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Effect of PLGA/lecithin hybrid microspheres and β-tricalcium phosphate granules on physicochemical properties, in vitro degradation and biocompatibility of calcium phosphate cement

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To improve the biodegradability and tissue ingrowth of calcium phosphate cement (CPC), poly(lactic-co-glycolic acid)/lecithin (PLGA/Lec) hybrid microspheres and isostatically-pressed high-strength beta-tricalcium phosphate (β-TCP) granules were incorporated into CPC matrix. The effect of them on the mechanical properties, setting time, microstructu and morphology of CPC was studied. Then three samples were selected to investigate the influence of the two additives on the biodegradability and biocompatibility of CPC. The results showed PLGA/Lec microspheres reduced the compressi strength of CPC to some extent, but extended the setting time obviously. Based on adding 10 wt% PLGA/Lec microspheres, 10 wt% β-TCP granules enhanced the compressive strength and shortened the setting time of CPC. Furthermore, PLGA/Lec microspheres improved the degradability of the cement and created macropores suitable for cells ingrowth in situ in the process of degradation. Both PLGA/Lec microspheres and β-TCP granules were tested to stimulate the proliferation o mouse bone marrow mesenchymal stem cells (mBMSCs)

Introduction

Calcium phosphate cement (CPC) is recognized as one of the most promising materials for bone repair, owing to its similar composition of natural bone minerals and excellent properties, such as being biocompatible and osteoconductive, easy-shaping and low heat liberating in the curing process [1-4]. However, because of the lack of macropores and relatively low porosity, the degradation behavior of apatitic CPCs is unsatisfactory when implanted into the body [5]. Increasing porosity and interconnectivity of CPC will promote the degradation and resorption of CPC. It allows nutrients transport if body fluids within the matrix and also improve the possibility that proteins and cells colonize into CPC. Besides, it has reported that the appropriate pore size for bone ingrowth should be larger than 100 μm [6].

Biodegradable PLGA microspheres can be used to impart macroporosity to the cement [7-10] and release drugs or growth factors for bone repair, such as gentamicin [11] and BMP-2 [12] . It has proved that the mechanical strength of CPC is reduced with the introduction of macropores into the cement matrix [13], but increases significantly when new bone grows into these pores [14]. Moreover, PLGA can degrade hydrolytically into its monomers lactic and glycolic acid. Then the acids acidify the surrounding area of the PLGA microspheres, leading to accelerated dissolution of CPC [15] as calcium phosphate ceramics generally dissolve faster under acidic conditions [16]. However, synthetic PLGA has some drawbacks. First, the lack of cell recognition sites hampers cell adhesion on PLGA. It can be modified by combining with lecithin through O/W emulsion method [17]. As a compone of cell membranes, lecithin has been proved to improve the biocompatibility and alleviate the inflammatory reactions of the PLLA scaffold [18]. Besides, the introduction of PLGA microspheres prolongs the setting time of CPC [19]. Beta-TCP is one of absorbable biological ceramics [20-23]. It can be resorbed completely within 12 months when implanted into bone defects [24, 25]. Previous study has revealed that high-strength β-TCP aggregates prepared through isostatic pressing can enhance the strength of CPC and shorten its setting time [26].

The relation between the degradation and strength of the CPC combining PLGA microspheres has been studied previously [27]. This work focused on regulating the balance between improving the degradation and maintaining appropriate physicochemical properties as well as biocompatible behaviors of CPC. The method was adding degradable PLGA/Lec microspheres to form macropores in situ and meanwhile dispersing high-strength β-TCP granules in CPC matrix to enhance the mechanical properties. The effect of simultaneou incorporation of PLGA/Lec microspheres and β-TCP granules o physicochemical properties, in vitro degradation and biocompatibility of CPC was investigated.

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Results and discussion

Mechanical test and setting time measurements

The effect of content of PLGA/Lec microspheres and β-TCP granules on the compressive strength of the cement is plotted in Fig. 1(a). The compressive strength, initial setting time and final setting time of CPC0 were 30.56 ± 2.17 MPa, 6 ± 0.5 min and 18 ± 1.3 min, respectively. If considering PLGA/Lec microspheres individually, the compressive strength of the cements decreased with the increment of microspheres, from 30.56 ± 2.17 MPa to 15.42 ± 0.84 MPa. Based on incorporating a certain amount of PLGA/Lec microspheres, the compressive strength showed a similar tendency with the augment of β-TCP granules. However, when adding 10 wt% PLGA/Lec microspheres, the compressive strength achieved an optimal value of 27.83 ± 1.33 MPa with further addition of 10 wt% β-TCP granules, but not significantly higher than that of CPC without β-TCP granules, which was 26.86 ± 1.42 MPa (Tukey's test, $p < 0.05$). Based on adding 20 wt% PLGA/Lec microspheres, the compressive strength slumped rapidly to lower than 10 MPa while the content of β-TCP granules increased to 8 wt% and remain stable with further addition of β-TCP granules.

