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Morphology and mechanical behavior of TTCP-derived calcium phosphate cement subcutaneously implanted in rats

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Abstract A pre-hardened, TTCP-derived CPC was immersed in Hanks' solution as well as subcutaneously implanted into abdomen of rats. The implant-soft tissue interfacial morphology was examined and properties of the CPC were evaluated and compared under in vitro and in vivo conditions. The results indicate that the surface of immersed samples appeared rougher and more porous than that of implanted samples and was covered with a layer of fine apatite crystals. The CPC samples implanted for 4 weeks or longer were surrounded by a layer of fibrous tissue, which was further surrounded by a soft tissue capsule comprising numerous fat cells. The soft tissue capsule had a non-uniform distribution in thickness, which increased most significantly between 4 weeks and 12 weeks after implantation. None of polymorphic cells, osteoblast cells or bone cells adjacent to the implant were observed. The majority of original TTCP powder was transformed into apatite after 1 day of either immersion in Hanks' solution or implantation. The average porosity values of samples immersed in Hanks' solution for 4 weeks or longer were significantly larger than those immersed for 1 day or 1 week. The porosity values of samples implanted for different times were not significantly different. The DTS values of Hanks' solution-immersed samples largely decreased after a few weeks of immersion. The implanted samples maintained their strengths throughout the study.

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Introduction

Calcium phosphate cement (CPC) was developed as early as 1975 by Driskell et al. [1]. Later on, Brown and Chow [2], indicated that mixing and reaction of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) powders in a diluted phosphate-containing solution led to formation of hydroxyapatite (HA). Since then a number of different CPC products were introduced into the market as a filling material for dental and orthopedic applications due to superior biocompatibility and osteoconductivity of the material [3–6]. One major advantage of CPC is its ability to be easily shaped in paste form during operation or injected into a cavity with a syringe without requiring an open way through tissues.

Although studies have repeatedly demonstrated favorable bone responses to CPC implant [7–14], it is worth noting that sometimes the implant material is not only in contact with bone, but also with the surrounding soft tissues, such as periosteum, connective tissue and muscle. Furthermore, operation-induced fragmented CPC could migrate to extravertebral site causing asymptomatic complication after surgery of osteoporotic vertebral compression fractures [15]. Apparently not only CPC-bone interaction, the interaction between CPC and soft tissue also needs to be evaluated.

Miyamoto et al. [16] reported that severity of soft-tissue reaction to CPC is dependent on the setting characteristics of the cement. They observed that CPC with a longer setting time resulted in a more profound tissue reaction. In addition, the dispersed fragments themselves could evoke an inflammatory response [17].

Yang et al. [18] indicated that bone formation in soft tissue is a complex process involving fibrous connective tissue invasion, polymorphic mesenchymal cell aggregation,

osteoblast differentiation and tissue remodeling. Interestingly, it was observed that bone formation did not occur in response to porous HA implanted extraskeletally in rats, rabbits, or goats, but did occur in dogs and pigs. Their study in dogs showed that polymorphic cells usually first appeared close to capillaries, and some of them were likely to migrate to the implant surface. Osteoblast differentiation occurred directly within the polymorphic cell clusters. In rats, rabbits or goats, however, cells which aggregated at the implant surface differentiated into other cell types than osteoblasts.

In their dog implantation study, Yuan et al. [19] mixed HA, α-TCP and DCPD powders in a weight ratio of 5:55:40 in a hardening solution containing K_2HPO_4 H_2O, NaH_2PO_4 H_2O and water to obtain a CPC. The CPC was then implanted into thigh muscle of dogs in pre-hardened form and into femoral bone and dorsal muscle in paste form. Observing bone formation in pores and deep rugged surface of implant in both dorsal and thigh muscle, the authors suggested that the CPC used in the study was both osteoconductive and osteoinductive in dogs. Despite the few studies and their interesting results, it seems that the knowledge regarding CPC-soft tissue interaction is still too limited for a solid conclusion to be drawn.

In the present study, a recently-developed single phase, pre-hardened TTCP-derived CPC [20] was immersed in Hanks' solution as well as subcutaneously implanted into abdomen of Wistar rats. The CPC-soft tissue interfacial morphology was examined and properties of the CPC were evaluated and compared under in vitro and in vivo conditions.

Materials and methods

The TTCP powder used for this study was fabricated in-house from the reaction of dicalcium pyrophosphate ($Ca_2P_2O_7$) (Sigma Chem. Co., St. Louis, MO, USA) and calcium carbonate ($CaCO_3$) (Katayama Chem. Co., Tokyo, Japan) with a weight ratio of 1:1.27. The powders were mixed uniformly in ethanol for 12 h, followed by heating in an oven at 50 °C for 1 day to let dry.

To obtain a CPC paste, the TTCP powder was mixed in diammonium hydrogenphosphate ($(NH_4)_2HPO_4$, 33.3 wt%) hardening solution with a pH value of 8.1 and liquid/powder ratio of 0.34 mL/gm. After mixing for 1 min, the cement paste was uniformly packed in a stainless steel mold which has an opening of 6 mm in diameter and 3 mm in depth under a pressure of 1.4 MPa. The hardened CPC was then removed from the mold and immersed in Hanks' physiological solution [21] at 37 °C for 1 day to increase its strength.

