Article ID: 1000-324X(2023)07-0830-09 **DOI:** 10.15541/jim20220662

In vitro Study of Biphasic Calcium Magnesium Phosphate Microspheres for Angiogenesis and Bone Formation

WU Wei[1, 2], BAKHET Shahd[2], ASANTE Naomi Addai[2], KAREEM Shefiu[2], KOMBO Omar Ramadhan[3], LI Binbin[2], DAI Honglian[1, 2]

(1. Shenzhen Institute of Wuhan University of Technology, Shenzhen 518000, China; 2. State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Biomedical Materials and Engineering Research Center of Hubei Province, Wuhan University of Technology, Wuhan 430070, China; 3. Department of Medical Science and Technology, Mbeya University of Science and Technology, Mbeya, Tanzania)

Abstract: Beta tricalcium phosphate (β-TCP) ceramic substituted materials have attracted a large amount of attention in the last decades because of their chemical similarity with bone inorganic components, good biocompatibility, and osteoconductivity. Such materials can be used for bone replacement and bone formation in various forms, such as nanoparticles, scaffolds and microspheres. In this study, five different microsphere materials of tricalcium phosphate/trimagnesium phosphate (TMP) (TCP, 25% TMP, 50% TMP, 75% TMP, and TMP) composites were prepared and characterized. With the increase of TMP content in the composite microspheres, the cumulative concentration of Mg^{2+} and Ca^{2+} released from the microspheres increased, indicating that TMP can regulate the degradation rate of the composite microspheres. The osteoblast precursor cell line (MC3T3-E1 cells) and human umbilical vein endothelial cells (HUVECs) were used as models to evaluate the biocompatibility, angiogenesis and osteogenesis of the composite microspheres. The results showed that compared with TCP, TMP and 75% TMP group, 25% TMP and 50% TMP composite microspheres had better cell compatibility and had a certain proliferative effect on HUVECs. Therefore, composite microspheres of 25% TMP and 50% TMP have more significant positive effects on angiogenesis and osteogenesis.

Key words: β-TCP; TMP; bone defect; ceramics; microsphere; bone tissue engineering

The bone provides body support, facilitates movement, protects vital organs, and transmits sound inside the ear[1]. It's a composite connective tissue of organic and inorganic phases. Its inorganic phase is close to hydroxyapatite, and the organic phase comprises type I collagen fibres, water and other essential proteins. Bone defect is usually caused by high-energy trauma, bone tumors, infection, or congenital deformity[2]. In orthopedic clinic, biological and synthetic bone grafts are usually used to repair bone defect. These grafts are categorized into autografts, allografts, and xenografts[3-4]. Current clinical challenge of using autografts and allografts in the regeneration of large bone defects has inspired the development of bone tissue engineering[1,5-10]. At present, synthetic bone grafts are mainly made of calcium phosphate ceramics and widely used for bone regeneration in dentistry and orthopedics. Calcium phosphate ceramics have excellent biocompatibility, osseointegration and osteoconduction[11-12]. Among them, tricalcium phosphate (TCP), especially β-tricalcium phosphate (β-TCP, β-$Ca_3(PO_4)_2$), has constantly been explored and applied in bone tissue regeneration for excellent biodegradability[13-15]. Nevertheless, pure β-TCP ceramics have uncontrolled degradation rate and no pro-angiogenesis effect, which is not conducive to tissue regeneration at the defective site.

Received date: 2022-11-05; **Revised date:** 2022-12-18; **Published online:** 2023-03-20
Foundation item: National Key Research and Development Program of China (2022YFB4601402); National Natural Science Foundation of China (32201109); Guangdong Basic and Applied Basic Research Foundation (2021A1515110557); Foshan Xianhu Laboratory of the Advanced Energy Science and Technology Guangdong Laboratory (XHT2020-008)
Biography: WU Wei (1998–), male, Master. E-mail: 2625276216@qq.com
吴 未(1998–), 女, 硕士. E-mail: 2625276216@qq.com
Corresponding author: DAI Honglian, professor. E-mail: daihonglian@whut.edu.cn
戴红莲, 教授. E-mail: daihonglian@whut.edu.cn

Partially substituting or doping ions in the β-TCP can improve its biological properties[16-26]. Magnesium ions have received much attention from researchers due to the unique biological properties. There are growing evidences that magnesium ions, released from magnesium-containing bone repair materials after implantation, can promote bone tissue regeneration in areas of bone defects and accelerate healing of bone injuries[27]. In addition, magnesium enhances the expression of vascular endothelial growth factors, which plays a central role in vascular development. Therefore, there is a non-negligible role for magnesium in promoting angiogenesis. It is strengthened because magnesium can directly promote blood vessel growth under ischemic conditions[28]. Numerous studies have shown that bone repair materials containing magnesium promote both osteogenesis and angiogenesis[29-31]. However, these effects of magnesium ions are strongly concentration dependent. Moderate amounts of magnesium ions promote the mineralization of bone marrow mesenchymal stem cells (BMSCs), while excess magnesium ions inhibit the mineralization[32]. Therefore, the right amount of magnesium ion release for bone implant materials is crucial for bone tissue regeneration and functional reconstruction. Furthermore, previous studies have shown that introducing magnesium into an existing biocompatible matrix material can further promote tissue regeneration in the defect[33]. The combination of β-TCP with magnesium matrix materials can regulate the degradation and further enhance the ability of angiogenesis[30-31].

Another critical factor to consider for bone implant materials is the shape of the material. Compared to powder materials and block ceramics, microspheres with good fluidity are less prone to agglomeration, and have a point contact between microsphere particles. When spherical particles fill the bone pore cavity, the spaces between their particles can constitute interconnected macropores, conducive to the growth of cells, bone tissue and blood vessels[34-35]. Based on the above, we think different dopant concentrations of trimagnesium phosphate (TMP) in TCP affects angiogenesis and bone regeneration. The pore structure on the surface of the microspheres is conducive to the growth of blood vessels so as to provide oxygen and nutrition for bone regeneration, further promoting tissue regeneration in the area of the bone defect.

Therefore, we prepared a biphasic composite microsphere composed of TCP and TMP, in which the content of TMP in TCP increased from 0 to 100%, to explore cytocompatibility, osteogenic differentiation, mineralization and angiogenesis of MC3T3-E1 and HUVECs.