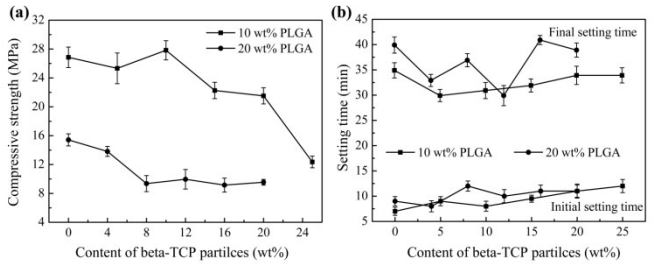

Figure 1. Influence of the content of PLGA/Lec microspheres and β-TCP granules on the compressive strength (a) and setting time (b) of the cement. Data are presented as mean ± standard deviation (n = 6)

The influence of content of PLGA/Lec microspheres and β-TCP granules on the setting time of CPC is detailed in Fig. 1(b). It can be seen that the addition of PLGA/Lec microspheres alone prolonged the setting time of CPC distinctively. With the increase of microspheres from 0 to 20 wt%, the initial setting time increased from 6 ± 0.5 min to 9 ± 0.9 min and the final setting time increased from 18 ± 1.3 min to 40 ± 1.6 min. Nevertheless, under the condition of mixing a fixed amount of PLGA/Lec microspheres, the incorporation of low-content β-TCP granules prolonged the initial time slightly, but decreased the final setting time obviously. While adding 10 wt% PLGA/Lec microspheres, the final setting time got shorter at first and then rose slowly with increased content of β-TCP granules. When adding 20 wt% PLGA/Lec microspheres, the final setting time fluctuated a little with incremental content of β-TCP granules, but not longer than 45 min.

The phase composition of these cements was determined by X-ray diffraction as shown in Fig. 2. It was evident that the composition of hydrated cements mainly was poorly crystalline hydroxyapatite (JCPDS 00-009-0432), along with a little amount of unreacted DCPA (JCPDS 00-009-0080). With the increase of β-TCP granules in the composites, the diffraction intensity of DCPA decreased slightly and the β-TCP (JCPDS 00-009-0169) phase was clearly detected. Compared with β-TCP granules, the content of PLGA/Lec microspheres had little effect on the phases of hydrated CPC.

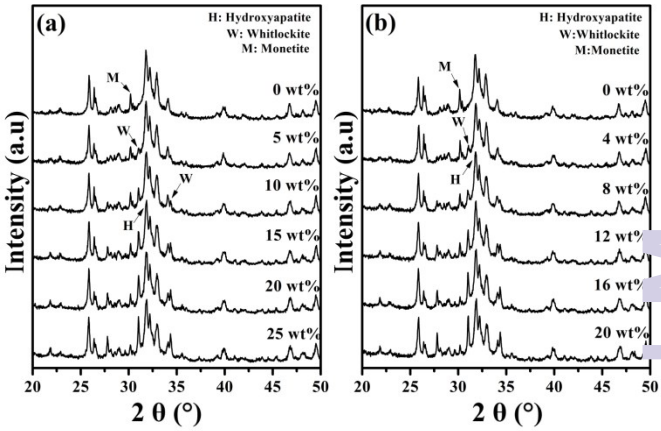

Figure 2. XRD patterns of CPC containing with different content of PLGA/Lec microspheres and β-TCP granules. (a) CPC containing 10 wt% PLGA microspheres and different content of β-TCP granules (0, 5, 10, 15, 20, and 25 wt%); (b) CPC concretes with 20 wt% PLGA microspheres and different content of β-TCP granules (0, 4, 8, 12, 16 and 20 wt%).

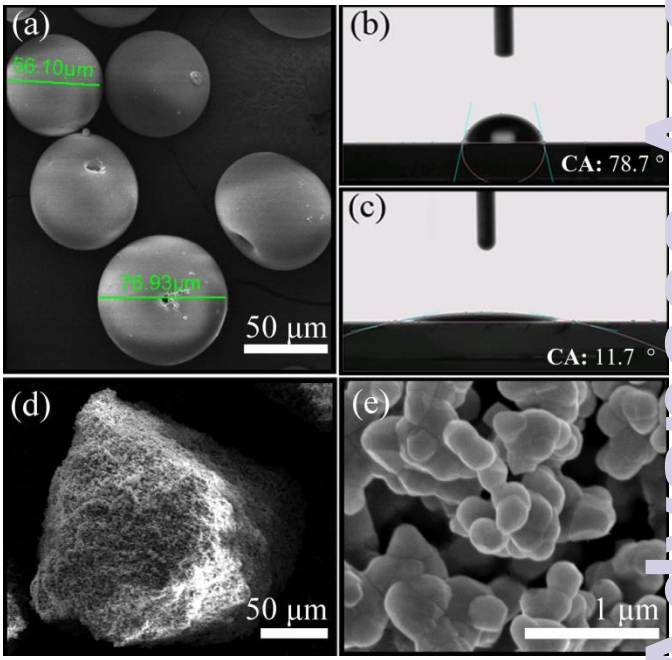

Figure 3. SEM photo of PLGA/Lec microspheres (a); the contact angle of PLGA membranes containing 10 wt% lecithin (b) and without lecithin (c); The morphology of the isostatically pressed and sintered β-TCP granules (d); the microstructure of β-TCP granules with higher magnification (e).