To compare morphology and strength under in vitro and in vivo conditions, the pre-hardened CPC samples were divided into two groups. The samples of in vitro group continued to be immersed in Hanks' solution (10 cc solution/g CPC) for 1 more day, 1 week, 4, 12 and 24 weeks. The solution was maintained at 37 °C and continually stirred to help maintain uniform ion concentrations. The pH value of the solution, which started from 7.05, was monitored using a pH meter (HM-20S, Tokyo TOA Electronics Ltd, Tokyo, Japan). The diametral tensile strength (DTS) of the samples was measured using a desktop mechanical tester (Shimadzu AGS-500D, Tokyo, Japan) at a crosshead speed of 0.5 mm/min. A one-way ANOVA method was used to evaluate the statistical significance of the strength. A Scheffe' multiple comparison test was used to determine the significance of the deviations in DTS. In all cases, the results were considered statistically different with $p < 0.05$.

The samples of in vivo group were subcutaneously implanted into the abdomen site of Wistar rats for designated durations at National Cheng-Kung University Medical College Animal Center. Five adult, healthy, male Wistar rats, each weighing about 500 g, were used for the study. The rats were housed individually in stainless-steel cages with free access to food and water. A minimum of 3 days was allowed between receipt of the animals and the start of operation. The animals were operated under general anesthesia (pentobarbital sodium 0.1 mL/100 g, Tokyo Kasei Kogyo, Tokyo, Japan). To implant CPC, the abdomen of the rat was first shaved, washed and disinfected with iodine. Two longitudinal incisions about 1 cm long were made through the full thickness of the skin and subcutaneous pockets lateral to the incisions were created by blunt dissection with scissors. In each pocket (about 2 cm × 2 cm) were implanted five samples for DTS testing and morphological examination. In addition, to reduce moisture effect, all samples of both in vitro and in vivo groups for DTS testing were oven-dried at 50 °C for 1 day before testing. After implantation the wounds were carefully closed. The animals recovered from anesthesia approximately 1 h after operation. To compare with in vitro data consistently, the animals were sacrificed after 1 day, 1 week, 4, 12 and 24 weeks.

The samples for histological examination were fixed in 4% formaldehyde diluted in a 0.1 M phosphate buffer (pH 7.4) for 3 days, dehydrated in increasing grades (70%, 95% and 100%) of ethanol, defatted and cleaned with xylene. Samples were then infiltrated and embedded in methylmethacrylate (MMA, Fluka 64200, Buchs, Switzerland) [22]. Non-decalcified, thin (40–50 μm) sections were prepared by first sectioning the harvested samples into approximately 750 μm thick sections in a transversal direction using a diamond blade, followed by grinding and mechanical polishing to the final thickness. At least three sections were prepared for each sample. All sections for

histological examination were stained with toluidine blue and examined using a light microscope (Leitz labrorlux 12 pols, Leica Co., Germany). The thickness of the capsule attached to the implant was determined by manually tracing the capsule and measured with aid of an imaging system (Image-pro plus version 4.5).

To eliminate the effect of the soft-tissue capsule on the strength of the present TTCP cement, prior to DTS testing of in vivo samples, the capsules surrounding the implants were removed. The DTS testing of in vivo samples was conducted using the same, earlier-mentioned method as for the testing of Hanks' solution-immersed samples.

X-ray diffraction (XRD) was carried out to evaluate phase changes of the pre-hardened CPC samples under both in vitro and in vivo conditions. A Rigaku D-MAX B X-ray diffractometer (Tokyo, Japan) with Ni-filtered CuKα radiation operated at 30 kV and 20 mA at a scanning speed of 1°/min was used for the study. Powder samples prepared from crushing the hardened CPC cylinders were used for XRD characterization. The various phases were identified by matching each characteristic X-ray diffraction peak with that compiled in JCPDS files. A Fourier transform infrared (FTIR) spectroscopy system (Jasco, FT/IR-460 Plus, Tokyo, Japan) in transmission absorption mode with a spectral resolution of 2 cm^{-1} was used to characterize the various functional groups of the TTCP powder and crushed pre-hardened CPC samples. A field-emission scanning electron microscope (SEM) (XL-40, Philips, Holland) operated at 15 kV was used to examine the morphology of both in vitro and in vivo samples. Samples for SEM examination were coated with platinum using an ion sputtering system (Hitachi E-1010, Tokyo, Japan). Porosity of the samples was measured according to ASTM C830 method.

Results and discussion

Histological observation

All pre-hardened CPC samples maintained their original cylindrical shape without noticeable dissolution or damage in structure, no matter they were immersed in Hanks' solution (Fig. 1) or were implanted (Fig. 2). This result demonstrates that the pre-hardened CPC prepared in this study has good mechanical stability in both environments. In these low magnification photographs the pre-hardened CPC samples immersed in Hanks' solution appear "clean and neat" throughout the test. The samples implanted into the rat abdomen site, however, were observed to be attached with a fibrous capsule after 4 weeks.

