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Addition of sodium hyaluronate and the effect on performance of the injectable calcium phosphate cement

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Abstract An injectable calcium phosphate cement (CPC) with porous structure and excellent anti-washout ability was developed in the study. Citric acid and sodium bicarbonate were added into the CPC powder consisting of tetracalcium phosphate (TTCP) and dicalcium phosphate dihydrate (DCPD) to form macro-pores, then different concentrations of sodium hyaluronate (NaHA) solution, as liquid phase, was added into the cement to investigate its effect on CPC's performance. The prepared CPCs were tested on workability (injectable time and setting time), mechanical strength, as well as anti-washout ability. The experimental results showed that addition of NaHA not only enhanced the anti-washout ability of the CPC dramatically but also improve its other properties. When NaHA concentration was 0.6 wt%, the injectable time elongated to 15.7 ± 0.6 min, the initial and final setting times were respectively shorten to 18.3 ± 1.2 and 58.7 ± 2.1 min, and the compressive strength were increased to 18.78 ± 1.83 MPa. On the other hand, Addition of NaHA showed little effect on porous structure of the CPC and enhanced its bioactivity obviously, which was confirmed by the apatite formation on its surface after immersion in simulated body fluid (SBF). In conclusion, as an in situ shaped injectable biomaterials, the CPC with appropriate addition of NaHA would notably improve its performance and might be used in minimal invasive surgery for bone repair or reconstruction.

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1 Introduction

Since CPC was reported by Brown and Chow in 1986 [1], it has been widely regarded and extensively studied. With nature of self-setting in low temperature and in situ, intimately adaption to neighboring bone even for irregularly shaped cavities and excellent surface bioactivity, injectable CPC has been developed as a promising bone repair or substitute material and could be used in minimally invasive surgery [2–5].

As thus, several formulations of CPCs have been developed for bone cement applications, and the final products are mainly hydroxyapatite (HAP), DCPD or amorphous calcium phosphate (ACP) [6–9]. It is known that HA have excellent biocompatibility and osteoconductivity due to its similarity to the mineral phase of bone, so the CPC has been increasingly regarded and used for treatment of vertebral body fracture, anterior cruciate ligament reconstruction, and some reconstructions of non-stress-bearing bone [10–12]. When used in clinic, the injectable CPC must have the following characteristics: better injectability while keeping the physico-chemical properties suitable for surgical use, proper setting time convenient for surgery, excellent anti-washout ability preventing the cement from crumbling in biological fluids, and good mechanical strength [13–15]. So far, some additives, such as critic acid, chitosan and sodium alginate, have been used to optimize the properties of CPC [7, 16, 17]. It has been reported that addition of a certain amount of citric acid could improve the injectability and compressive strength of CPC, but its anti-washout ability was still poor [7, 18, 19]. On the other hand, some documents have confirmed that the anti-washout ability of CPC could be effectively improved by the addition of chitosan and sodium alginate. However, these two

biopolymers would inhibit the setting process and the formation of HAP, leading to decrease of the mechanical strength [13, 16, 17, 20, 21]. Furthermore, the addition of them would influence the cements' rheology and reduce the injectability.

NaHA, a polysaccharide having a disaccharide repeating unit (D-glucuronic acid and N-acetyl-D-glucosamine), is a sort of water soluble polymer and known to be a bioactive biomaterial. Besides, NaHA has good viscoelastic and hydrating properties when it is in the dissolved state. In extracellular matrix, hyaluronate participates in a hydrated network and acts as an organizing core in the intercellular matrix, connecting complex intercellular aggregates [22–24]. If the special network is introduced into CPC, the viscosity and other properties of the cement might be enhanced. In this study, NaHA, as the liquid phase, was introduced into the CPC system and its effect on the workability, mechanical strength, anti-washout ability and bioactivity of the cements was investigated systematically.

2 Materials and methods

2.1 Material preparation

All chemicals of analytical grades were commercially available from domestic companies except those mentioned specially. The CPC powder was prepared by mixing TTCP and DCPD at a molar ratio of 1:1. TTCP powder was prepared by us according to previous report [25]. 13.3% of HAP powders (supplied by Engineering Research Center for Biomaterial, Sichuan University, China), 5% of sodium bicarbonate ($NaHCO_3$) powders and 4% of citric acid powders at mass fraction were added into the CPC powders and uniformly mixed.

