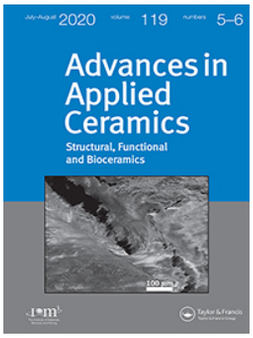

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Effect of monocalcium phosphate on the physical, chemical, mechanical and biological properties of calcium silicate cement: an *in vitro* study

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ABSTRACT

This study evaluated the effect of monocalcium phosphate (MCP) on the mechanical and biological properties of calcium silicate (CS) cement. Cements composed of CS with different weight ratios (0–40%) of MCP were prepared and their pH and compressive strength were tested using the pH detector and Universal Testing Machine, respectively. Cell viability, migration, mineralisation and differentiation were evaluated using the Cell Counting Kit 8 (CCK-8), Transwell, alkaline phosphatase activity assay, Alizarin Red-S staining and real-time polymerase chain reaction, respectively. With the MCP content increase, the pH of the cements decreased from 11.5 to 8 and the compressive strength decreased from 25.81 to 3.45 MPa. The extracts of cements promoted cell proliferation, migration, osteoblastic differentiation and mineralisation when MCP was 10–20 wt-%. These suggested that MCP powder can regulate the properties of CS cement. Composite cement containing 10–20 wt-% MCP showed improved biocompatibility and mineralisation and promising for bone defect restoration.

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KEYWORDS
Bone cement; calcium silicate; monocalcium phosphate; osteogenesis

Introduction

The restoration of critical bone defects using bone graft materials is important in cases of trauma, disease and surgery. Synthetic bone graft substitutes manufactured from raw mineral materials are of increasing interest because of their precisely divined compositions unlimited availability, no risk of pathogen transmission and no ethical concerns [1]. Bioactive ceramics such as tricalcium phosphate (TCP), hydroxyapatite (HA), various biphasic combinations of TCP and HA and bioactive glasses have been well-studied over the past two decades because of their biocompatibility, biodegradability and osteoconductivity [2], and TCP is generally used in bone regeneration clinically [3]. However, some studies have demonstrated that pure TCP shows slow bone formation with suboptimal osteointegration *in vivo* [4]. Furthermore, it also lacks osteoinductive properties [5] and mechanical strength [6].

The past decade years have seen an increase in clinical using calcium silicate (CS) cements as root-end filling, pulp capping, root repair and other dental repair materials [7–9]. Biological characteristics of new CS-based materials have been optimised by adding additional components such as setting modulators, radiopacifying agents and drugs [10]. Recently, the application of CS in bone repair has drawn more interest [11], since it shows self-setting properties at body temperature, high mouldability, excellent bioactivity and osteostimulatory and biodegradable properties [12–14]. It has also been shown that silicon (Si) ions released from CS could provide an ideal extracellular environment for directing osteogenic differentiation of bone mesenchymal stem cells [15]. However, a large amount of calcium hydroxide [$Ca(OH)_2$] is formed during the hydration of dicalcium silicate ($2CaO{\cdot}SiO_2$) and tricalcium silicate ($3CaO{\cdot}SiO_2$), which could cause a highly alkaline environment *in situ* [16]. Monocalcium phosphate (MCP) is the most acidic calcium orthophosphate and the most soluble calcium orthophosphate at almost the entire range of pH values [17]. The acidity and solubility make MCP not biocompatible and impossible to be used alone as a bone substitute [17]. On the other hand, these properties of MCP make it promising to prepare a kind of composite cement composed by CS and MCP (CS/MCP), whose pH value and degradation rate could be regulated [15]. In addition, MCP can promote the formation of HA and regulate the osteogenesis of CS cement [10]. However, studies on biological properties of this type of composite cement are quite limited, especially its biological effects on bone marrow stromal cells [16,18,19].

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Therefore, the purpose of this study is twofold: the effect of MCP on CS cement was investigated through a serial of CS/MCP composite cements with a wide range of mass fraction of MCP (from 10 to 40%) in aspects of microstructures, mechanical and chemical properties. Thereafter, the biological effects of CS/MCP composite cements on bone marrow stromal cells were also investigated, compared with traditionally clinical used TCP cement.

