta$ =27.769°, 31.026°, 35.597°, and 41.186° matched with other CPCs, and whitlockite is generated through the conversion of calcium phosphate from amorphous to crystalline state.<sup>24,25</sup> This indicates that a fraction of  $\alpha$ -TCP was not transformed into HAp. Moreover, 8.5tSF/CPC had higher extent of HAp transition than tSF/CPC.

## Mechanical properties of CPCs

The compressive strength of CPCs was remarkably improved with tSF solution as curing liquid (Figure 4). Specifically, the compressive strength of 8.5tSF/CPCs reached 56.3±1.1 MPa. However, all groups using PBS as curing liquid treated with calcium hydroxide solution showed no significant difference from CPCs using PBS. In addition, the compressive strengths are all lower than the SF/CPC groups. The pH of curing liquid is not the determining factor of the enhancement of SF/CPC. The results indicate that the replacement of PBS with SF solution increases the mechanical property of CPCs. Moreover, the compressive strength could be further improved by treating SF with calcium hydroxide.

The micromorphology of CPCs is shown in SEM images (Figure 5A). CPC exhibits many plate-like crystals with some large grains. Many needle-like crystals with large grains were also found in the ALK/CPC group. However, the crystals

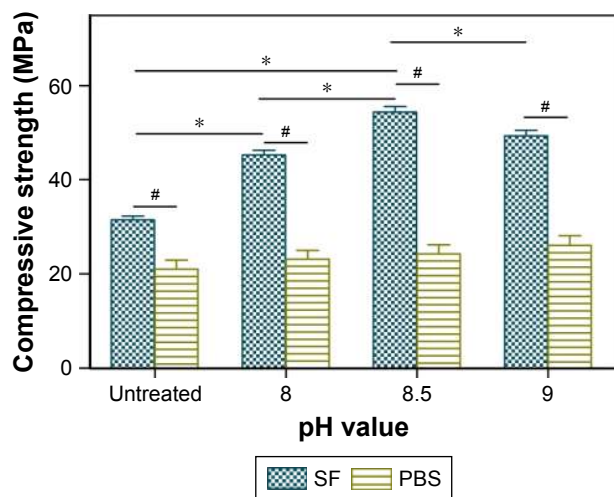




**Figure 3** XRD patterns of CPC, 8.5ALK/CPC, SF/CPC, and 8.5tSF/CPC.

**Abbreviations:** ALK, alkaline; CPC, calcium phosphate cement; HAp, hydroxyapatite; tSF, silk fibroin treated with calcium hydroxide; XRD, X-ray diffraction; SF, silk fibroin.

are surrounded by a mass of clusters for SF/CPC when SF is added. Plate-like crystals are found in SF/CPC, but more needle-like in tSF/CPC. The clusters in tSF/CPC are closer than in SF/CPC. Figure 5B demonstrates that higher carbon content is found in the clusters and the amount is much greater than in the crystals. According to the results, the clusters are more similar to a mixture of SF and CPCs.