Images of the morphology of PLGA/Lec microspheres and sintered β-TCP granules are given in Fig. 3. The microsphere

were smooth and globose with a diameter of larger than 50 μm [Fig. 3(a)]. The contact angles of PLGA membranes with and without lecithin were respectively 11.7° and 78.7° [Fig. 3(b, c)], demonstrating the incorporation of lecithin greatly improved the hydrophilicity of PLGA microspheres. The sintered β-TCP granule sized ranging in 106–212 μm showed a very rough morphology [Fig. 3(d)] with numerous submicropores [Fig. 3(e)]. The micrographs of fracture surface of CPC1 are presented in Fig. 4. As shown in Fig. 4(a), the sample displayed a typical coarse surface with some microspheres and granules homogeneously fractured and well embedded in the CPC matrix, indicating that PLGA/Lec microspheres and β-TCP granules could participate in the applied forces. Importantly, as showed by the black arrows, the crack occurring in the matrix hindered by the microsphere and stopped by the granule, indicating the good combination of PLGA/Lec microspheres and β-TCP granules with the CPC matrix. Higher magnification SEM photos of the sectional interface between the high-strength β-TCP granule and CPC matrix can be seen in Fig. 4(c, d). Along the interface, hydroxyapatite crystallite appeared in the surface [Fig. 4(d)] of the rough granule, revealing the intimate contact and bonding between the CPC matrix and the granules. Furthermore, along the interface of the PLGA/Lec microsphere and the CPC matrix [Fig. 4(e, f)], thinner HA crystallite appeared in the surface of the microsphere, indicating the fine contact and bonding of the CPC matrix with PLGA/ Lec microspheres.

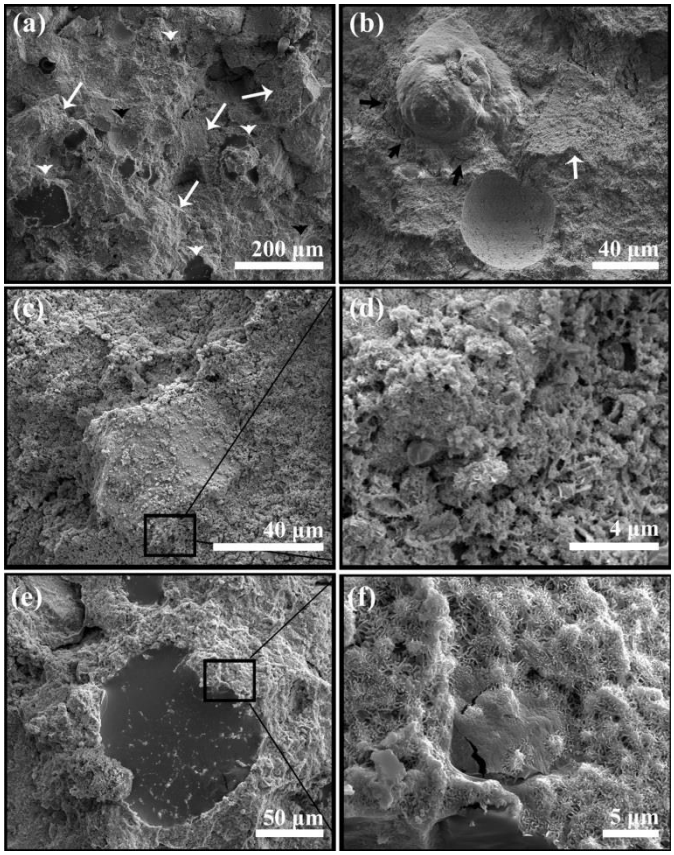

Figure 4. SEM photo of cross section of CPC1 (a); fracture surfaces of CPC1 with cracks in the matrix (b); fracture surfaces of CPC1 showing good bonding with β-TCP granules (c) and the higher magnification images of the cross-section of the CPC-β-TCP granules interface (d); fracture surfaces of CPC1 showing good bonding with PLGA/Lec microspheres (e) and the higher magnification images of the cross-section of the CPC-PLGA/Lec microspheres interface (f). White arrows: PLGA/Lec microspheres; black arrows: crack; white heads: β-TCP granules; black heads: pores left by PLGA/Lec microspheres.