Materials and methods

Preparation of calcium phosphate silicate cement and extract

CS powder mainly contains $2CaO{\cdot}SiO_2$ and $3CaO{\cdot}SiO_2$, which was provided by Innovative BioCeramix Inc. (Vancouver, Canada). Serial CS/MCP composite cements with 10, 20, 30 and 40 wt-% MCP (Biorular, Danbury, USA) were prepared, while pure CS cement and a commercial TCP (HydroSet Injectable HA bone substitute; Sryker Leibinger GmbH& Co. KG, Freiburg, Germany) were used as control groups. The prepared materials and the liquid to powder (L/P) ratio are shown in Table 1. After being mixed with deionised water, cements were inserted into a mould (diameter: 10 mm, height: 1 mm) for 24 h. The superficial morphology as well as the presence of Ca, Si, P elements in the cement was detected by scanning electron microscopy (SEM; 10.0 kv) and by energy dispersive X-ray spectroscopy (EDS; 25 kv; take-off angle 3.7) (JEOL JSM-7500F, Japan).

Extracts of the various cements were prepared according to the International Organisation for Standardisation (ISO 10993-12) at a ratio of 1.25 cm^2 mL^{-1}. Briefly, a stock solution was first prepared by adding the specimens to α-minimum essential medium (α-MEM, Sigma, USA). Supernatant was collected after incubation at 37°C for 24 h. Subsequently, extracts were sterilised by filtration through 0.22 μm filter membranes (Merck Millipore, USA) and stocked for further experiments.

pH measurement of α-MEM extracts

Cements were immersed in α-MEM with a final liquid-to-powder ratio according to the ISO, and the pH value of the suspension supernatants was recorded by a pH metre (PHB-2, Runsun Instruments Inc, China) by a every 15 min over a period of 1 h. Cements were then immersed in the same medium and stored at 37°C and 100% humidity for 24 h, after which their pH were recorded again.

Compressive strength

After the powder was mixed with deionised water, cements were placed into a customised cylindrical mould (diameter: 4 mm) for 24 h. After being removed from the mould, the specimens were kept at room temperature for 1 day. Compressive strength testing was conducted on a Universal Test Machine (Instron, USA) at a loading rate of 1 mm min^{-1}. A total of 10 specimens were examined for each of the materials.

Cell culture and osteogenic characteristics

Human bone marrow stromal cells (hBMSCs) were used in this study. The use of hBMSCs was approved by Peking University. The hBMSC growth medium consisted of α-MEM plus 10% foetal bovine serum (FBS, Hyclone, USA) and 1% penicillin–streptomycin (PS, Gibco, USA). The osteogenic medium for hBMSCs consisted of hBMSC growth medium or extracts plus 100 nM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 0.05 mM ascorbic acid (Sigma, USA).

After culturing in growth medium to 80–90% confluency, cells were detached and passaged. Passage 4–6 cells were used in the experiments.

Cell proliferation

Cell proliferation was determined using a Cell Counting Kit 8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), with cell culture time of 1, 3 and 5 days, after hBMSCs were seeded in 96-well plates at a concentration of 3000 cells 100 μL^{-1} and cultured in the extracts described above. Plates were incubated at 37°C and 100% humidity with 5% CO_2 in culture medium, and the medium was replaced every two days. At specific time points, 10 μl CCK-8 solution was added into each well. Plates were then incubated in a cell culture incubator for 2 h, after which they were read at 450 nm using a microplate reader (ELX080, Bio Tek,

Table 1. Nominal composition, liquid-to-powder ratio (L/P ratio) and diametral tensile strength (mechanical strength) of the cements.

Material	CS:MCP(weight ratio)	L/P ratio(mL g^{-1})	Compressive strength(MPa)
CS	100:0	0.35	25.81±6.72
CS/10%MCP	90:10	0.37	11.82±7.12
CS/20%MCP	80:20	0.40	4.37±1.35
CS/30%MCP	70:30	0.43	3.45±2.42
CS/40%MCP	60:40	0.48	4.05±1.78
TCP	–	0.40	33.41±11.03

USA). Each group had five parallels and the experiment was repeated three times.