NaHA solution, which was prepared by dissolving sodium hyaluronate with a molar mass of Mn = 1.0–1.5 × 10^6 g/mol in 0.2 M PBS, was used as the liquid phase in this study. PBS without NaHA (0%) was used as control. Different concentrations of NaHA solutions (from 0.1% to 1% with 0.1% as interval) were made. The liquid to solid ratio of 0.35 ml/g was selected in this study.

2.2 Morphology and phase characterization

The CPC powder was homogeneously mixed with NaHA solution using a mortar to form a paste, then the paste was injected into a stainless-steel mold (6 mm in diameter and 12 mm in height) and incubated at 37°C and 100% relative humidity for 24 h. Then the hardened cement samples were removed and characterized by X-ray diffraction (XRD, Y-200, China). The fractured surfaces of the hardened cement samples were observed by a scanning electron microscope (SEM, JSM-5900LV, Japan).

2.3 Setting time measurement

The setting time of the CPC was measured using Gillmore needles according to ASTM C191. After the newly prepared CPC paste was poured into the stainless-steel mold, the initial setting time and final setting time were determined by the light needle (113.4 g in weight and 2.13 mm in diameter) and the heavy needle (453.6 g in weight and 1.06 mm in diameter), respectively. Each test was performed in triplicate.

2.4 Injectability test

A 5 ml syringe, which was fitted with a needle of 1.6 mm inner diameter, was used to test the injectability of the cement. The newly prepared CPC paste was poured into the syringe, and the injectable time was determined by the time interval from the starting point of mixing the paste to the time when the paste can be hardly pushed out through the needle under the pushing force of 20 N. The tests were performed under the condition of 37°C and 100% relative humidity. Each measurement was carried out in triplicate.

2.5 Porosity and compressive strength measurement

The newly prepared CPC paste was injected into the stainless-steel mold and incubated at 37°C and 100% relative humidity for 24 h. After hardening, the porosity (P) of the samples was defined as follow:

$$P = (d_{HAP} - d_{measured})/d_{HAP} \quad (1)$$

where d_{HAP} is the density of fully dense hydroxyapatite which is 3.14 g/cm^3, and $d_{measured}$ was the specimen's density which was measured as mass/volume [10].

The hardened specimens were subject to compressive strength measurements, which were performed by a computer-controlled universal testing machine (WDW-50, China) at a crosshead speed of 1 mm/min. The compressive strength was calculated by using the fracture load divided by the cross-sectional area of the specimen. Each test was performed in triplicate.

2.6 Anti-washout ability observation

The newly prepared CPC paste was poured into the syringe and immediately injected into the distilled water at 37°C, and then the behavior of the cement was observed at 5 min, 1 h, and 24 h, respectively. After immersion in water for 24 h, the non-decay parts of the cements were collected and freezing-dried. The percentage of remaining cements

was calculated, and each measurement was performed in triplicate.

2.7 Incubation in simulated body fluid (SBF)

The paste of CPC without and with 0.6% NaHA were injected into stainless mold, then the cement samples were removed after setting for 3 h and incubated in 28 ml of SBF at 37°C. The SBF was prepared according to kokubo's method [26], and changed every 2 days. The surface morphology of the samples was observed after incubation for 1d, 3d, 7d and 14d.

3 Results

3.1 Morphology observation

SEM images of the fractured surface of the CPCs without and with 0.6% NaHA concentration are shown in Fig. 1. A lot of macro-pores with the diameter of 100 ~ 300 μm distributed in both CPCs can be seen in Fig. 1a and b. Besides, the higher magnification SEM images (Fig. 1c and d) show that both CPCs are composed of reticulations, in which a lot of micro-pores occur, and the crystals are interlocked each other strongly. Besides, comparing with each other, there were not obvious morphological differences in both CPCs, proving that addition of NaHA had little influence on the structure of the CPC.