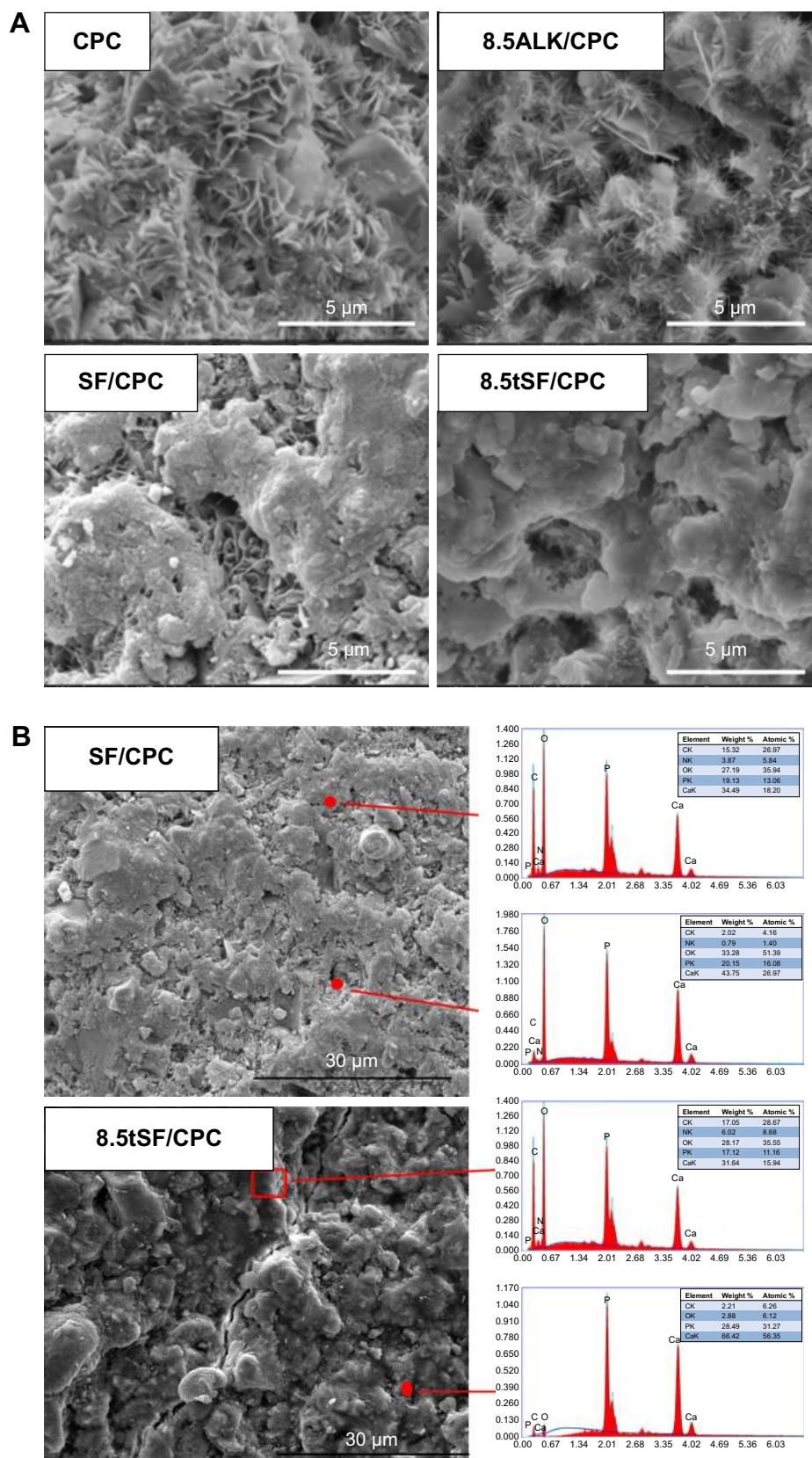
**Figure 4** The compressive strength of CPCs using PBS and SF with different pH values as curing liquids.

**Notes:** \*, significant difference between the pH value using SF as curing agent. ( $P < 0.05$ ). #, significant difference between the SF and PBS at the same pH value ( $P < 0.05$ ).

**Abbreviations:** CPC, calcium phosphate cement; PBS, phosphate buffer saline; SF, silk fibroin.

## Handling properties of CPCs

Based on the mechanical test results, tSF/CPC possesses the highest compressive strength and ALK/CPC, which has the same pH as tSF/CPC, was selected to test the handling properties of cements. The setting times of different bone cements are summarized in Table 3. The setting time of 8.5tSF/CPC (I-ST,  $24.53 \pm 0.88$  minutes; F-ST,  $41.03 \pm 1.06$  minutes) is much shorter than that of CPC (I-ST,  $41.67 \pm 1.32$  minutes; F-ST,  $62.04 \pm 1.66$  minutes) and 8.5ALK/CPC (I-ST,  $34.23 \pm 1.55$  minutes; F-ST,  $54.23 \pm 1.78$  minutes), but longer than SF/CPC (I-ST,  $17.69 \pm 1.72$  minutes; F-ST,  $32.00 \pm 0.95$  minutes) in both the initial and final settings. Clearly, the setting time was prolonged in ALK condition. This is because the hydration reaction slowed down when more  $\text{OH}^-$  was added, which retarded the solidification process. However, with the addition of SF, the setting time could be shortened, likely because that SF might act as an adhesive network to solidify CPC and prevent CPC particles from moving around. In the anti-washout tests, the CPC paste collapsed into powder and could not maintain the pellet shape; ALK/CPC paste also collapsed but still maintained the pellet shape, SF/CPC paste showed peeling edges and strongly maintained the pallet shape, but no collapse was observed in tSF/CPC (Figure 6A). The 8.5tSF/CPC still possessed decent injectability. It could be easily ejected from a syringe and well maintained the original shape in PBS (Figure 6B). In addition, it could also be injected from



**Figure 5 (A)** SEM images of CPC, 8.5ALK/CPC, SF/CPC, and 8.5tSF/CPC. **(B)** SEM images and EDS spectra of SF/CPC and 8.5tSF/CPC. Scale bars: 5  $\mu\text{m}$  **(A)** and 30  $\mu\text{m}$  **(B)**. **Abbreviations:** ALK, alkaline; CPC, calcium phosphate cement; EDS, energy dispersive spectrometer; SEM, scanning electron microscopy; SF, silk fibroin; tSF, silk fibroin treated with calcium hydroxide; XRD, X-ray diffraction.