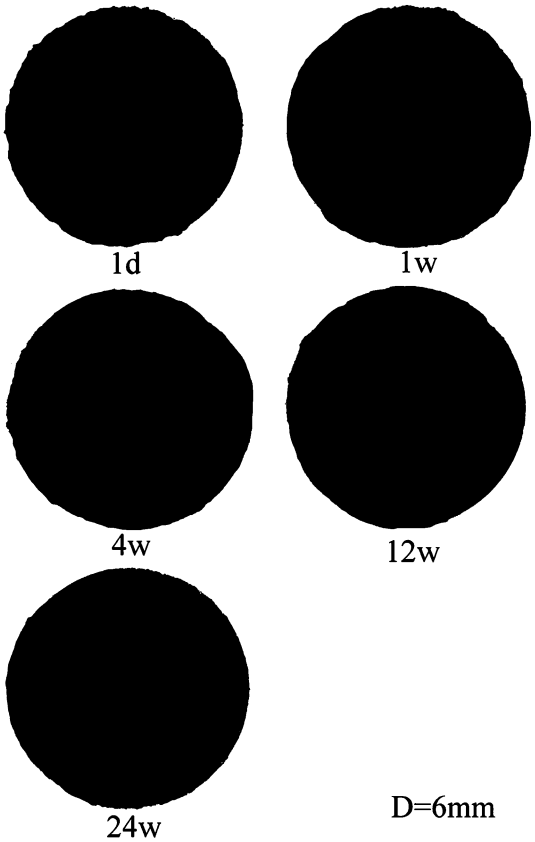

Fig. 1 Transverse-directional photographs of pre-hardened CPC immersed in Hanks' solution

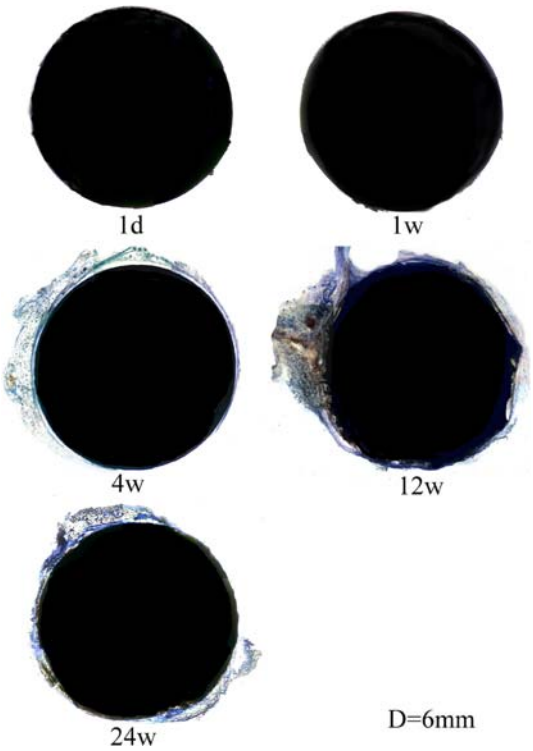

Fig. 2 Transverse-directional photographs of pre-hardened CPC implanted into abdomen of rats

Histological examination revealed the morphology in implant-tissue interface regime in greater detail. As shown in Fig. 3, the pre-hardened CPC samples implanted for 4 weeks or longer were surrounded by a layer of fibrous tissue, wherein fibers were oriented substantially parallel to the implant surface. The fibrous tissue was further surrounded by a soft tissue capsule comprising numerous fat cells. Throughout the entire implantation period (24 weeks), the surface of the pre-hardened CPC implant remained substantially intact without any noticeable cracking, chipping or dissolution. Another conclusion from the present histological observation is that none of polymorphic cells, osteoblast cells or bone cells adjacent to the implant were observed throughout the study, in agreement with the observation of Yang et al. [18].

The fibrous capsule surrounding a subcutaneous implant could be an indication of biocompatibility of the implant. Ooms et al. [17] suggested two mechanisms to account for the subcutaneous resorption of calcium phosphate ceramics: solution-mediated process and cell (giant cell, osteoclast)-mediated process. The authors also indicated that the formation of capsule is considered to be a healing reaction to the surgical trauma and the continued presence of the implant. Ratner et al. [23] revealed that small insoluble fragments separating from an implant can activate macrophages and the release of such pro-inflammatory kinins as fibroblast growth factor and platelet-derived growth factor, which, in turn, can influence fibroblast behavior. According to the authors, such kinins could also induce thickening of the fibrous capsule. In their study of subcutaneous implantation of carbonate apatite in Wistar rats, Barralet et al. [24] suggested that formation of fibrous capsule is an attempt of the body to immobilize the implant and counter the locally increased calcium and phosphate ion concentrations. Therefore, thicker encapsulation often forms as a result of higher local ionic concentrations.