In vitro degradation

Fig. 5 shows the development of the weight loss [Fig. 5(a)] of CPC0, CPC1 and CPC2 immersed in PBS solution and the pH value of PBS solution [Fig. 5(b)]. During the second week, the pH value of all samples decreased below 7, resulting from the dissolution of residual DCPA after hydration. After 4 weeks, CPC1 presented accelerating degradation and a distinct drop in pH when compared with those of other two samples. This can be explained as the erosion of microspheres acidified the solution and increased the mass loss as well as the dissolution rate of CPC. After immersion in PBS for 8 weeks, the total weight loss of CPC1 reached 23.53 ± 1.13 wt%, significantly higher than that of the control, which was 17.73 ± 0.47 wt%.

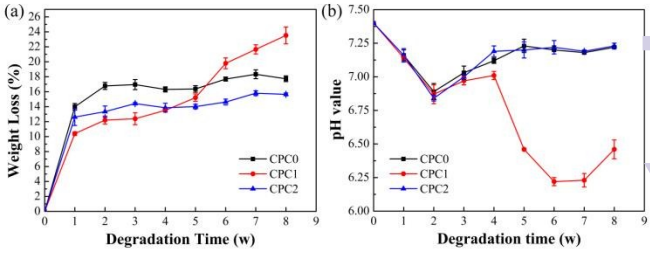

Figure 5. The weight loss of degrading CPCs (a) and pH value of the soaking solution (b) in each week.

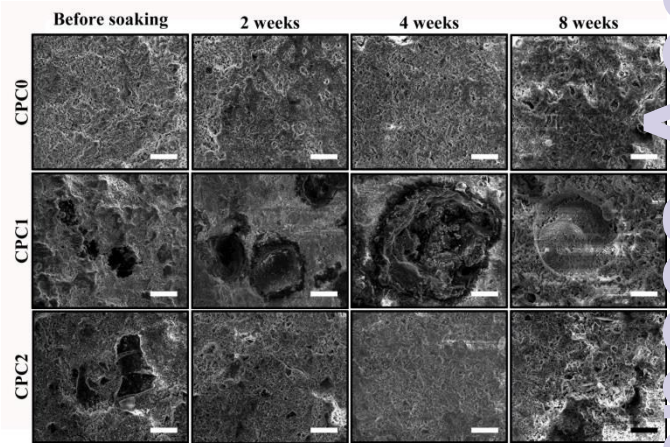

Figure 6. SEM photos of morphologies of CPC0, CPC1 and CPC2 after immersion in phosphate buffer solution after different weeks. All the column bars are equal to 50 μm.

The morphology of the cement composites after soaking in PBS for different time is presented in Fig. 6. Due to dissolution of the CPC matrix, all samples with compact structure turned int being porous with the prolongation of immersion time. Before immersed in PBS, the PLGA/Lec microspheres were intact and tightly embedded in the CPC matrix. After immersion in PBS fo 2 weeks, surface erosion of PLGA/Lec microspheres occurre After immersion in PBS for 4 weeks, bulk erosion of the microspheres became visible in the CPC matrix. In the samp immersed in PBS for 8 weeks, there was an overall macropo with a diameter greater than 100 μm generated in situ by th

degradation of PLGA/Lec microspheres, indicating the microspheres in CPC1 were almost completely degraded. As shown in Table 1, during the degradation, the porosity of all samples generally increased over time. In addition, there was mineralization occurring on the surface of degraded PLGA/Lec microspheres during the soaking (Fig. 7).

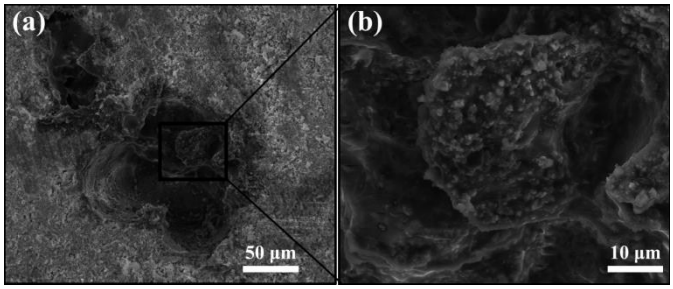

Figure 7. SEM images of morphology of CPC after immersion in phosphate buffer solution for 6 weeks (a) and the surface morphology of the degraded PLGA/Lec microspheres with higher magnification (b).