**Table 3** The initial and final setting times of CPCs

Sample	CPC	8.5ALK/CPC	SF/CPC	8.5tSF/CPC
Initial setting time (min)	41.67±1.32	34.23±1.55	17.69±1.72	24.53±0.88
Final setting time (min)	62.04±1.66	54.23±1.78	32.00±0.95	41.03±1.06

**Abbreviations:** ALK, alkaline; CPC, calcium phosphate cement; SF, silk fibroin; tSF, silk fibroin treated with calcium hydroxide.

11 to 13G needle. All these results indicate that 8.5tSF/CPC may be an ideal candidate filler material for VP/KP surgeries.<sup>25</sup>

## Biocompatibility of CPCs

There was no difference in the CCK-8 results observed between the control group, SF/CPC and 8.5tSF/CPC, and as shown in Figure 7A, the OD value of all 3 groups increased in a stable manner on day 1 to day 5. This result showed that both SF/CPC and 8.5tSF/CPC have low cytotoxicity. The MC3T3-E1 cell can attach and grow on the surface of the 8.5tSF/CPC cements from the SEM images in Figure 7B. The attached cells showed good morphology and stretch on the surface of 8.5tSF/CPC.

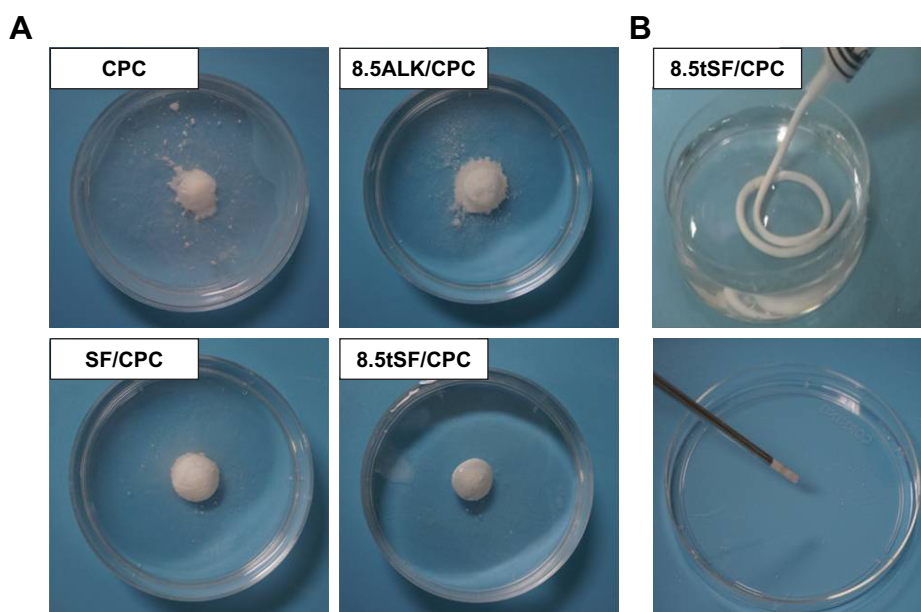
## Sheep vertebra augmentation tests with CPCs

The mechanical properties of CPC-filled sheep vertebrae are shown in Figure 8. Compared with those of drilled vertebrae,

both the compressive strength and stiffness of CPC-filled vertebrae were increased. The replacement of CPC with SF/CPC also contributes to higher mechanical properties. The 8.5tSF/CPC group showed the highest compressive strength at 20.02±1.73 MPa and stiffness at 2.75±0.47 N/mm, which were comparable with those of native vertebrae.