3.2 Phase transformation of CPC

Figure 2 shows the XRD patterns of the CPCs with different concentrations of NaHA after setting for 24 h. For comparison, the XRD patterns of the starting solid powder are also shown (pattern a). Obviously, there was a phase transformation occurred during the hardening process of the CPC paste. It should be clearly observed that the peaks of DCPD in the hardened CPCs (pattern b, c and d) almost disappeared, and the intensity of TTCP decreased sharply. Besides, the peaks of HAP presented higher diffraction intensity, indicating that new HAP phase formed during the hardening process and there was still a few TTCP and DPCD left. Basically, the three types of CPC showed almost the same XRD patterns, meaning that addition of NaHA hardly changes the formation of HAP.

3.3 Setting time and injectability

Results of the setting time and injectable time of CPC with different concentrations of NaHA are shown in Fig. 3. Obviously, the addition of NaHA cut down the setting time of the CPC. The final setting time of the CPC paste without NaHA was 80.7 ± 3.5 min, and it decreased to the shortest value (55.3 ± 4.0 min) when the concentration of NaHA was 0.5%. Later, the setting time increased gradually with the increasing of NaHA concentration from 0.5% to 1.0%. It should be noted that the initial and finial setting times of

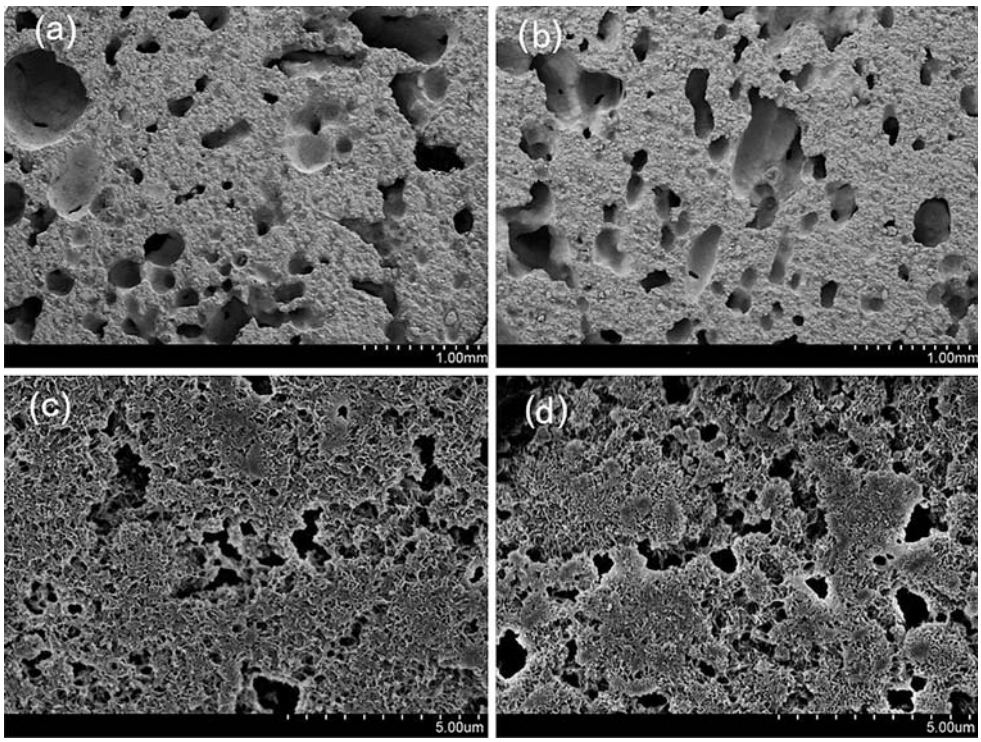

Fig. 1 SEM images of the fractured surface of the CPCs without and with 0.6% NaHA: **a** and **c** for CPC without NaHA; **b** and **d** for CPC with 0.6% NaHA