Table 1. Porosity of CPCs after soaking in PBS for different time

Sample	Porosity (%)				
	0 w	2 w	4 w	6 w	8 w
CPC0	37.38 ± 0.04	40.93 ± 0.06	48.32 ± 0.83	62.25 ± 0.17	64.61 ± 0.18
CPC1	39.11 ± 0.21	47.08 ± 0.12	42.53 ± 0.21	65.99 ± 1.39	66.03 ± 0.31
CPC2	41.01 ± 0.07	41.27 ± 0.39	47.66 ± 2.5	58.70 ± 0.35	64.55 ± 0.15

In vitro biocompatibility

The quantitative analysis of proliferation and ALP activity of mBMSCs seeded on the cements are shown in Fig. 8. The cell number on all samples clearly increased with prolongation of time [Fig. 8(a)]. On day 1, cell attachment on all samples was similar. On day 3, cell numbers increased on all samples compared to day 1. And the cell numbers on CPC1 and CPC2 were significantly higher than that on CPC0, indicating the superior compatibility of β-TCP granules. On day 7, the proliferation of mBMSCs showed the similar trend as that on day 3. But the greatest amount of cell proliferation was detected on CPC1 with a marked difference compared with that on CPC0 and CPC1, demonstrating that PLGA/Lec microspheres improved the cellular affinity of CPC. These were supported by the results of Calcein-AM staining (Fig. 9). It indicated that the cell number was notably higher on day 3 compared with day 1. Fig. 9 also shows the SEM photos of mBMSCs attached onto the surface of CPC0, CPC1 and CPC2 after 1 day of culture. The cells displayed elongated and flattened morphology on all samples. ALP activity is a marker of early osteoblastic differentiation and commitment of stem cells towards the osteoblastic phenotype. Though the ALP activity of cells on CPC0 and CPC2 declined slightly in day 14, all samples clearly increased from day 7 to day 10 [Fig. 8(a)]. There was no significantly difference among the three samples on each time point. These together demonstrated the addition of PLGA/Lec microspheres and β-TCP granules improved the cell affinity of CPC.

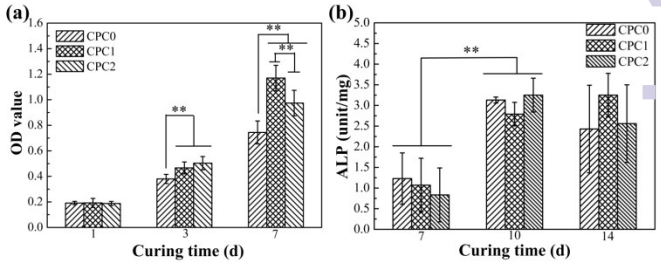

Figure 8. Increase in viable cell numbers (a) of mBMSCs cultured on CPCs by CCK-8 assay after 1, 3 and 7 days and ALP activity (b) of mBMSCs cultured on CPCs after 7, 10 and 14 days. Data are presented as mean ± standard deviation (n = 6). ** and * mean statistical significant difference between the marked samples, $p<0.01$ and $p<0.05$ respectively.

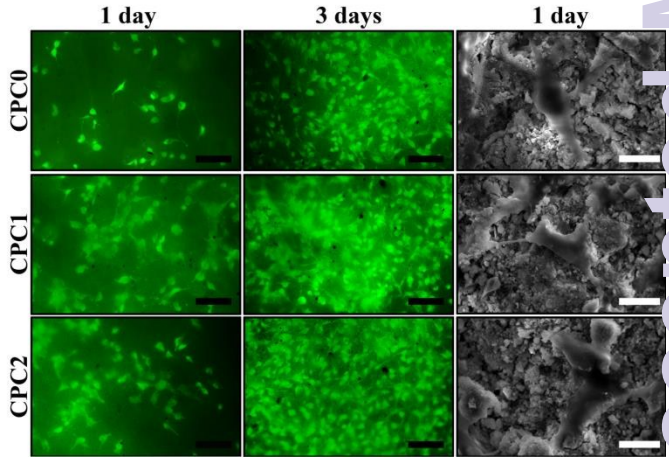

Figure 9. The fluorescence images and SEM photos of mBMSCs cultured on CPCs. The black and white column bars are equal to 100 μm and 10 μm, respectively.

Discussion

The objective of the current study is to investigate the influence of co-addition of PLGA/Lec microspheres and β-TCP granules on the properties of CPC. In this work, these two additives can help CPC achieve a relative balance between biodegradability and other physicochemical performances as well as the biocompatibility of CPC.

Increasing the porosity of CPC can facilitate bone tissue ingrowth and raise its degradation rate [28, 29]. With PLGA/Lec microspheres incorporated into CPC, the degradation rate and porosity of CPC were improved. Moreover, the macropores created by the degradation of PLGA/Lec microspheres were appropriate for cell migration and bone tissue ingrowth. During the degradation, thin HA crystallite [Fig. 4(f)] and rod-like apatite formation (Fig.7) was seen on the surface of PLGA/Lec microspheres, which could be explained as that the lecithin improved the hydrophilicity of the microspheres, resulting in helping the mineralization [30] on the surface of microspheres. Although β-TCP granules had little effect on degradation of CPC, β-TCP granules could improve the resorption rate of CPC after PLGA/Lec microspheres completely degraded as β-TCP was more resorptive than HA [31].