## Discussion

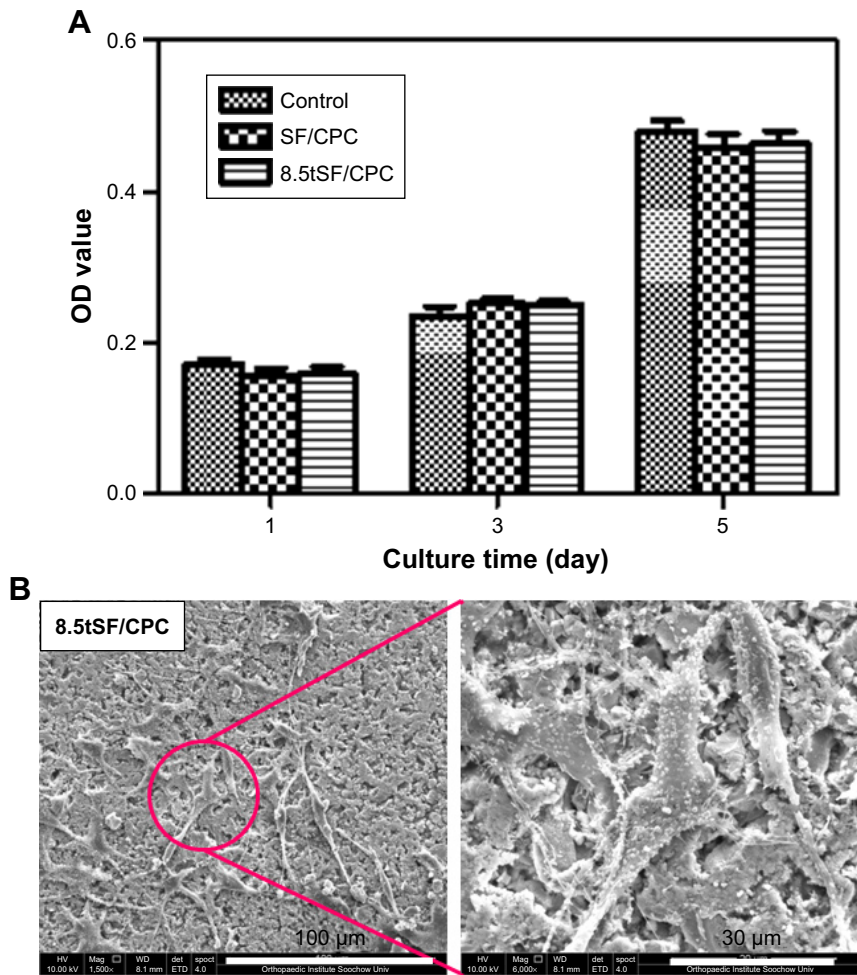
CPCs have been studied for decades since it was invented. Due to the limited mechanical properties, they are usually used for repairing defects of nonload-bearing bones, such as radius,<sup>44</sup> or for load-bearing bone repair in combination with fixation systems.<sup>44</sup> They cannot be used alone in minimally invasive spine surgeries, such as VP and KP.<sup>45</sup> Recently, increasing studies have composited CPCs with other materials to enhance their mechanical and handling properties. Among these materials, polymers, both natural and synthetic, are attracting increasing interest.<sup>31–33,46</sup> The structure and functional groups of polymers dramatically impact the mechanical properties of CPC composites. Taking advantage of the massive functional groups, such as –COOH and –OH in its molecular chain, SF has been composited with CPCs in many studies. The compressive strength of CPC/SF composites has been reported to be as high as 50 MPa.<sup>24,25</sup> However, CPC-based composite cements remain to be improved so that they are comparable with PMMA cements, whose compressive strength exceeds 70 MPa.



**Figure 6** The anti-washout property (A) of different CPCs and injectable performance (B) of 8.5tSF/CPC.

**Abbreviations:** ALK, alkaline; CPC, calcium phosphate cement; SF, silk fibroin; tSF, silk fibroin treated with calcium hydroxide.