In the present study the soft tissue capsule had a non-uniform distribution in thickness, which increased most significantly between 4 weeks and 12 weeks after implantation (Fig. 3). To be specific, the 4-week sample indicated an average capsule thickness of about 90 μm, while the 12-week sample demonstrated an average thickness of about 150 μm. After 12 weeks, the capsule

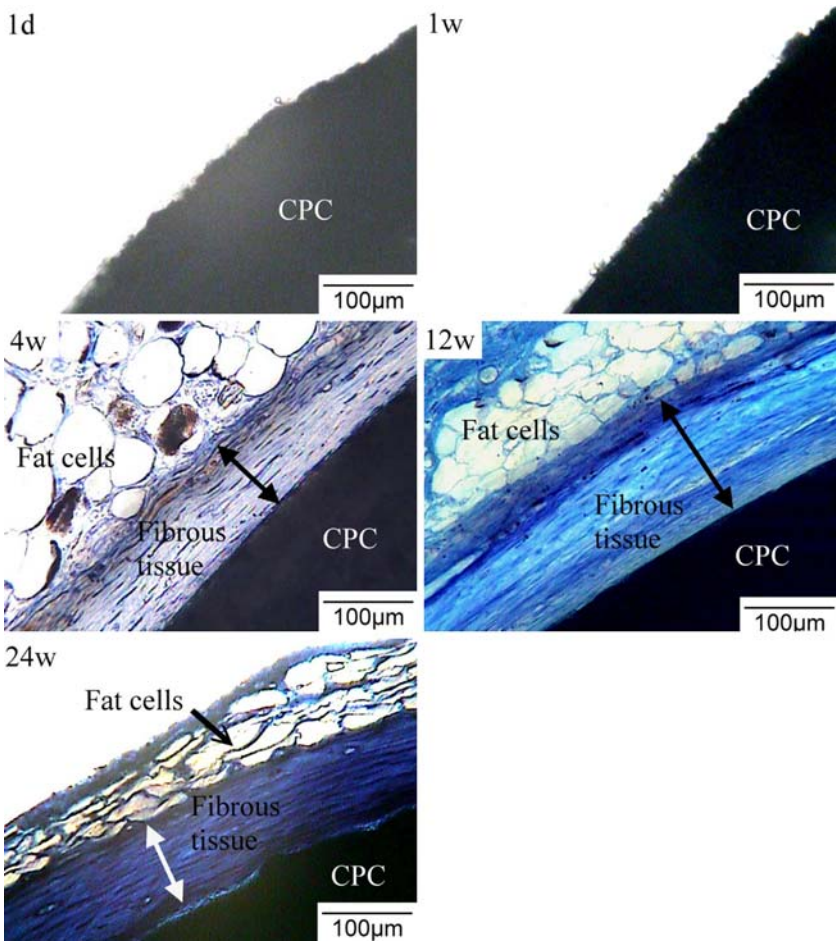

Fig. 3 Transverse-directional histological micrographs of pre-hardened CPC implanted into abdomen of rats

thickness did not change much, indicating that the capsule had been largely mature after 12 weeks.

SEM examination

Figures 4 and 5 demonstrate typical surface morphologies of Hanks' solution-immersed and rat-implanted pre-hardened CPC, respectively. As shown in these figures, the surface of the implanted samples always appears flatter and smoother than that of Hanks' solution-immersed samples due to the "protection" of the surrounding soft tissue capsule from dissolution of the implant. According to Szivek et al. [25], this encapsulation can create a locally high calcium concentration environment and thus reduce dissolution rate of calcium ions from implant into body fluids.

The surface of pre-hardened CPC samples immersed in Hanks' solution, on the other hand, appears rougher and more porous from a microscopic point of view. Without shielding of the soft tissue capsule, the solution-immersed surface became more reactive and dissolvable. The SEM micrographs also show that the Hanks' solution-immersed surface was covered with a layer of tiny crystals, which, according to the later-mentioned XRD result, are agreed to be apatite crystals. Such apatite crystals are most commonly observed in various forms of whiskers and formed as a result of surface dissolution-precipitation processes in many calcium phosphate-based systems [20, 26–29].

XRD and FTIR analyses

Figures 6 and 7 are XRD patterns of immersed and implanted pre-hardened CPC, respectively, while Figs. 8 and 9 are FTIR profiles of immersed and implanted samples, respectively. As indicated in Figs. 6 and 7, the pre-hardened (in Hanks' solution for 1 day) TTCP cement (see Materials and methods) has been transformed into apatite after one more day of either immersion in Hanks' solution or implantation into rat abdomen, although small amounts of TTCP phase were still recognizable after 1 week.