1　Materials and methods

1.1　Preparation of TCP with different amount TMP

Gelatin/β-TCP/TMP microspheres were prepared by water-in-oil emulsification and cross-linking method. Paraffin was the oil phase, Span 80 the emulsifier, and 1-ethyl-3-(3-dimethylamino) propyl carbodiimide, hydrochloride (EDC) the cross-linking agent. In a typical preparation, 50 mL of paraffin and 5 mL of Span 80 were mixed at 50 ℃ and used as the oil phase for the further use 25% (in mass) gelatin solution was prepared and mixed uniformly at 50 ℃ with different compositions (TCP, 25% TMP, 50% TMP, 75% TMP, and TMP) of β-TCP/TMP powders and mixed homogeneously to obtain an aqueous phase used for further processing. The aqueous phase was added dropwise to the oil phase with constant stirring. When the temperature of the above system dropped below 20 ℃, an amount of EDC binder was added and stirred for 30 min. The prepared microspheres were washed with isopropanol, filtered, and dried at room temperature. The dried gelatin/β-TCP/TMP microspheres were placed in a crucible, and calcined in a muffle furnace to remove the gelatin to obtain β-TCP/TMP microspheres.

1.2　Characterization techniques

1.2.1　X-ray powder diffraction

The chemical composition and crystal structures of the samples were carried out using a Bruker D8 Advance X-ray diffractometer (Germany) equipped with a 1.8 kW CuKα ceramic X-ray tube, operating at 40 kV and 40 mA. The data analysis was performed using χ'Pert Highscore Plus software from PANalytical.

1.2.2　Scanning electron microscopy

Microstructures were detected using a JEOL-7100F scanning electron microscope (SEM, JSM-6701F, JOEL Co., Ltd., Japan). The SEM sample to be observed was deposited onto the SEM sample holder and sputtered with gold to enhance the electrical conductivity.

1.2.3　Thermo gravimetric analysis

Thermo gravimetric analysis of the samples was performed using a synchronous thermal analyzer (STA503, NETZSCH, Germany) with conditions of nitrogen as protective gas, heating rate of 10 ℃/min, and test temperature range from 25 ℃ to 1000 ℃.

1.3　Ionic release behaviors of the materials

The bidirectional microspheres were immersed in 0.05 mol/L Tris-HCl buffer solution (pH 7.4) with the mass ratio of sample to buffer solution at 0.2 g/g, and then placed in a constant temperature oscillator (37 ℃). The

supernatant was sampled and compensated to the same volume by fresh Tris-HCl solution on the 3rd, 7th and 14th day after soaking. The contents of Ca and Mg ions in the sampled solution were measured by ICP-OES (Prodigy 7).

1.4 *In vitro* cell culture experiments

The MC3T3-E1(Lot number: BNCC333989) and HUVECs (Lot number: BNCC342438) purchased from BNCC Company (China). Endothelial cell medium (ECM) and alpha-minimum essential medium (α-MEM) were purchased from HyClon Company (China). The prepared composite microspheres of different components were weighed 20 mg, respectively and placed in 1 mL culture medium (α-MEM for MC3T3-E1 and ECM for HUVECs), then placed in an incubator under constant temperature at 37 ℃ for 24 h. Later, the supernatant was filtered. Finally, the medium was stored in an environment condition of 4 ℃ for further use.

1.4.1 Cytotoxicity of the microspheres

The MC3T3-E1 and HUVECs (α-MEM for MC3T3-E1 and ECM for HUVECs) were seeded in different 24 well plates at the density of 3×10^4 cells/well and incubated at 37 ℃. After 24 h, the medium were replaced by different individual microsphere samples with the prepared amount of TCP, 25% TMP, 50% TMP, 75% TMP, and TMP extract separately and incubated again at 37 ℃ (n=3 for each group, including the control). After 1, 3 and 5 d, 100 μL of the medium from each well plate was transferred into a 96-well plate, and 10 μL of CCK-8 (biosharp life science) solution was added and incubated for 2 h. The optical density (OD) values were measured on the Thermo Lab Systems microplate reader (MK3, USA) at the wavelength of 450 nm.

1.4.2 Alkaline phosphatase activity

The MC3T3-E1 cells with their α-MEM were seeded in 24 well plates at the density of 3×10^4 cells/well and incubated at 37 ℃. After 24 h, the medium was replaced by osteoblasts-inducing medium and incubated at 37 ℃, with the medium being changed every two days. Consequently, an Alkaline phosphatase (ALP) test was carried out on the 7th and 14th day. The cells were fixed with 4% paraformaldehyde for 30 min and then washed with phosphate buffered saline (PBS). Later, the cells were stained with an ALP staining kit (Beyotime Biotechnology) for 10–15 min and then observed by a fluorescence microscope.

1.4.3 Alizarin red S staining

Alizarin red S staining (ARS, Sigma-Aldrich, Germany) staining was used to indicate bone-nodule formation. The test was carried out on the 14th day using the same fixation method mentioned at the section 1.4.2. The fixed sample was stained with an ARS staining kit (Beyotime Biotechnology) for 1–2 h, then washed with PBS. A fluorescence microscope was used to examine the samples. Finally, different images were also captured from the fluorescence microscope.

1.4.4 Angiogenic effect assay

The MC3T3-E1 and HUVECs with their normal cell culture media (α-MEM for MC3T3-E1 and ECM for HUVECs) were seeded in different 6 well plates at the density of 3×10^4 cells/well and incubated at 37 ℃. After 24 h, the medium was replaced by the osteoblasts-inducing medium with different amounts of prepared TMP/TCP microspheres, extracted separately, and incubated again at 37 ℃ (n=3 for each group, including the control). Total mRNA was extracted from HUVECs seeded in the well plates for 1 and 3 day of culture. Moreover, MC3T3-E1 cells were cultured for 3 and 7 d. The well plates were washed with PBS, and then 1 mL of RNAiso Plus was added. Then the standard testing centre protocol was used to carry out the procedure. Table 1 describes the primers used in PCR for gene expression analysis.

1.5 Statistical analysis

Results were analyzed with OriginLab Pro 2021 and the comparative studies of means were done by one-way analysis of variance (ANOVA) with the quantitative data expressed as the mean ± standard deviation (SD). A statistical significance was considered at $^*p < 0.05$.