The simplified schematic diagram of the degradation process of CPC combined with PLGA/Lec microspheres and β-TCP granules

is shown in Fig. 10. When the solution permeated into the CPC matrix through the inner pores in CPC, the soluble salt dissolved and the PLGA/Lec microspheres started to disintegrate into small pieces, resulting in increasing porosity. The porosity of CPC1 decreased on week 4, which may be explained as the disintegrated pieces of microspheres blocked some pores. This could also explained why porosities of CPCs in Table 2 were almost the same. Besides, during the degradation, calcium ions and phosphate group in PBS solution slowly co-precipitated on the surface of the matrix, especially on PLGA/Lec microspheres, which might occupied some pores. Futhermore, the microspheres showed firmed bonding with the matrix during the degradation. After the microspheres degraded almost completely in week 8, CPC with high porosity and macropores was obtained and the retained bioresorbable β-TCP granules can help maintain the strength of CPC and keep improving the resorption rate of CPC.

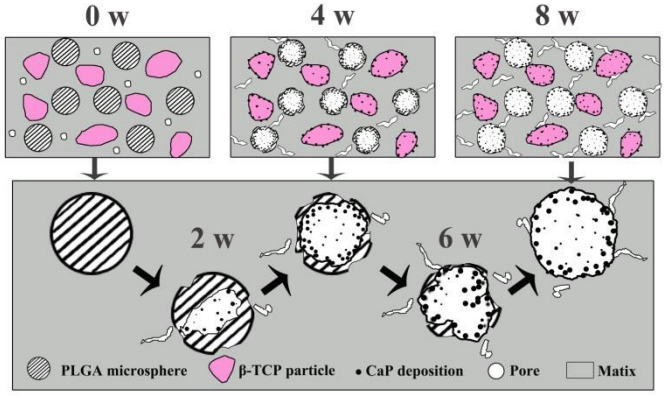

Figure 10. The schematic diagram of PLGA/Lec microspheres degrading in CPC.

However, the high macroporosity would render the CPC implants mechanically weaker, thereby raising the possibility of failure and limiting its wide application. With the incorporation of 10 wt% PLGA microspheres, the compressive strength of CPC decreased slightly from 30.56 ± 2.17 MPa to 26.86 ± 1.42 MPa, but increased to 27.83 ± 1.33 MPa with further addition of 10 wt% β-TCP granules. The PLGA/Lec microspheres and β-TCP granules embedded in CPC matrix could bear the applied stresses and resist further opening and propagation of cracks via plastic deformation of PLGA/Lec microspheres and crack pinning by β-TCP granules. The fractured composites still maintained the CPC-PLGA and CPC-β-TCP bonds, indicating the strong CPC-PLGA and CPC-β-TCP bonding.

Owing to the porous structure of the β-TCP granules, during the hydration, small fraction of liquid could infiltrate into the inner of granules, thereby the actual L/P ratio of the CPC matrix drop slightly, which may be one of the reasons for the decrease in the setting time [Fig.1 (b)]. When the total mass fraction of PLGA/Lec microspheres and β-TCP granules was increased to a certain value, the setting time prolonged because the amount of CPC was less in the composites and therefore the actual L/P ratio relatively increased.

The cell culture studies suggest that our cement containing PLGA/Lec microspheres and β-TCP granules is biocompatible. For clinical application of biomaterials, both physical and chemical properties of the surface of materials were important for surface bioactivity. The rough surface is helpful to promote numerous protein interactions and thereby aid cells adhesion [32]. In the present work, a rough surface structure was formed by introducing coarse β-TCP granules and PLGA/Lec microspheres in CPC matrix, which would definitely contribute to the good surface-cell interactions. Surface chemical properties of biomaterials also play a very important role in good surface bioactivity. Lecithin can supply PLGA microspheres with cell recognition sites. The good interaction of lecithin with the integrins on the surface of cells [33] contributed to better cellular proliferation of CPC1 compared with that of the control. After being incubated 1 day, mBMSCs were able to adhere, spread and remain viable on CPC0, CPC1 and CPC2 while observed by either fluorescence microscopy or SEM (Fig. 9). The results have also shown that both the cell numbers on the CPC with β-TCP granules and PLGA/Lec microspheres were higher than that on the control.

ALP activity was not significantly different between CPC incorporated with PLGA/Lec microspheres and/or β-TCP granules and CPC alone. In addition, the bioactivity of the cement composites can be further enhanced by incorporating other bioactive cytokines, such as bone morphogenetic protein 2 [34], or doping some bioactive ions, such as Zn2+ [35]. The PLGA/Lec microspheres can function as a carrier for the controlled release of biological cues to guide cell response and the β-TCP granules or the PCCP can be doped with bioactive ions to stimulate the cell differentiation. Taken together, the results suggest that CPC with PLGA/Lec microspheres and β-TCP granules was expected to promote osteogenesis due to their stimulatory effects on osteoblastic cells proliferation and bone formation [36], as well as balance the degradability and mechanical properties of the grafts.