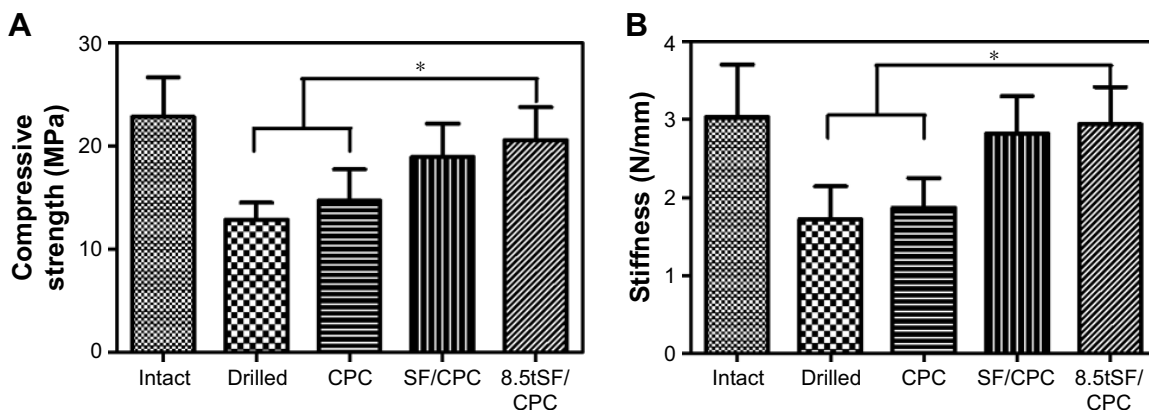




**Figure 7 (A)** Proliferation of MC3T3-E1 cell on different bone cements. **(B)** SEM images of MC3T3-E1 cell adhesion on 8.5tSF/CPC bone cement. **Abbreviations:** CPC, calcium phosphate cement; SF, silk fibroin; OD, optical density; SEM, scanning electron microscopy; tSF, silk fibroin treated with calcium hydroxide.

It has been reported that the interfacial binding between organic and inorganic phases in composited CPCs could be enhanced by introducing functional groups to the additives. Since the structure and surface charge of SF is affected by the pH of solution,<sup>34,35</sup> in this study, we treated SF with calcium hydroxide to obtain tSF with different structures and surface

charges so that it could be used as a reinforcing additive in CPCs. We found that particle size of SF increased with the pH of solution (Figure 1). In contrast, the zeta potential of SF solutions showed different behaviors. The charges of the SF chains became more negative when pH increased from 7 to 8.5 and then decreased. These results imply that with the



**Figure 8** The compressive strength **(A)** and stiffness **(B)** of intact, drilled, CPC, SF/CPC and 8.5tSF/CPC filled vertebrae. \* $P < 0.05$ . **Abbreviations:** CPC, calcium phosphate cement; SF, silk fibroin; tSF, silk fibroin treated with calcium hydroxide.



addition of calcium hydroxide, the SF chains first began to carry more negative charge and grew larger. However, as the content of calcium hydroxide increased, the superfluous  $\text{Ca}^+$  ions blocked negative charges.<sup>34</sup> In addition, more random SF chains were found after they were treated with calcium hydroxide, which may imply a more stretched morphology of SF chains in tSF solution. These results agree with previous studies about the conformation of SF under ALK conditions.<sup>36</sup> Hence, tSF solutions with more random SF chains and negative charges were used in our next study.

The low mechanical strength of CPCs is a major problem to be solved. Previously, reinforcing agents, such as carbon nanotubes/HAp composites were added to CPCs for mechanical reinforcement.<sup>14</sup> In a report, acid-modified SF was used to reinforce magnesium phosphate cements (MPCs).<sup>24</sup> With this method, the mechanical property of MPCs improved, since the interfacial combination between SF and MPCs was enhanced. In our study, ALK-treated SF solution was used as curing liquid to prepare CPCs. When tSF is composited with  $\alpha$ -TCP, significant enhancement is obtained, with the compressive strength reaching as high as  $56.3 \pm 1.1$  MPa. In addition, the compressive strength of CPCs increased with pH, proving that tSF could indeed improve the mechanical property of CPCs. However, for the control group with PBS solution as curing liquid, regardless of pH, the compressive strength was not significantly different and was indeed significantly lower than that of SF groups. Based on the zeta potential and dynamic light scattering results, the more negative charges and more stretched structure of SF might play critical roles in this reinforcing effect. These negative charges might originate from the hydrolyzed  $-\text{COOH}$  groups on SF chains, which could fortify the interfacial interactions between SF and  $\alpha$ -TCP, and thereby improve the mechanical property of the latter.