2 Results

2.1 Characterization of β-TCP/TMP composite microspheres

2.1.1 Phase composition analysis

After preparation, the β-TCP/TMP composite microspheres of each group display roughly the same size (Fig. S1). Fig. 1 shows the X-ray diffraction patterns of the prepared β-TCP/TMP composite microspheres. The resu

Table 1 Primer sequences used in RT-qPCR

Gene	Primer sequence
VEGF	AGGAGTACCCCGACGAGATAGA
	CACATCTGCTGTGCTGTAGGAA
FGF	ACAGGAGCGACCAGCACATT
	TTGGTGTCTGCGAGCCGTAT
COL I	CACTGCAAGAACAGCGTAGC
	AAGTTCCGGTGTGACTCGTG
OPN	ACACTTTCACTCCAATCGTCCCTAC
	GGACTCCTTAGACTCACCGCTCTT

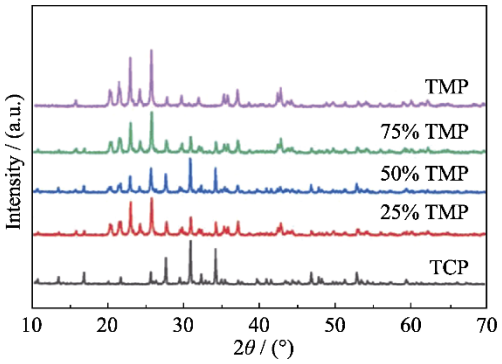

Fig. 1　XRD patterns of prepared TCP/TMP composite materials

lts revealed a slight shift and transformation in diffraction peaks as the number of TMP increases. Additionally, Fig. S2(a, b) in the supporting information shows the XRD patterns of the synthesized β-TCP and TMP with relatively sharp peaks, which indicate high crystalline phases of the starting materials β-TCP and TMP. The positions of the diffraction peaks are consistent with the PDF standards of two powders (11-0234, 09-0169 for TMP and β-TCP), respectively.

2.1.2　Surface structure morphology

SEM images (Fig. 2) show the surface structure morphology of the prepared composite microspheres TCP, 25% TMP, 50% TMP, 75% TMP, and TMP. Pure TCP microspheres (Fig. 2(a)), showed particulate morphology with particle size distribution of (0.342±0.099) μm. Pure TMP microspheres showed particulate morphology and size distribution of (0.933±0.615) μm (Fig. 2(e)). Besides, the intermediate TMP percentages showed mixtures of fine and coarse morphology, and increased large-coarse particles as TMP increases (Fig. 2(b-d)).

2.1.3　Thermo gravimetric property

Fig. 3 shows the TG/DTA/DTG curves of the prepared β-TCP/TMP microspheres prepared by water-in-oil emulsion cross-linking method. As can be observed from (Fig. 3(a, b, e)), the first weight loss of microspheres occurred between 0–100 ℃, which is attributed to the loss of physically adsorbed water. The second weight loss occurred between 300 and 400 ℃, which is attributed to the EDC decomposition and the beginning of the decomposition of some gelatin. There was a third weight loss in the pure TMP ceramic microspheres compared with other microspheres, mainly due to gelatin's high decomposition (Fig. 3(e)). The 50% TMP group and the 75% TMP group (Fig. 3(c, d)) had only one mass change process, and the weight loss were both higher than 40%. The experiment showed a complete absence of weight loss after 600 ℃, indicating a complete decomposition of the gelatin and EDC in the composite microspheres. The DTA curve also showed a solid exothermic peak between 450 and 500 ℃, which is attributed to the exothermic decomposition of gelatin. All results of DTA and DTG were consistent with those of TG.

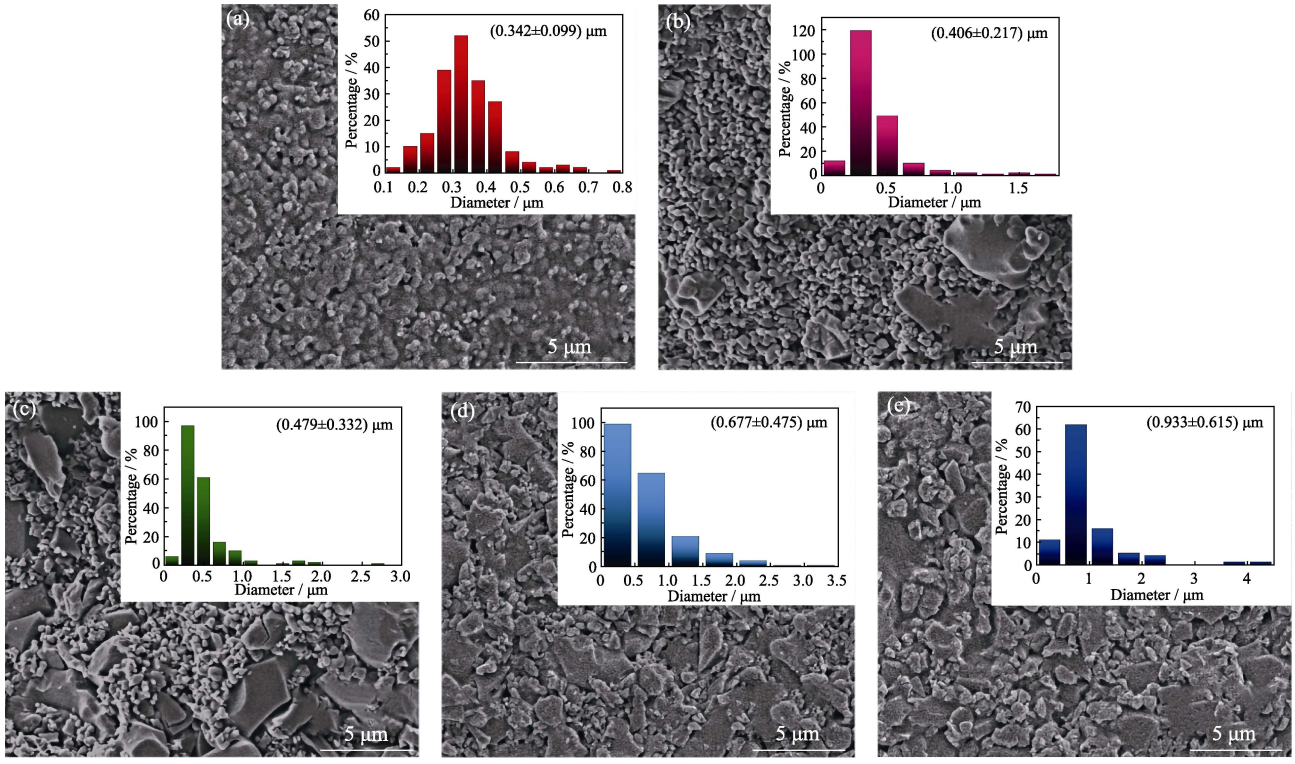

Fig. 2　SEM images of different microsphere composites with insets showing their corresponding particle size distributions
(a)TCP; (b) 25% TMP; (c) 50% TMP; (d) 75% TMP; (e) TMP