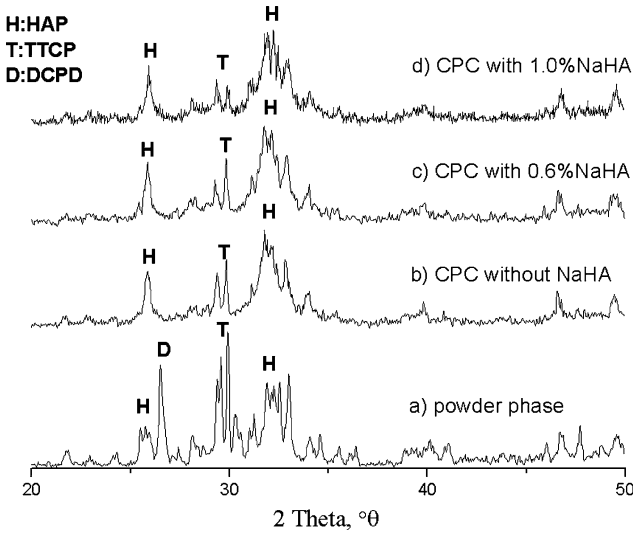

Fig. 2 XRD patterns of the starting powder and the CPCs

the CPC with NaHA were obviously shorter than those without NaHA.

Similar with the variation tendency of the setting time, the injectable time of the CPC paste declined when NaHA concentration increased from 0% to 0.4%, and then it increased with the increasing of NaHA concentration from 0.4% to 1.0%. The percentage of the cements expelled in the injection time was above 90% for the cement with every concentration of NaHA. Besides, in the entire process of injecting, the cements were homogenous and there was no powder–liquid phase separation during the injection process.

3.4 Porosity and mechanical strength

The porosity and compressive strength of CPC with different concentrations of NaHA are shown in Fig. 4. It could

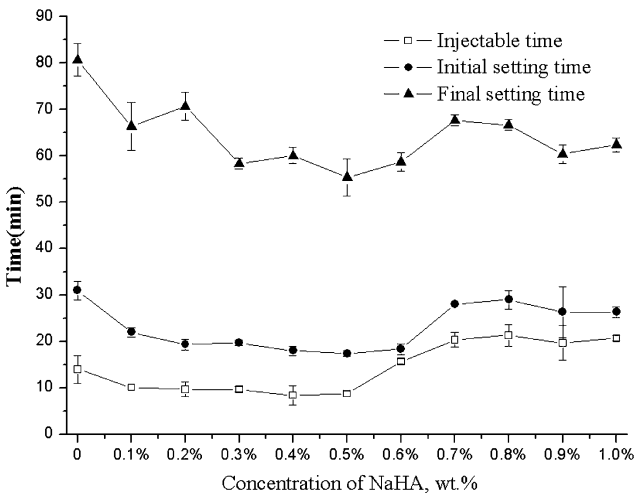

Fig. 3 Influence of the concentration of NaHA on the setting times and injectable time of the pastes

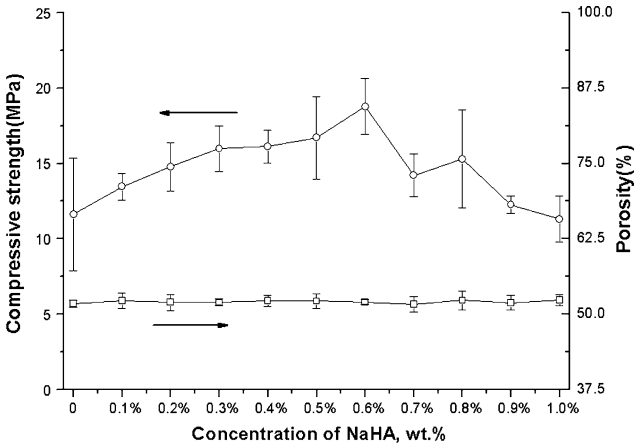

Fig. 4 Influence of the concentration of NaHA on the porosity and compressive strength of the cements

be found that addition of NaHA had little influence of the porosity of the cements, and the porosity stayed between 55% and 60%. The compressive strength of the hardened CPC increased with the increasing of NaHA concentration at the starting stage, and it reached to the biggest value (18.78 ± 1.83 MPa) when the concentration of NaHA was 0.6%. But later, the compressive strength declined when NaHA concentration increased from 0.6% to 1.0%.

3.5 Anti-washout ability

Figure 5a shows the behaviors of the cements with different concentrations of NaHA, which were injected into distilled water at 37°C for 5 min, 1 h and 24 h. The cement without NaHA started to decay after immersion in water for 5 min, and the extent of decay enlarged with the increase of immersion time. However, after addition of NaHA, the anti-washout ability of the cements became stronger than before, and the cements could almost remain its initial shape and no obvious decay was observed when NaHA concentration reached to 0.3% or more. After immersion in water for 24 h, the mass percentages of the remaining cements with different concentrations of NaHA are presented in Fig. 5b. The mass percentage of the remaining cement without NaHA was below 50%, but with addition of NaHA, it increased sharply and maintained above 90% when NaHA concentration increased to 0.3% or more.