In addition, if using only PLGA/Lec microspheres, improvement of in vitro degradation and biocompatibility of CPC may be dose dependent, which will be conducted in further experiment.

Experimental

Materials preparation

The CPC powders used in this work were a mixture of partially crystallized calcium phosphate (PCCP) and anhydrous dicalcium phosphate (DCPA, $CaHPO_4$) at a mass ratio of 1:1 as developed in our previous work [37]. PLGA (MW: 30 000, 50:50) microspheres containing 10 wt% lecithin were obtained through an O/W emulsion method and vacuum drying. The microspheres were sieved in the range of 53-212 μm and stored at -20 °C. Beta-TCP particles were prepared via a solid-phase reaction. Calcium carbonate (CC, $CaCO_3$) and DCPD were mixed at a molar ratio of 1:2 with deionized water and milled into slurry, then dried at 80 °C in an oven for 24 h and finally pressed isostatically at 200 MPa for 2 min into compacted cakes. The as-prepared cakes were crushed through roll milling and oval-like granules with a diameter ranging in 106–212 μm were sieved. The obtained particles were calcined in a furnace at 900 °C for 2 h to get β-TCP granules.

The CPC powder was uniformly mixed with PLGA/Lec microspheres and β-TCP granules at different proportions as shown in Table 2. CPC alone samples (CPC0) were considered as control. All samples were obtained by mixing powders with deionized water under a liquid to powder (L/P) ratio of 0.4 mL/g at 24–26 °C.

Table 2. Content of PLGA/Lec microspheres and of β-TCP granules in CPC*

Sample	PLGA/Lec microspheres (wt%)	β-TCP granules (wt%)	CPC matrix
1	0	0	100
2	10	0	90
3		5	85
4		10	80
5		15	75
6		20	70
7	20	0	80
8		4	76
9		8	72
10		12	68
11		16	64

* The total content of CPC including the matrix was defined as 100 wt% in the table. It means that when the content of PLGA/Lec microspheres and β-TCP granules were assumed as 10 wt% and 10 wt%, that of the matrix would be 80 wt%.

Physicochemical and mechanical characterization of cements

The cement paste was pouring into steel molds (Φ6 × 12 mm) and loaded with 700 kPa pressure for 5s in order to remove the bubbles. Afterwards, the samples were demoulded, then cured under 37 °C and 95% humidity for three days. The ends of hydrated columns were polished for mechanical test. The compressive strength of the columns was measured by a universal material testing machine (Instron 5567, Instron, USA) at a crosshead speed of 0.5 mm/min. Each measurement was repeated six times and the average value was calculated.

The setting time of CPC was measured according to ASTM CPC191-03 with a Vicat apparatus which has a movable rod of 300 ± 0.5 g in mass and a removable needle of 1 ± 0.05 mm diameter, fixed at the end of the rod. The Vicat needle was carefully lowered vertically onto the surface of the newly shaped cement samples and kept there for 5 s, applying an equivalent static pressure of 3.7 MPa. The indentation was repeated at intervals of 30 s until the cement was hardened. Initial setting occurred when a 1 mm needle penetrated 25 mm into cement paste and final setting occurred when there was no visible penetration. The setting time of the cement was measured in a humidity chamber at 37 °C and 97% humidity. Each measurement was also performed six times and the average value was calculated.

The porosity of CPC was measured by the Archimedes method. Briefly, the hydrated CPC column was initially weighted as W0, then placed into a pycnometer which was weighted W1 when full of alcohol and vacuumized until no more obvious bubbling from the columns was observed. Subsequently, the pycnometer with columns was weighted W2 with alcohol filling up again. In the end, the samples were taken out, wiped by filter papers steeping with alcohol and weighted W3.The porosity was evaluated by the equation 1:

$$P (\%) = 100 * (W3 - W0) / (W1 + W3 - W2) \quad (1).$$

The hydrated CPC samples were milled into powder and analyzed using a X-ray diffractometer (XRD; X'Pert PRO, PANalytical, Netherlands) with CuKα radiation (λ = 1.5418Å). The data were collected for 2θ from 10° to 60° with a step size of 0.016° and a scan step time of 10 s. The surface and cross-section of the samples with gold-coating were observed by a scanning electron microscope (SEM; Nova NanoSEM 430, FEI, USA).