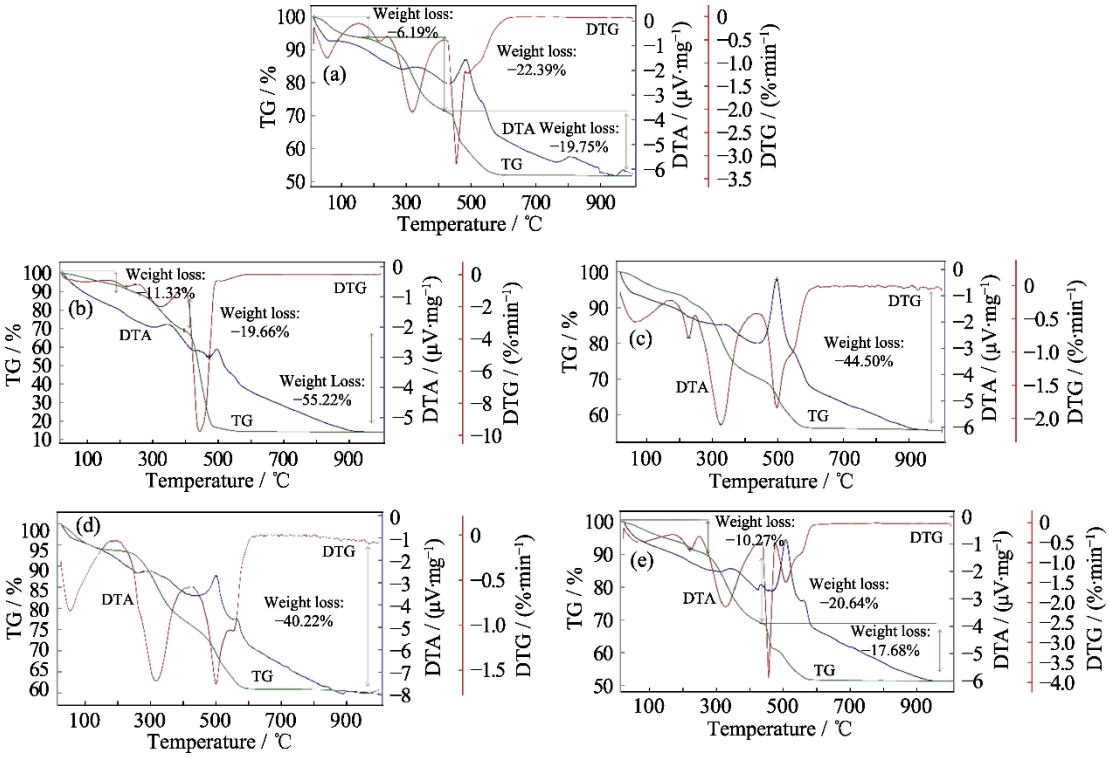

Fig. 3 TG/DTA/DTG curves of microspheres prepared by water in oil emulsion cross-linking method
(a) TCP; (b) 25%TMP; (c) 50%TMP; (d) 75%TMP; (e) TMP

2.2 Ionic release property

Fig. 4 showed the release behaviors of Ca^{2+} and Mg^{2+} from different microspheres. Higher cumulative concentration of released Mg^{2+} was detected when the microspheres initially contained a higher fraction of TMP. The tendency of the Ca^{2+} concentration was similar to that of the Mg^{2+}.

2.3 Cytocompatibility of the microspheres

CCK8 assay was used to evaluate cytocompatibility of the microspheres. Fig. 5(a) shows the effects of different microspheres concentration extracts on cell viability of MC3T3-E1. There is no significant difference in OD value between different treatment groups on the 1st day. However the TMP, 25% TMP, 50% TMP and 75% TMP groups showed successively enhanced OD value compared to the control and TCP groups on the 3rd day, with the increase of TMP content, indicated that TMP enhances cell viability in a dose-dependent manner. On the 5th day, TCP and 75% TMP groups showed lower OD value compared to the other groups, indicated that both TCP and excessive TMP can inhibit cell viability of MC3T3-E1. As for HUVECs in Fig 5(b), on the 3rd and 5th day, TMP, 25% TMP, 50% TMP showed enhanced OD value compared to the control group, while TCP and 75% TMP groups showed lower OD value compared to the other groups, indicating that both TCP and excessive TMP can inhibit cell viability of HUVECs. All these results demonstrated that TCP/TMP microspheres can enhance cell viability in a dose-dependent manner. Notably, 25% TMP and 50% TMP microspheres have higher ability to promote cell proliferation.

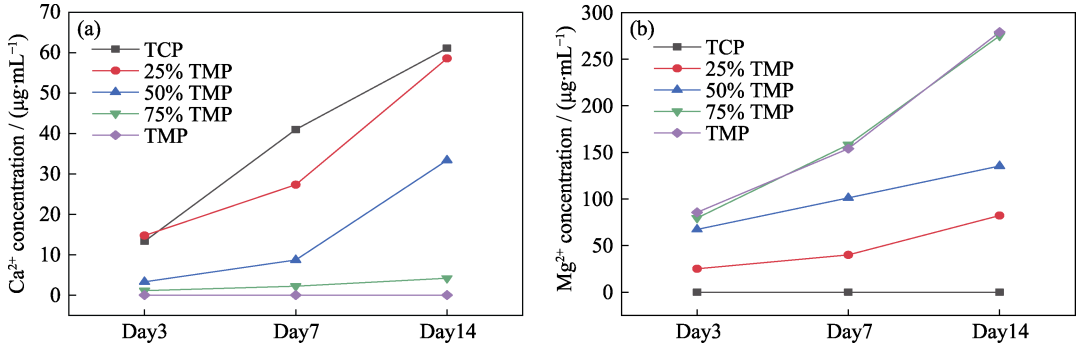

Fig. 4 Released $Ca^{2+}$and Mg^{2+} concentrations from materials immersed in tris-HCl solution

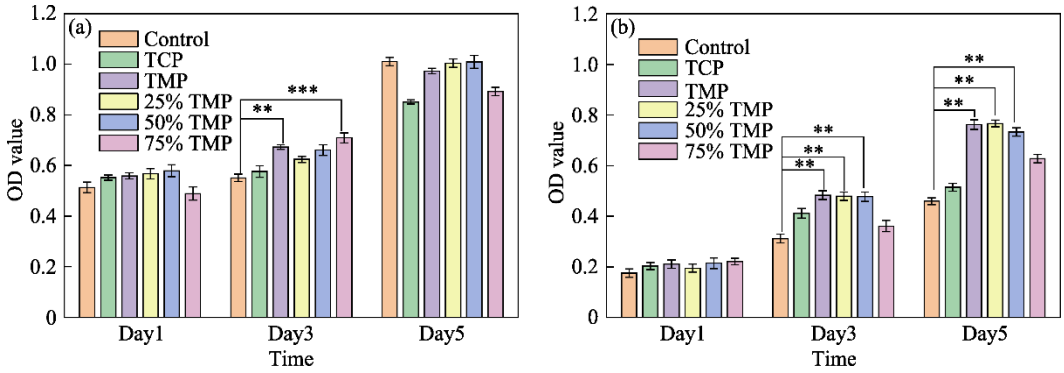

Fig. 5 Cell viabilities of (a) MC3T3-E1 and (b) HUVECs assessed by CCK-8 assay
(a) MC3T3-E1 and (b) HUVECs assayed on day 1, 3, 5 cultured with different microspheres concentration extracts
*: $p < 0.01$; **: $p < 0.005$; ***: $p < 0.0002$; Colorful figures are available on website