Further, XRD analysis results show that a stronger HAp peak exists in the spectrum of tSF/CPC instead of other 3 groups, indicating high extent of HAp transition in this group. As reported in a previous study, high HAp transition implies more complete CPC hydration and results in better formation of HAp crystals and more stable structure of CPCs.<sup>41</sup> This appears also true in our study, as can be seen from the morphology of tSF/CPC, which shows a compact structure with many clusters and needle-like crystals (Figure 5). The clusters were also found in SF/CPC groups. In addition to Ca and P elements, a massive C element exists in the clusters, which indicate that this cluster could be a mixture of SF and CPCs. In the ALK condition, the flower-like crystal changes to a needle-like form. This phenomenon echoes the findings in the study of Tanahashi et al, in which

needle-like HAp crystals were observed in  $\alpha$ -TCP cured in an ALK environment (pH=10).<sup>42</sup> In our study, the needle-like crystal section occupied less space than the flower-like crystal section in tSF/CPC, resulting in fewer microgaps and more compact morphology of CPC than those in other groups, which strengthened the CPCs.<sup>43</sup> Using sheep vertebra as a model for vertebra augmentation tests, we found that tSF/CPC-filled vertebrae had highest mechanical properties compared with other CPC-filled ones. Moreover, the vertebrae filled with tSF/CPC showed no statistical difference with native vertebrae, suggesting that tSF/CPC may be a promising load-bearing filler material for vertebral augmentation. The similar stiffness between tSF/CPC-filled vertebrae and intact vertebrae demonstrated that the filling material in the vertebra could play the same role in mechanical function. It should be noted that while mechanical recovery has been reached by tSF/CPC injection, there is still potential risk from the stiffness mismatch between tSF/CPC and local tissue of the vertebra. According to a previous study, the relative higher stiffness of filling material within vertebrae could affect the microphysiological activity of them and might result in new vertebra fracture or deterioration.<sup>28</sup> Therefore, other effects of tSF/CPC-augmented vertebrae should also be checked in our further study.

The handling property is another concern for clinical applications of CPCs. The setting times and anti-washout property of currently used CPCs are inferior compared with conventional PMMA bone cements.<sup>37</sup> Ideally, the I-ST should be no  $<5$  minutes and the F-ST should be no  $>30$  minutes.<sup>25</sup> In our work, the hardening time will be shorter when CPC were mixed with SF. Using ALK-treated SF as a curing liquid could further reduce the hardening time. It is known that hydration of  $\alpha$ -TCP is more favorable in an ALK condition, where abundant HAp is produced and adhere to HAp nuclear sites to continue growing.<sup>38</sup> This process may accelerate the hardening of CPCs. As has been previously reported, polymers, such as chitosan and silk could shorten the setting time of CPCs when composited with them.<sup>26</sup> However, in our study tSF indeed slowed down the hardening process compared with untreated SF. This might be attributed to the special rheological property of tSF solution, which had lower viscosity compared with untreated SF solution.<sup>35</sup> The water in SF solution with lower viscosity could fast diffuse in mixed CPCs paste and accelerate the hydration process during the initial setting and hardening periods. As cement leakage can result in severe complications, such as fatal pulmonary embolism, the anti-washout property is another important factor and greatly affects the clinical applications of CPCs.<sup>39</sup> We found that the anti-washout property of CPCs

was markedly improved by tSF. This is in agreement with an earlier study, in which addition of chitosan was able to prevent CPCs from collapse in water.<sup>40</sup>

## Conclusion

In this study, composite CPCs have been prepared using  $\alpha$ -TCP powder and tSF, that is, calcium hydroxide-treated SF solution. The tSF/CPC cement showed significantly higher compressive strength than CPC. Particularly, 8.5tSF/CPC, that is composite of  $\alpha$ -TCP and tSF at pH of 8.5, showed the highest compressive strength ( $56.3 \pm 1.1$  MPa). The enhancement of CPCs may be attributed to the following 2 facts. First, tSF might enhance the interfacial bonding between SF, the organic phase, and CPC, the inorganic phase of composite cement. Second, the conversion rate of HAp from  $\alpha$ -TCP was also improved by tSF. The tSF/CPC showed considerable biocompatibility in vitro. The drilled sheep vertebrae filled with tSF/CPC possessed comparable mechanical properties as native vertebrae. Therefore, calcium hydroxide-treated SF solution as curing liquid may be an effective approach to enhance the mechanical properties of CPCs. However, the stiffness of tSF/CPC composite cement is still 1 limitation for its application in VP/KP. The curing time should also be optimized to meet the clinical requirement. Moreover, the in vivo effects of tSF/CPC are to be evaluated. Our following study will address these issues.

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## Author contributions

MH, ZH, PZ, and FL performed all the experiments. XZ and HY supervised the ex vivo experiments. MH, FH, CS, and BL analyzed the data and wrote the manuscript. All authors reviewed and approved the final version of manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare no conflicts of interest with this work.

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