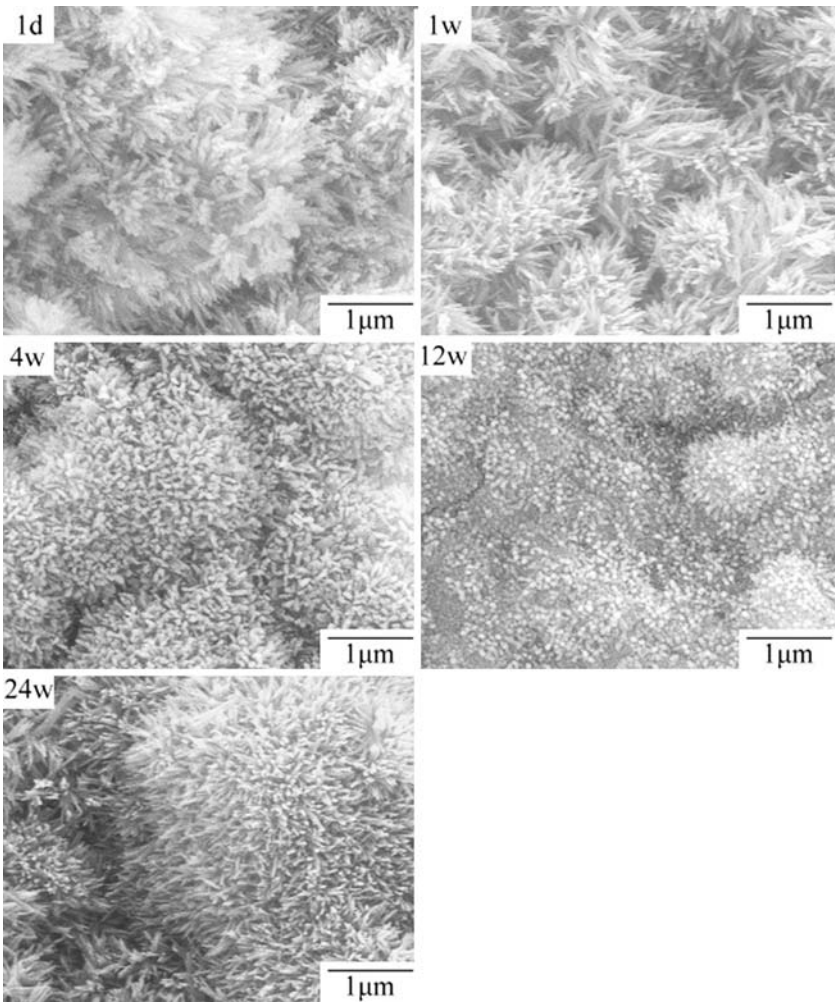

Fig. 4 SEM micrographs of surface of pre-hardened CPC immersed in Hanks' solution

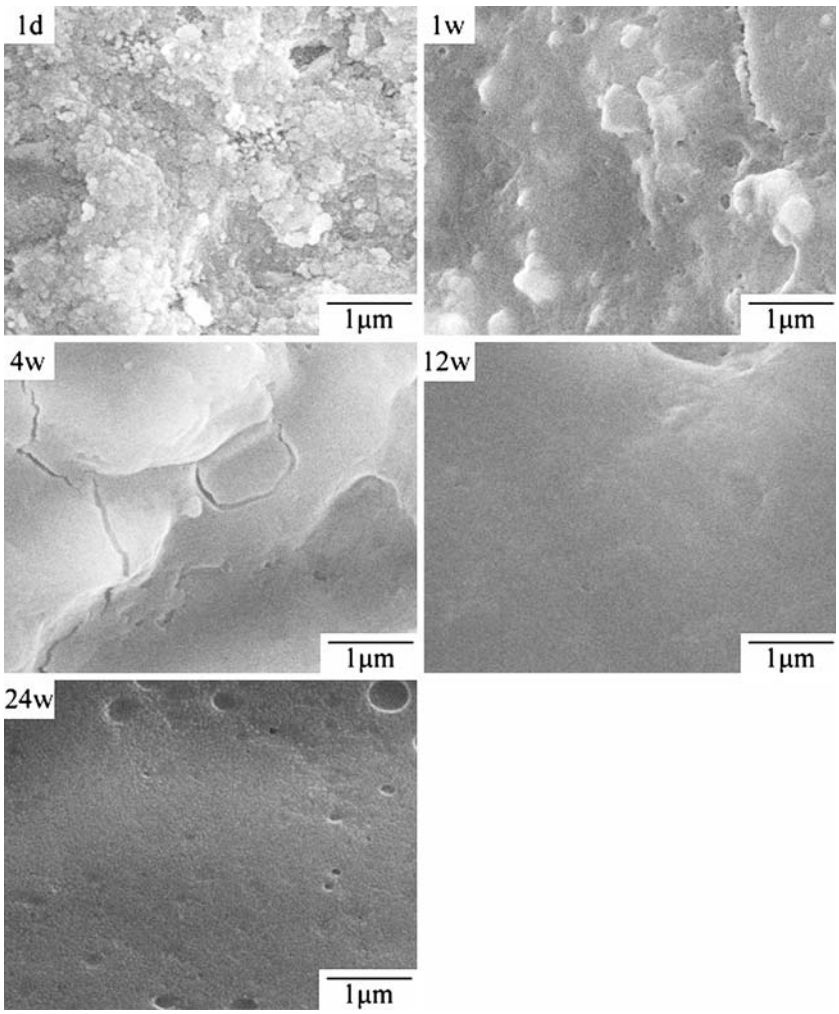

Fig. 5 SEM micrographs of capsule-free surface of pre-hardened CPC implanted into abdomen of rats

The XRD patterns of in vitro and in vivo samples are similar, indicating a similar phase transformation process in both environments, once the TTCP cement was pre-hardened in Hanks' solution for 1 day. The broadness of apatite peaks is a direct result of fine apatite crystals, in agreement with earlier studies [30, 31]. As shown in Figs. 8 and 9, the FTIR profiles of in vitro and in vivo samples are also similar without any significant differences in their surface functional groups. The two clusters of peaks, around 900–1,150 and 550–600 cm^{-1}, demonstrate a typical apatite spectrum [32, 33]. The formation of the soft tissue capsule surrounding implanted samples does not seem to have a significant effect on the phase transformation process.