In vitro degradation test

The samples of CPC alone (CPC0), CPC containing 10 wt% PLGA/Lec microspheres and 10 wt% β-TCP granules (CPC1), and CPC with 11.1 wt% β-TCP granules (CPC2) were first weighed Ws. Then they were immersed in phosphate buffer solution (PBS, pH 7.4) at a liquid to initially weighed solid ratio of 50 ml/g. Finally they were incubated at 37 °C in a shaking water bath (770R, APlus, America) at 60 rpm for up to 8 weeks. The immersion solution was refreshed every 1 week. For each datum point, the samples were taken out and weighted as Wd after drying. The weight loss (WL) of samples was calculated by the equation 2:

$$WL (\%) = 100 * (Ws - Wd) / Wd \quad (2).$$

The morphology and microstructure of the samples after incubation were observed using SEM. The pH value of the immersion solution was measured by an acidimeter (PB-10, Sartorious, Germany) immediately after the samples were taken out. All samples were run in triplicate and data points were shown as mean ± standard deviation (SD).

In vitro biocompatibility study

The CPC0, CPC1, CPC2 samples were subjected to biocompatibility assessment under in vitro condition. Generally, the hydrated cements were sterilized by gamma irradiation. Mouse bone marrow mesenchymal stem cells (mBMSCs; ATCC, Cat. No. CRL-12424) were used to carry out the cell experiment. The culture medium for mBMSCs curing contained 90% high-glucose Dulbecco's Modified Eagle's Medium (H-DMEM; Gibico, No. 11965-092) and 10% fetal bovine serum (FBS; Hyclone, Cat. No. NWJ0473). The medium for osteogenic induction was further supplemented with 10 mM sodium β-glycerophosphate, 0.1 μM dexamethasone and 50 mg/L vitamin C. All samples were placed in a 48-well plate (Corning Incorporated, USA) and immersed in culture medium 12 h prior for cell culture. Then, 1×10^4 cells and 1×10^5 were respectively seeded on each sample in 500 μL medium for proliferation test and alkaline phosphatase (ALP) activity measurement. Then they were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Culture media was changed every other day, and samples were removed at a planned time point for different evaluation.

The viability of cells was quantified by Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay at three time points (day 1, 3 and 7). In detail, the samples seeded with cells were moved to a new 48-well plate and 250 μL medium with 10% CCK-8 was added to each well. After incubating for 1 h, 100 μL CCK-8 solution was moved to each well of a 96-well plate for the optical density measurement by a spectral scanning multimode reader (Thermo 3001; Thermo Scientific, USA) at a wavelength of 450 nm. After being cured 1 day and 3 days, mBMSCs/materials collected were rinsed twice with PBS and then stained with Viability Assay Kit for Animal Live Cells (Calcein-AM; Biotium, USA). The system was allowed to incubate at room temperature for 45 min, then washed with PBS for 3 times and finally observed by the fluorescence microscope (40FL Axioskop; Zeiss, Germany).

The morphology of adhered cells after 1 day was observed by SEM. mBMSCs/materials collected were first rinsed twice with PBS, then fixed by 2.5% glutaraldehyde solution for 4 h and finally dehydrated with graded ethanol.

ALP activity was calculated from a standard curve after normalizing to the total protein content at three time points (day 7, 10 and 14). Briefly, 200 µL p-Nitrophenyl Phosphate Disodium Salt (PNPP) solution was added to each well with cell lysate and incubated at room temperature for 15min for the ALP activity detection. This was followed by adding 100 µL of 1 M NaOH solution to terminate the reaction of p-nitrophenol conversion to p-nitrophenylate. The total protein was measured by incubating the cell lysate with 160 µL BCATM protein assay kit (Thermo Scientific, USA) for 15 min. The spectral scanning multimode reader was used to determine the activity of ALP at the wavelength of 405 nm and the concentration of total protein at the wavelength of 562 nm. All samples were run in sextuplicate, and data points were shown as mean ± SD.

Statistical analysis

Quantitative data are presented as mean ± standard deviation and statistical analysis was performed using a one-way analysis of variance (one-way ANOVA). A comparison between two means was made using the Tukey's test, with statistical significance set at $p < 0.05$.

Conclusions

In the present work, we demonstrated that PLGA microspheres modified by lecithin promoted the cell affinity and improved the degradation performance of calcium phosphate cement along with β-TCP granules. With the co-incorporation of PLGA/Lec microspheres and β-TCP granules, the mechanical strength and setting time of CPC were appropriate for clinical applications. Moreover, the macropores created by the relatively quick degradation of PLGA/Lec microspheres were suitable for cell migration and potential for bone tissue ingrowth, which would contribute to faster repair of bone defects and the complete replacement of CPC by newly formed bone. Although β-TCP granules showed little contribution to the degradation to CPC in vitro, they had the potential to regulate the rate of CPC resorption and new bone formation in vivo after PLGA/Lec microspheres completely degraded. Future research will focus on transferring and evaluating these findings to the in vivo situation.

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