2.4 Osteogenic effects of the microspheres

ALP is the early signature protein for osteogenic differentiation. ALP activity assay was used to evaluate the osteogenic effects of the microspheres. The staining of MC3T3-E1 after being cultured with different microsphere concentration extracts has shown almost no difference in density for all groups (Fig. 6). Nevertheless, ALP staining in all material groups was stronger than those in the control on the 14th day and TCP group showed the highest ALP staining. ARS staining was used as indicator of bone-nodule formation. The ARS staining was performed after 14 d culture. As shown in (Fig. 6), there is more calcium deposition in the group of 25% TMP than the others.

2.5 Gene expression

2.5.1 Expression levels of VEGF and FGF

Expression levels of VEGF and FGF were determined by using RT-PCR after HUVECs cultured in different concentration extracts of different microspheres on 1st and 3rd day. There was no significant difference in the VEGF levels on the 1st day, which indicated that gene expression started after a few days of the cell culture. The VEGF levels of 50% TMP group were slightly higher on the 3rd day culture than the other groups (Fig. 7(a)). On the 3rd day, the FGF level of 100% TMP was slightly higher than the other groups(Fig. 7(b)). These results indicated that 25% TMP and 50% TMP of biphasic composite microspheres have greater potential to promote HUVECs angiogenesis.

2.5.2 Expression levels of collagen and osteopontin

Expression levels of collagen (COLI) and osteopontin (OPN) genes of MC3T3-E1 cells were determined quantitatively using RT-PCR after culture with the different concentration extracts on the 3rd and 7th day (Fig. 8). Although COLI expression levels on the 7th day was significantly lower, the expression levels were

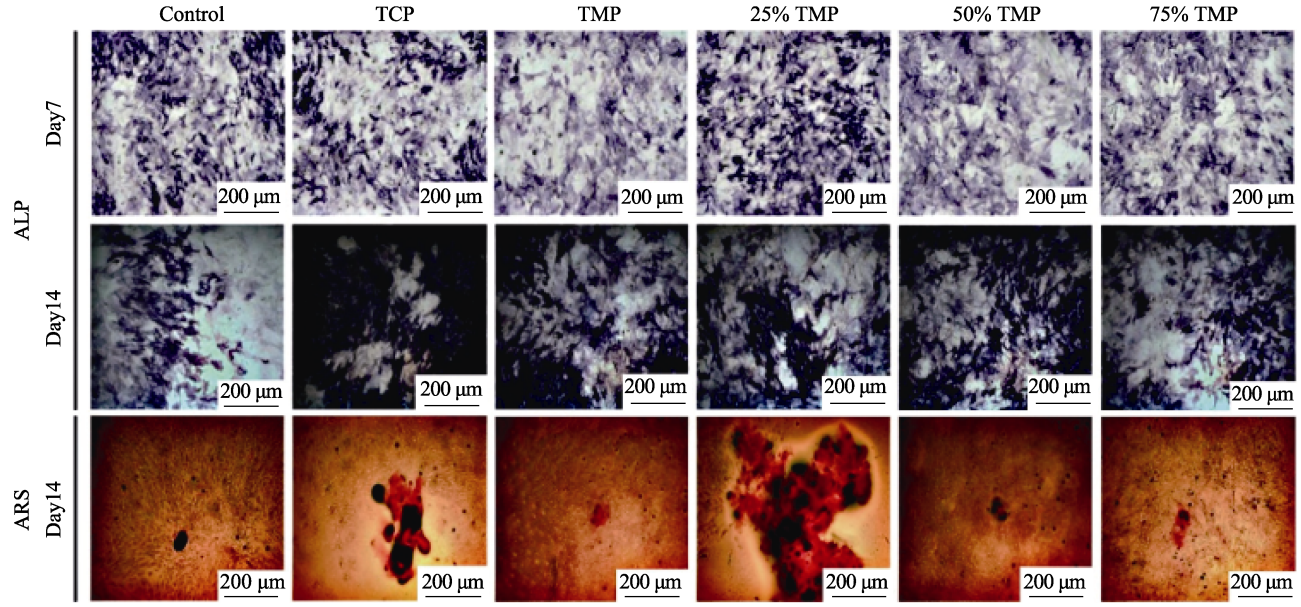

Fig. 6　ALP activity and ARS staining of MC3T3-E1 cells cultured with microspheres extract compared with the control on the 7th and 14th day

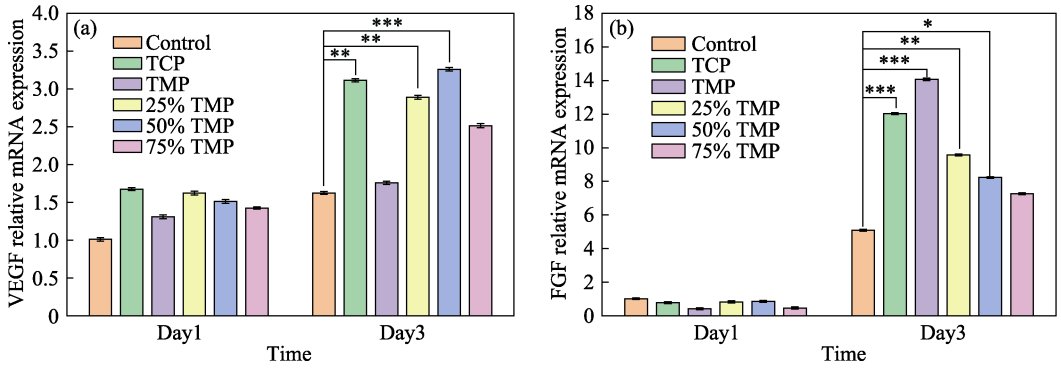

Fig. 7 (a) VEGF and (b) FGF of HUVECs cultured with microspheres extracts on the 1st and 3rd day