3.6 Incubation in SBF

Figure 6 shows the SEM photographs of the cements without and with 0.6% NaHA after soaking in SBF for 1d, 3d, and 7d at 37°C. As shown in Fig. 6a and e, after soaking in SBF for 1d, no precipitation formed on the surface of the former while reticulations appeared on

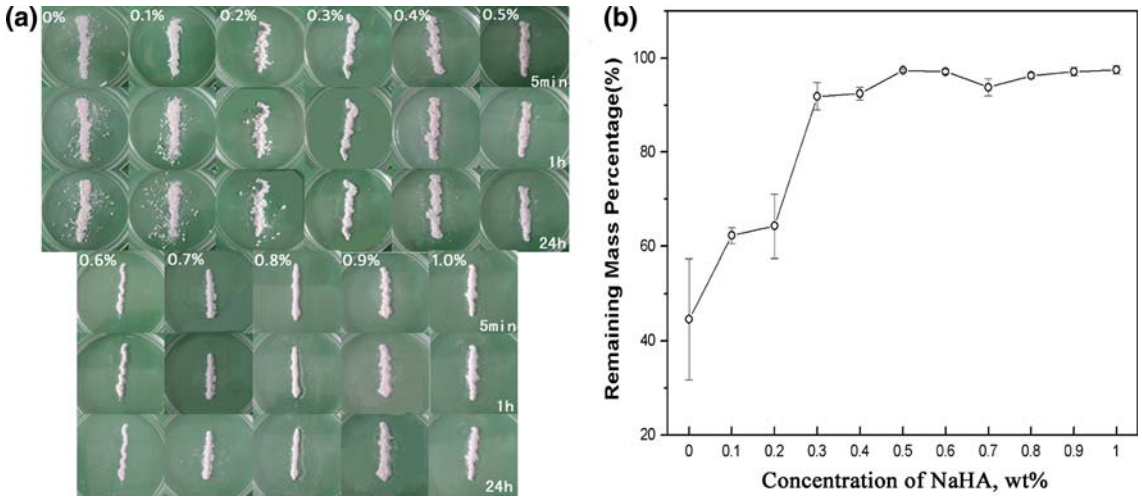

Fig. 5 **a** The behaviors of the cements with different concentrations of NaHA injected into distilled water at 37°C for 5 min, 1 h and 24 h. **b** The mass percentages of remaining cements with different concentrations of NaHA after immersion in 37°C water for 24 h ($n = 3$)

the surface of the latter. When the soaking time was 3d, the surface of the cement with 0.6% NaHA was covered by ball-like particles (Fig. 6f), and only a few net-like textures appeared on the surface of the cement without NaHA (Fig. 6b). After soaking in SBF for 7d, both cements were covered completely by those ball-like particles (Fig. 6c and g), proving that the deposition of bone-like apatite layer [26]. Besides, it should be noted from Fig. 6d and h that the lower magnification photos confirmed that the apatite layer formed on the cement with 0.6% NaHA was thicker than that on the cement without NaHA, which showed the faster growth of bone-like apatite on the cement with NaHA.

4 Discussion

A major disadvantage of current bone implants such as HA ceramics is that they are pre-shaped and generally need to be carved to match with the shape of the surgical site. An injectable CPC setting in situ can overcome the problem and show great advantage in complex shaped defects for minimal invasive surgery. In the present study, an injectable CPC, which might be used in minimally invasive surgery for bone repair or substitution, was developed by addition of some extra additives.

An appropriate porous structure is favorable for ingrowth of bone tissue and can promote the degradation of the cement after implantation. It is known that calcium phosphate ceramics with appropriate porous structure had excellent osteoconduction and even osteoinduction [27–31]. The results of SEM observation (Fig. 1) and the porosity (Fig. 4) confirmed that the hardened CPCs all had the porous structure, whose formation should be ascribed to the solid components of citric acid and sodium bicarbonate. When contacting with the liquid phase, they would react with each other to form CO_2. Moreover, addition of NaHA showed no adverse effect on the porous structure formation in the hardened body.