Porosity measurement

Presented in Fig. 10 are the porosity values (in volume fraction) of pre-hardened CPC samples immersed in Hanks' solution and implanted in rats for different periods of time. As indicated in the bar graph, the average porosity values of the samples immersed in Hanks' solution for 4 weeks or longer (38–40%) are significantly ($p < 0.001$) larger than those immersed for 1 day or 1 week (31–33%). This result is consistent with the earlier-mentioned SEM observation that pre-hardened CPC samples immersed in Hanks' solution always appear rougher and more porous.

Statistical analysis shows that the porosity values of implanted samples for different implantation times are not significantly different ($p > 0.05$). These almost-unchanging porosity values are also consistent with the SEM observation that the surface of implanted samples always appears flat and smooth due to a shielding effect of soft tissue capsule.

DTS assessment

One major finding of this research is the dramatically different trends in the variation in DTS with immersion/implantation time between Hanks' solution-immersed and

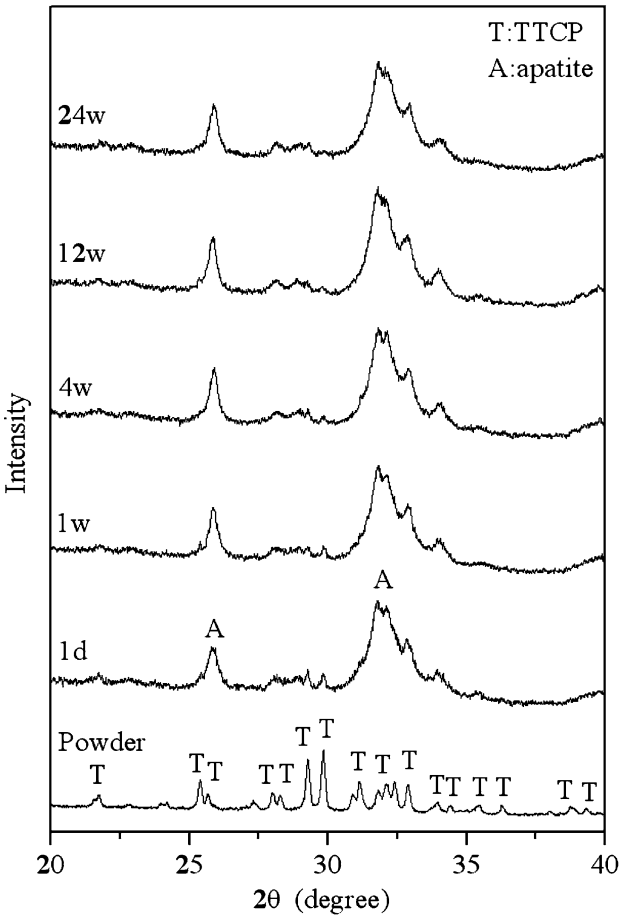

Fig. 6 XRD patterns of pre-hardened CPC immersed in Hanks' solution

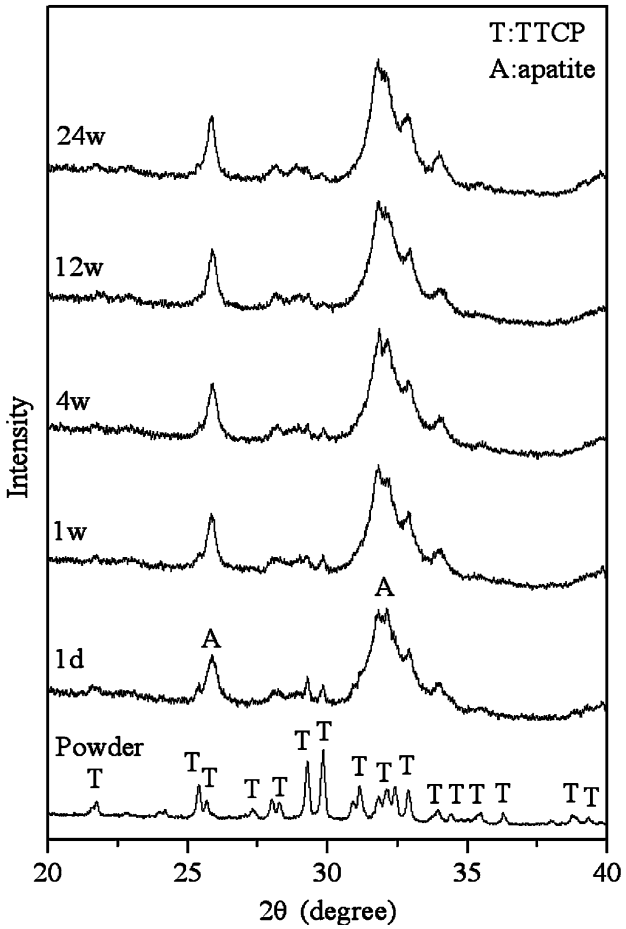

Fig. 7 XRD patterns of capsule-free surface of pre-hardened CPC implanted into abdomen of rats