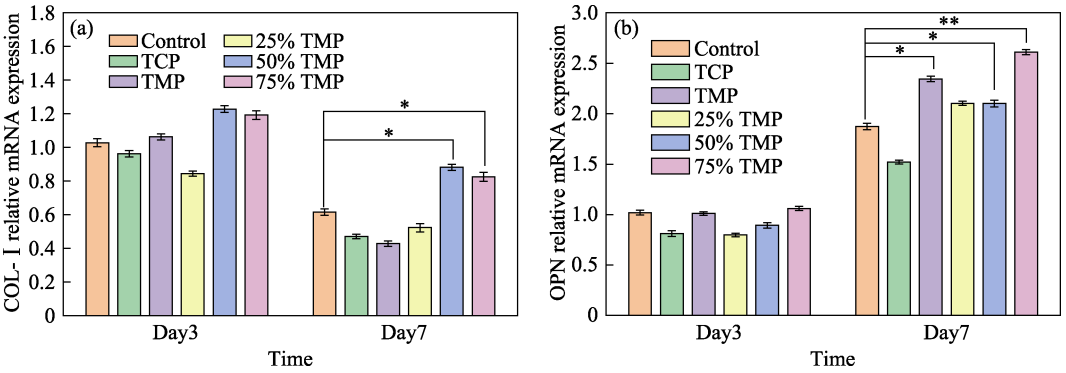

Fig. 8 (a) COLI and (b) OPN genes expression of MC3T3-E1 which cultured with microspheres extract compared to the control on the 3rd and 7th day

up-regulated in both 50% TMP and 75% TMP groups when compared with the control. As for OPN, the expression level of all material treatment groups was almost the same as that of the control group on the 3rd day. However, compared with the control group, 75% TMP and TMP groups, the expression level in TCP group was significantly decreased on the 7th day.

3 Discussion

Tricalcium phosphate (TCP), especially β-tricalcium Phosphate (β-TCP, β-$Ca_3(PO_4)_2$), is one of the important compounds of calcium phosphate with much application in bone tissue formation and regeneration. This study has tackled the biomaterials intended for promoting bone formation and the composite evaluation, biocompatibility process and their effect on MC3T3-E1 cells and HUVECs. The TMP/TCP microspheres were prepared using the cross-linking method[29]. An appropriate combination of the TMP and the TCP ceramic can obtain a material suitable for bone formation and regeneration[30]. From our results, the composite microspheres have shown a mixture of fine and cores morphology compared with the pure material[29]. This can explain our findings why the cytocompatibility have a significant enhancement, and the differentiation of MC3T3-E1cell on the 25% TMP and 50% TMP composite microspheres were enhanced compared to 75% TMP and the control[28]. The extracts of 25% TMP and 50% TMP composite microspheres could provide more favorable conditions for cell proliferation.

New vessel formation requires the integrated actions of several angiogenic growth factors[36]. The angiogenic-associated genes (VEGF and FGF) are among the significant proangiogenic growth factors which can regulate angiogenesis. VEGF is a major angiogenic modulator signal protein produced by the cells that stimulates blood vessel formation[37]. It also regulates several developmental processes such as angiogenesis, lymphangiogenesis and neuronal development[38]. FGF is a signal protein involved in various biological processes, including cell growth and tissue repair. It regulates the proliferation, differentiation, and migration of endothelial cells[39-40]. Moreover, it also regulates endothelial cell proliferation, differentiation, and migration[39-40]. In this study, the 25% TMP, 50% TMP microspheres were noticed promoting the specific angiogenic and osteogenic gene expression, such as the VEGF, FGF, OPN and COLI. The findings of the study demonstrated that the TCP/TMP microspheres of a moderate concentration can interact more efficiently with MC3T3-E1 and HUVECs in expediting osteogenic and angiogenesis[41-42]. Also, the high magnesium environment created by the rapid degra-

dation of the TMP/TCP composite may result in the dysfunction of calcium-dependent physiology processes and be disadvantageous to MC3T3-E1 cell physiology. However, here the 25% TMP microspheres are more suitable for the growth and differentiation of the cells accordingly[43].

The current results demonstrate that the composite microspheres of a moderate concentration of TCP/TMP can interact more efficiently with MC3T3-E1 and HUVECs in expediting osteogenic and angiogenesis. Taking all of these characterizations of TCP/TMP composite microspheres under consideration, it is suggested that the 25% TMP and 50% TMP composite microspheres can effectively promote nutrient transport and aniogenesis, thus suitable for designing high-performance systems for the formation and engineering of bone tissues.

4 Conclusions

This study aims to reveal the effects of the different phase composition of TCP/TMP composite microspheres (25% TMP, 50% TMP, 75% TMP) on the behavior of MC3T3-E1 and HUVECs cells. The cytocompatibility tests have confirmed that 25% TMP and 50% TMP groups were more likely to promote cell proliferation, and MC3T3-E1 cells differentiation. In addition, the above two groups had a positive effect on the expression of genes related to bone formation and angiogenesis. However, the biological properties and mechanism of these composites still need further *in vivo* study.

Supporting materials:

Supporting materials related to this article can be found at https://doi.org/10.15541/jim20220662.

References:

[1] NARITA K, KOBAYASHI E, SATO TJMT. Sintering behavior and mechanical properties of magnesium/*β*-tricalcium phosphate composites sintered by spark plasma sintering. *Materials Transactions*, 2016, **57(9):** 1620.

[2] CHUTHATHIP M, AHMAD-FAUZI M N, YANNY-MARLIANA B I, *et al.* Effect of magnesium oxide on physical and biological properties in *β*-tricalcium phosphate ceramic. *Journal of Physics Conference Series*, 2018, **1082(1):** 012026.

[3] BASU S, BASU B. Doped biphasic calcium phosphate: synthesis and structure. *Journal of Asian Ceramic Societies*, 2019, **7(3):** 265.

[4] GHIȚULICĂ C D, CUCURUZ A, VOICU G, *et al.* Ceramics based on calcium phosphates substituted with magnesium ions for bone regeneration. *International Journal of Applied Ceramic Technology*, 2020, **17(1):** 342.

[5] MAJI K, DASGUPTA S. Effect of *β*-tricalcium phosphate nanoparticles additions on the properties of gelatin-chitosan scaffolds. *Bioceramics Development & Applications*, 2017, **7(2):** 1000103.

[6] MURAKAMI S, MIYAJI H, NISHIDA E, *et al.* Dose effects of beta-tricalcium phosphate nanoparticles on biocompatibility and bone conductive ability of three-dimensional collagen scaffolds. *Dental Materials Journal*, 2017, **36(5):** 573.

[7] FANG Z Z. Sintering of advanced materials. Cambridge: Elsevier, 2010**:** 33–85.