In the CPC system, after mixing with liquid phases, TTCP and DCPD powders will react to transform to HAP. A thoroughly transformation to hydroxyapatite is one of the guarantees for the strong mechanical strength of CPC[7]. The XRD analysis (Fig. 2) of the cements confirmed this transformation. Either with or without NaHA, most of the starting powders had transformed into HAP, and there was no obvious difference in XRD patterns between the cements without and with NaHA. It had been reported that some extra organic phases, such as chitosan, would influence this transformation process [21, 25]. However, little inhibition for HAP transformation was observed in this CPC system, as might be ascribed to the relatively lower content of NaHA in the cement. On the other hand, there were a few of TTCP and DCPD phases left in the hardened CPC due to the short reaction time, and the transformation would complete finally after implantation and exposed to body fluids [21, 32].

As the hardening reaction happens in a low temperature (below 40°C) and the components in the CPC system are hydrophilic and lack of chemical bonding, the surrounding water is likely to invade the paste and destroy the connection among the crystals thus lead it to decay [16, 17, 33]. It is known that hyaluronate has the intrinsic feature to form a network with water and then to enhance the viscosity of the liquid phase to form gels [34, 35]. When NaHA was introduced into the CPC system, a network spreading all over the paste could be formed, resulting in the good maintenance of its shape even exposing to water. The high viscosity and the chelate reaction between