implanted samples. As clearly indicated in Fig. 11, the DTS values of Hanks' solution-immersed samples largely decreased after a few weeks of immersion. Specifically, the DTS values of samples immersed for 4 weeks or longer became only about one-half that immersed for 1 day or 1 week. This large decrease in material strength of Hanks' solution-immersed samples might be explained by their immersion-induced increases in porosity level, as mentioned earlier. Again, this increase in porosity value is considered to be a direct result of the earlier-mentioned absence of the "protective" soft tissue capsule surrounding the immersed samples. The fact that porosity and DTS values both significantly change in the same time frame (between 1 week and 4 weeks) seems to favor this interpretation. Since the XRD and FTIR results did not reveal any significant differences among samples immersed for different periods of time, crystal structure or surface chemistry (specifically surface functional groups) does not seem to have a major effect on strength of the material.

Interestingly, this large decrease in strength was not observed in the implanted samples. As indicated in Fig. 11, the DTS values of in vivo samples did not decrease with implantation time. Although the average DTS values of in vivo samples slightly increased with time after 1 week of implantation, one-way ANOVA analysis showed that the increases were not significantly ($p > 0.05$). While 1-day and 1-week DTS values of in vitro and in vivo samples are similar, the average 4-week DTS value of implanted samples is larger than that of 4 week-immersed samples by 122%. After 24 weeks, the difference increases to 146%. These results suggest that in vitro mechanical properties of the pre-hardened CPC cannot represent in vivo properties of the material, and it is highly likely that the mechanical properties of an implanted pre-hardened CPC can be underestimated based on their in vitro data. Again, the non-decayed strength observed in implanted samples is considered to be a direct result of the presence of the soft tissue capsule surrounding the implant.

Yamamoto et al. [34] had compared compression strengths of an α-TCP/DCPD-based CPC under in vitro (immersed in pseudo-extracellular fluid) and in vivo (subcutaneously implanted into the back of rabbit) conditions