[8] KAUR I, ELLIS L J, ROMER I, *et al*. Dispersion of nanomaterials in aqueous media: towards protocol optimization. *Journal of Visualized Experiments*, 2017, **130:** e56074.

[9] XUE W, DAHLQUIST K, BANERJEE A, *et al.* Synthesis and characterization of tricalcium phosphate with Zn and Mg based dopants. *Journal of Materials Science: Materials in Medicine*, 2008, **19(7):** 2669.

[10] GUO X, LONG Y, LI W, *et al.* Osteogenic effects of magnesium substitution in nano-structured *β*-tricalcium phosphate produced by microwave synthesis. *Journal of Materials Science*, 2019, **54(16):** 11197.

[11] ELIAZ N, METOKI N J M. Calcium phosphate bioceramics: a review of their history, structure, properties, coating technologies and biomedical applications. *Materials*, 2017, **10(4):** 334.

[12] RAO R R, ROOPA H N, KANNAN T S. Solid state synthesis and thermal stability of HAP and HAP–*β*-TCP composite ceramic powders. *Journal of Materials Science: Materials in Medicine*, 1997, **8(8):** 511.

[13] RUIZ-AGUILAR C, OLIVARES-PINTO U, AGUILAR-REYES E A, *et al*. Characterization of *β*-tricalcium phosphate powders synthesized by Sol-Gel and mechanosynthesis. *Boletín de la Sociedad Española de Cerámica y Vidrio*, 2018, **57(5):** 213.

[14] ANDO J. Tricalcium phosphate and its variation. *Bulletin of the Chemical Society of Japan*, 1958, **31(2):** 196.

[15] OLSSON M. Chemical stability of grain boundariesin *β*-tricalcium phosphate ceramics: *β*-TCP as bone substitute material. *Department of Chemistry-Ångström*, 2012, 42586904.

[16] SGLAVO VM, FRASNELLI M. Effect of Mg^{2+} doping on beta–alpha phase transition in tricalcium phosphate (TCP) bioceramics. *Acta Biomaterialia*, 2016, **33:** 283.

[17] MA Y, DAI H, HUANG X, *et al.* 3D printing of bioglass-reinforced *β*-TCP porous bioceramic scaffolds. *Journal of Materials Science*, 2019, **54(14):** 10437.

[18] GALLO M, SANTONI B L G, DOUILLARD T, *et al.* Effect of grain orientation and magnesium doping on *β*-tricalcium phosphate resorption behavior. *Acta Biomaterialia*, 2019, **89:** 391.

[19] TAVARES D D S, CASTRO L D O, SOARES G D D A, *et al.* Synthesis and cytotoxicity evaluation of granular magnesium substituted *β*-tricalcium phosphate. *Journal of Applied Oral Science*, 2013, **21(1):** 37.

[20] LEE D, SFEIR C, KUMTA P N J M S, *et al.* Novel *in-situ* synthesis and characterization of nanostructured magnesium substituted *β*-tricalcium phosphate (*β*-TCMP). *Materials Science*, 2009, **29(1):** 69.

[21] MARCHI J, DANTAS A, GREIL P, *et al.* Influence of Mg-substitution on the physicochemical properties of calcium phosphate powders. *Materials Research Bulletin,* 2007, **42(6):** 1040.

[22] RYU H-S, HONG KS, LEE J-K, *et al.* Magnesia-doped HA/*β*-TCP ceramics and evaluation of their biocompatibility. *Biomaterials*, 2004, **25(3):** 393.

[23] ZHANG X, JIANG F, GROTH T, *et al.* Preparation, characterization and mechanical performance of dense *β*-TCP ceramics with/without magnesium substitution. *Journal of Materials Science: Materials in Medicine,* 2008, **19(9):** 3063.

[24] ONUMA K, IIJIMA M J C. Nanoparticles in *β*-tricalcium phos-

phate substrate enhance modulation of structure and composition of an octacalcium phosphate grown layer. *CrystEngComm*, 2017, **19(44):** 6660.

[25] SADER M S, LEGEROS R Z, SOARES G A. Human osteoblasts adhesion and proliferation on magnesium-substituted tricalcium phosphate dense tablets. *Journal of Materials Science: Materials in Medicine*, 2009, **20(2):** 521.

[26] LIN L C, CHANG S J, KUO S M, *et al.* Preparation and evaluation of *β*-TCP/polylactide microspheres as osteogenesis materials. *Journal of Applied Polymer Science*, 2008, **108(5):** 3210.

[27] YUAN Z, WEI P, HUANG Y, *et al.* Injectable PLGA microspheres with tunable magnesium ion release for promoting bone regeneration. *Acta Biomaterialia.* 2019, **85:** 294.

[28] WANG J, XU J, HOPKINS C, *et al.* Biodegradable magnesium ased implants in orthopedics: a general review and perspectives. *Advanced Science,* 2020, **7(8):** 201902443.

[29] LIN S, YANG G, JIANG F, *et al.* Bone regeneration: a magnesiumnriched 3D culture system that mimics the bone development microenvironment for vascularized bone regeneration. *Advanced Science*, 2019, **6(12):** 1900209.

[30] PAN C, SUN X, XU G, *et al.* The effects of *β*-TCP on mechanical properties, corrosion behavior and biocompatibility of beta-TCP/Zn-Mg composites. *Materials Science & Engineering C*, 2020, **108:** 110397.

[31] ZHANG H, SHEN Y, XIONG Y, *et al.* Microstructural, mechanical properties and strengthening mechanism of DLP produced *β*-tricalcium phosphate scaffolds by incorporation of MgO/ZnO/58S bioglass. *Ceramics International*, 2021, **47(18):** 25863.

[32] ZHANG J. TANG L, QI H, *et al.* Dual function of magnesium in bone biomineralization, *Advanced Healthcare Materials*, 2019, **8(21):** 1901030.

[33] LIN X, GE J, WEI D, *et al.* Surface degradation-enabled osseointegrative, angiogenic and antiinfective properties of magnesium-modified acrylic bone cement. *Journal of Orthopaedic Translation.* 2019, **17:** 121.

[34] HE F, TIAN Y, FANG X, *et al.* Porous calcium phosphate composite bioceramic beads. *Ceramics International*, 2018, **44(11):** 13430.

[35] HO V H, TRIPATHI G, GWON J, *et al.* Novel TOCNF reinforced injectable alginate/*β*-tricalcium phosphate microspheres for bone regeneration. *Materials & Design*, 2020, **194:** 108892.

[36] MURAKAMI M, NGUYEN L T, HATANAKA K, *et al.* FGF-dependent regulation of VEGF receptor 2 expression in mice. *The Journal of Clinical Investigation*, 2011, **121(7):** 2668.