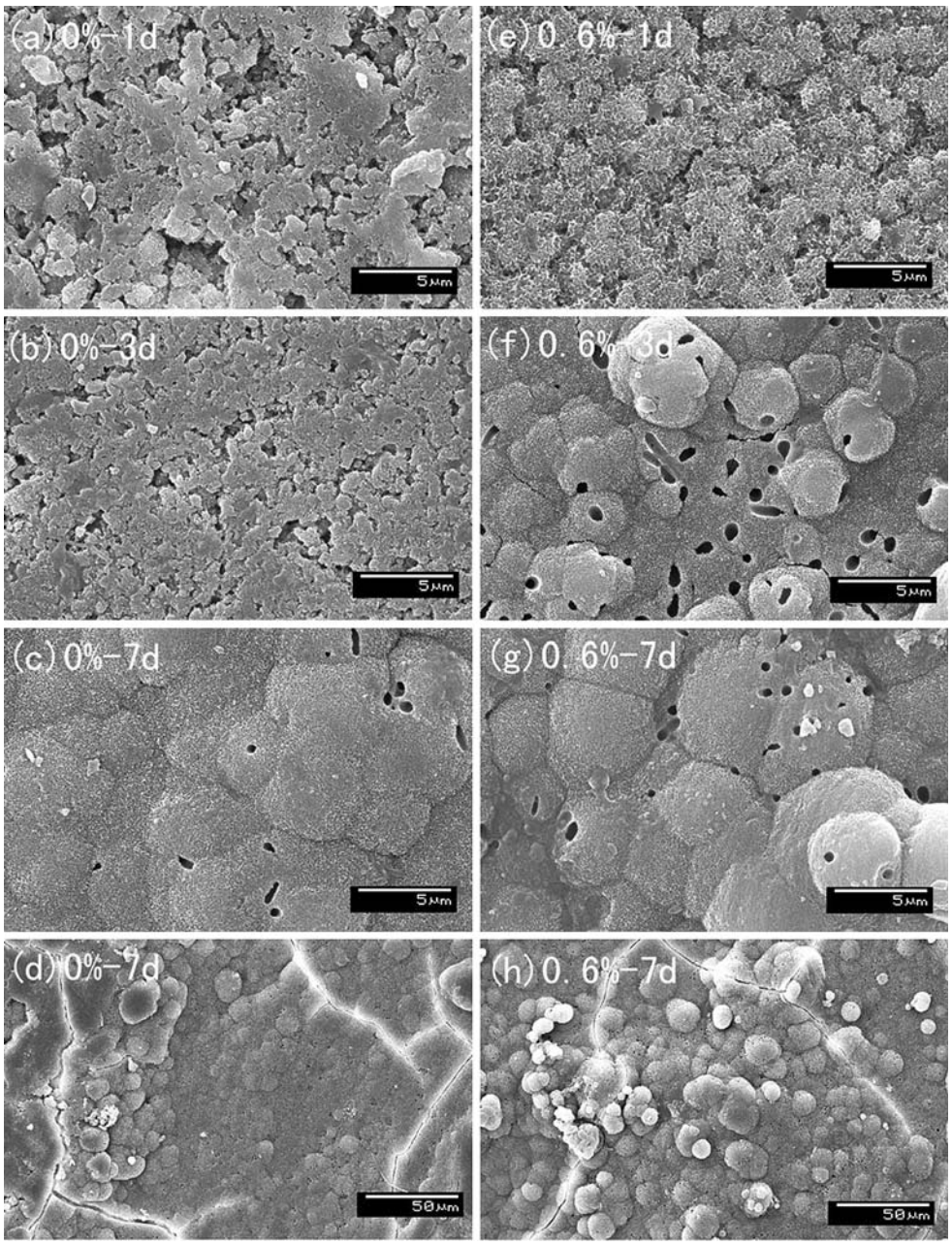

Fig. 6 SEM photographs of the CPCs without and with 0.6% NaHA after soaking in SBF for 1d, 3d, and 7d at 37°C. Mark '0%' for CPC without NaHA and '0.6%' for CPC with 0.6% NaHA

hyaluronate and Ca^{2+} would make HAP crystal nucleus in position, which promoted HAP crystal interlocking. As a result, the anti-washout ability of CPC was enhanced dramatically with addition of NaHA.

The injectable time, setting time and compressive strength all had a similarly trend of variation: there was a gradually decrease (or increase) in the beginning stage and later an adverse increase (or decrease) with the increasing of NaHA concentration. The reduction of the setting and injectable times at the low concentrations of NaHA could be explained by the viscosity increasing and the chelate reaction between hyaluronate in the liquid phase with Ca^{2+} dissolved from the powder phase happened during the initial hardening reaction of the cements. The binding between Ca^{2+} and the N-acetyl and carboxylate groups in hyaluronate resulted in the increase of the paste viscosity and formation of NaHA's network structure, which would promote the hardening of the CPC paste. When NaHA content further increased, free Ca^{2+} bound with hyaluronate decreased, and the excessive hyaluronate molecules might hold a lot of hydroxy groups by hydrating and absorb water molecules, leading to elongation of the setting time as well as injectable time.

The variation of compressive strength from the initial increasing and later decrease could also be explained from the reasons mentioned above. The chelate reaction and the

network links formed by hyaluronate would make more crystals interlocked tightly, so the compressive strength would firstly increase by addition of NaHA [7, 18]. However, when NaHA increased to a higher concentration, as mentioned above, the excessive hyaluronate molecules might hold a lot of hydroxy groups and water molecules that might inhibit the formed HAP crystals to interlock so as to decrease the compressive strength.

Whether a material has the ability to form bone-like apatite layer on its surface has been regarded as a key criterion to evaluate the bioactivity of the material [26]. According to Fig. 6, bone-like apatite formed on CPC with NaHA was faster than that on the CPC without NaHA after incubation in SBF. This could be explained by the reason that Ca^{2+} ions in the CPC binding with hyaluronate would release quickly with the dissolution of NaHA after soaking in SBF, leading to the increased concentrations of Ca^{2+}, HPO_4^{2-} and PO_4^{3-} around the samples. These supersaturated ions would congregate to reach the threshold of the apatite precipitate quickly and then form apatite crystal on the surface. Therefore, the CPC with NaHA might have better bioactivity than that without NaHA.

5 Conclusion

In this study, the performance of the CPC containing citric acid and sodium bicarbonate was significantly improved by using NaHA solutions as the liquid phase. Addition of NaHA could hardly affect the transformation of HAP and could maintain the porous structure of the CPC. Besides, the injectability, setting time and mechanical strength could be adjusted by using different concentrations of NaHA. Especially, the anti-washout ability and bioactivity of the CPC were significantly enhanced by the addition of NaHA. Therefore, the injectable CPC with NaHA might be a promising bone repair materials used in minimal invasive surgery.

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