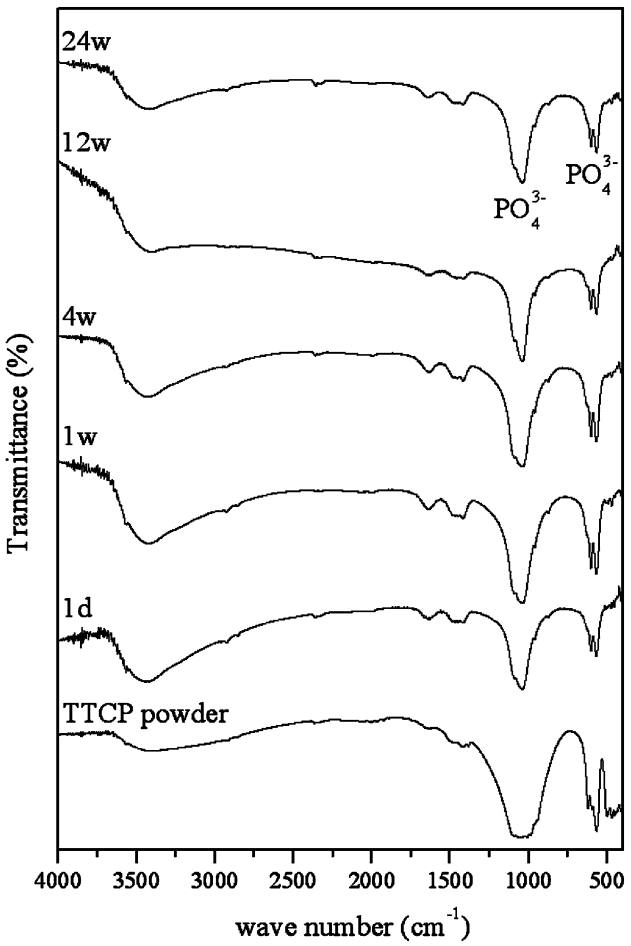

Fig. 8 FTIR profiles of pre-hardened CPC immersed in Hanks' solution

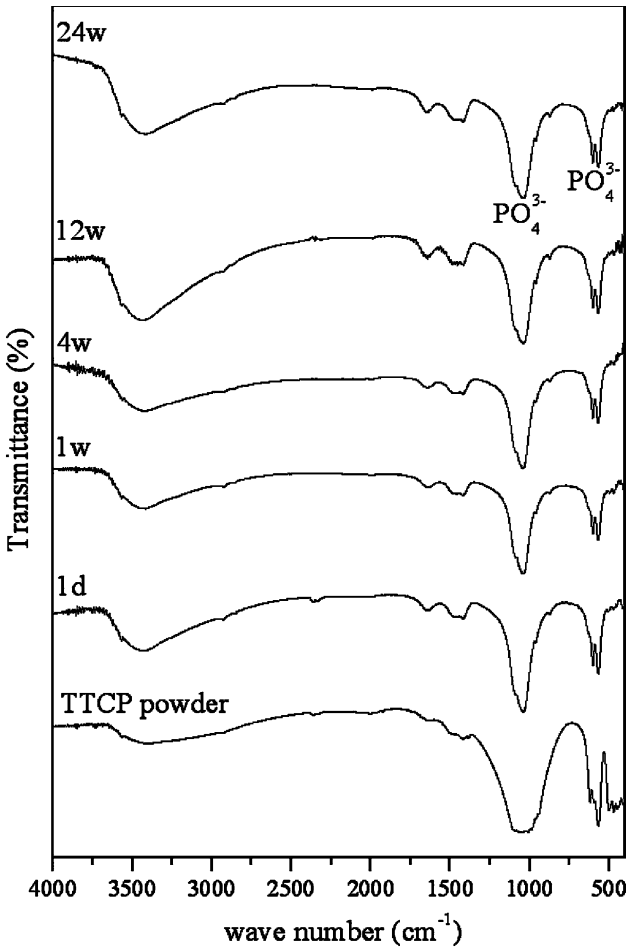

Fig. 9 FTIR profiles of capsule-free surface of pre-hardened CPC implanted into abdomen of rats

and obtained an opposite result. Their in vitro samples were found twice stronger than in vivo samples. A possible explanation for the opposite result of Yamamoto et al. might be that a sufficient strength had not yet been established in their CPC (being hardened for a relatively short time) before the material was implanted. Under this condition, the strength of the CPC could more easily be affected by the surgery procedure and/or the in vivo environment.

Conclusions

1. The surface of pre-hardened CPC samples immersed in Hanks' solution appeared rougher and more porous than that of implanted samples, and was covered with a layer of fine apatite crystals. The CPC samples implanted for 4 weeks or longer were surrounded by a layer of fibrous tissue, which was further surrounded by a soft tissue capsule comprising numerous fat cells. The soft tissue capsule had a non-uniform distribution in thickness, which increased most significantly between 4 weeks and 12 weeks after implantation. After 12 weeks, the capsule thickness did not change much.

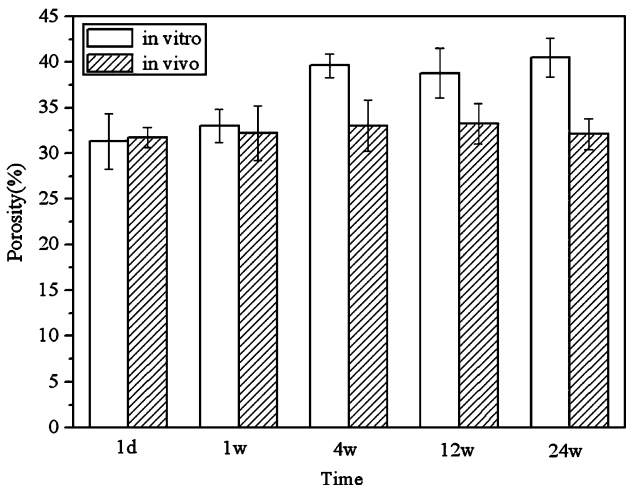

Fig. 10 Porosity values of pre-hardened CPC immersed in Hanks' solution and implanted into abdomen of rats ($n = 6$)

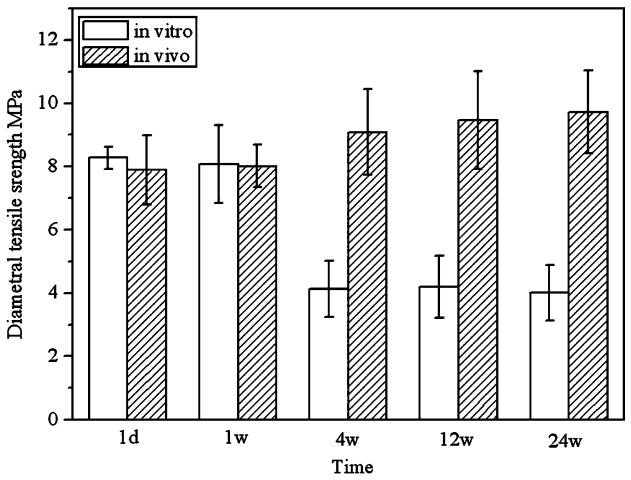

Fig. 11 DTS values of pre-hardened CPC immersed in Hanks' solution and implanted into abdomen of rats ($n = 6$)

The surface of the implant itself remained substantially intact without noticeable cracking, chipping or dissolution. None of polymorphic cells, osteoblast cells or bone cells adjacent to the implant were observed.

2. The pre-hardened TTCP cement has been transformed into apatite after one more day of either immersion in Hanks' solution or implantation into rat abdomen, although small amounts of TTCP phase were still recognizable after 1 week. The XRD and FTIR profiles of in vitro and in vivo samples are similar, indicating a similar phase transformation process in both environments.
3. The average porosity values of samples immersed in Hanks' solution for 4 weeks or longer were significantly larger than those immersed for 1 day or 1 week. The porosity values of samples implanted for different times were not significantly different.
4. The DTS values of Hanks' solution-immersed samples largely decreased after a few weeks of immersion. The implanted samples maintained their strengths throughout the study.

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