[37] OLIVARES-NAVARRETE R, HYZY S L, GITTENS R A, *et al.* Rough titanium alloys regulate osteoblast production of angiogenic factors. *The Spine Journal*, 2013, **13(11):** 1563.

[38] MATKAR P N, ARIYAGUNARAJAH R, LEONG-POI H, *et al.* Friends turned foes: angiogenic growth factors beyond angiogenesis. *Biomolecules*, 2017, **7(4):** 74.

[39] CHIM S M, TICKNER J, CHOW S T, *et al.* Angiogenic factors in bone local environment. *Cytokine Growth Factor Reviews*, 2013, **24(3):** 297.

[40] TAN A W, LIAU L L, CHUA K H, *et al*. Enhanced *in vitro* angiogenic behaviour of human umbilical vein endothelial cells on thermally oxidized TiO_2 nanofibrous surfaces. *Scientific Reports*, 2016, **6(1):** 21828

[41] PRZYBYLSKI M. A review of the current research on the role of bFGF and VEGF in angiogenesis. *Journal of Wound Care*, 2009, **18(12):** 516.

[42] CHEN Y, OU Y, DONG J, *et al*. Osteopontin promotes collagen I synthesis in hepatic stellate cells by miRNA-129-5p inhibition. *Experimental Cell Research*, 2017, **363(1):** 343.

[43] BHASKAR B, OWEN R, BAHMAEE H, *et al*. Composite porous scaffold of PEG/PLA support improved bone matrix deposition *in vitro* compared to PLA-only scaffolds, *Journal of Biomedical Research Part A*, 2018, **106(5):** 1334.

双相磷酸镁钙微球体外成血管和促成骨研究

吴 未[1, 2], BAKHET Shahd[2], ASANTE Naomi Addai[2], KAREEM Shefiu[2], KOMBO Omar Ramadhan[3], 李宾斌[2], 戴红莲[1, 2]

(1. 武汉理工大学 深圳研究院, 深圳 518000; 2. 武汉理工大学 材料复合新技术国家重点实验室, 湖北省生物材料工程研究中心, 武汉 430070; 3. 姆贝亚科技大学 医学科学与技术系, 姆贝亚, 坦桑尼亚)

摘 要: 磷酸三钙(*β*-TCP)陶瓷替代材料由于其与骨矿物成分相近及良好的生物相容性和骨传导性, 近年来被广泛关注, 常以纳米颗粒、支架和微球等形式用于骨修复。本研究制备了五种不同的磷酸三钙/磷酸三镁(TMP) (TCP、25% TMP、50% TMP、75% TMP 和 TMP)复合微球并作了相应表征。随着复合微球中 TMP 含量增加, 微球释放的 Mg^{2+}和 Ca^{2+}的累积浓度增加, 且 TMP 可以调节复合微球的降解速率。以小鼠胚胎成骨细胞前体细胞(MC3T3-E1)和人脐静脉内皮细胞(HUVECs)为模型, 评价了该复合微球的生物相容性、成血管和成骨作用。结果表明, 与 TCP、TMP 和 75% TMP 相比, 25% TMP 和 50% TMP 复合微球具有更好的细胞相容性, 对 HUVECs 有一定的促增殖作用。因此, 含 25% TMP 和 50% TMP 的复合微球对血管生成和成骨具有更积极的作用。

关 键 词: *β*-TCP; TMP; 骨缺损; 陶瓷; 微球; 骨组织工程

中图分类号: TB321; R318 **文献标志码:** A

Supporting Information

In vitro Study of Biphasic Calcium Magnesium Phosphate Microspheres for Angiogenesis and Bone Formation

WU Wei[1, 2], BAKHET Shahd[2], ASANTE Naomi Addai[2], KAREEM Shefiu[2],
KOMBO Omar Ramadhan[3], LI Binbin[2], DAI Honglian[1, 2]

(1. Shenzhen Institute of Wuhan University of Technology, Shenzhen 518000, China; 2. State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Biomedical Materials and Engineering Research Center of Hubei Province, Wuhan University of Technology, Wuhan 430070, China; 3. Department of Medical Science and Technology, Mbeya University of Science and Technology, Mbeya, Tanzania)

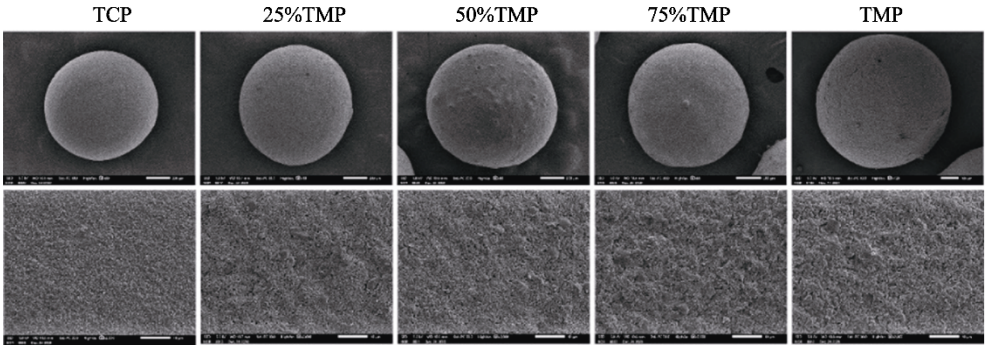

Fig. S1　SEM images of different microsphere composites with Bars at 200 μm (up row) and 10 μm (down row)

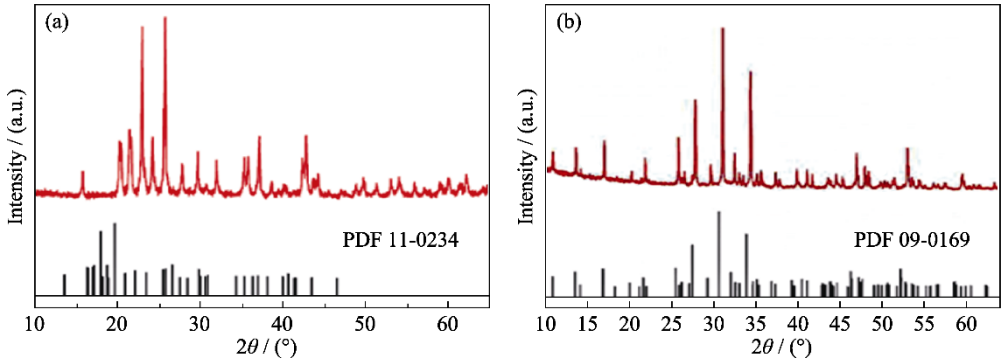

Fig. S2　XRD patterns of TMP (a) and β-TCP (b)