Transwell migration assay

Cell migration assays were evaluated using a two-chamber Transwell system (8 μm pore size and 6.5 mm diameter, Transwell BD Matrigel 3422, Corning, USA). Briefly, 1×10^4 hBMSCs were resuspended in 100 μl serum-free α-MEM and seeded into the top chamber of the Transwell plate, and 500 μl of α-MEM and other extracts with 10% serum was added to the lower chambers. After 16 h incubation at 37°C in 5% CO_2, cells remaining on the top surface were removed with a cotton swab, after which hBMSCs that had traversed the membrane to the bottom chamber were fixed for 20 min with 4% paraformaldehyde (Solarbio, China) and stained with crystal violet (Solarbio, China) for 10 min. To quantify the migrated cells, five random microscopic fields at 200× magnification were selected per filter for a cell count. Measurements were performed in triplicate and means were calculated in each experiment. The experiment was repeated three times.

Alkaline phosphatase staining and activity assay

Alkaline phosphatase (ALP) staining was performed using a BCIP/NBT ALP kit (Beyotime, Shanghai, China). Briefly, hBMSCs were plated in 24-well plates and cultured for 7 days with different osteogenesis extracts. After fixation with 4% paraformaldehyde for 20 min, cells were incubated in a mixture of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. ALP activity was assessed using the ALP Detection Kit (Jiancheng Technology, Nanjing, China). Briefly, hBMSCs were seeded in 24-well plates and cultured in the presence of extracts for 7 days. ALP activity was examined according to the manufacturer's instructions and normalised to the total protein content determined using the BCA method. Each group had three parallels and the experiment was repeated three times.

Alizarin red S staining

Osteogenic differentiation was induced by culturing hBMSCs for 21 days in osteogenesis extracts and assessed using an alizarin red S stain, which is an indicator of extracellular matrix (ECM) calcification. Briefly, cells were washed twice with phosphate-buffered saline followed by fixation with 4% paraformaldehyde for 20 min at room temperature. After washing in distilled water twice, cells were stained with 1% (w/v) alizarin red S (pH 4.1–4.5, Sigma, USA) for 15 min at room temperature and then washed twice with distilled water. For quantification, the area of membrane-bound staining was eluted with 10% cetylpyridinium chloride (Sigma, USA), and the absorbance of supernatants was measured at 405 nm. Each group had five parallels and the experiment was repeated three times.

Table 2. Sequence of primers.

Gene	Primer sequence (Forward/Reverse)
Runx2	F: 5′-TCCTATGACCAGTCTTACCCCT-3′ R: 5′-GGCTCTTCTTACTGAGAGTGGAA-3′
OCN	F: 5′-CACTCCTCGCCCTATTGGC-3′ R: 5′-CCCTCCTGCTTGGACACAAAG-3′
COL-1	F: 5′-CTGACCTTCCTGCGCTGATGTTC-3′ R: 5′-TTGGACGTTGGTGCCCCAGAC-3′
GAPDH	F: 5′-ATGGGGAAGGTGAAGGTCG-3′ R: 5′-GGGGTCATTGATGGCAACAATA-3′

Reverse transcription-polymerase chain reaction and quantitative-polymerase chain reaction

Total RNA of hBMSCs cultured in media with different osteogenesis extracts was isolated using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) after cells cultured for 7 or 14 days. Equivalent amounts of RNA sample were reverse-transcribed for first strand cDNA synthesis using oligo (dT) as a reverse transcription primer. cDNA was then used in gene-specific PCR targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH), runt-related transcription factor 2 (Runx2), osteocalcin (OCN) and collagen type I (COL-1). The qPCR was performed using a real-time PCR machine (ABI 7500, Applied Biosystems, Foster City, CA, USA) and a real-time PCR kit (SYBR Premix EX Taq, TaKaRa, Dalian, China) with GAPDH as the housekeeping gene for normalisation. Primer sequences are shown in Table 2. Each group had 5 parallels and the experiment was repeated three times.

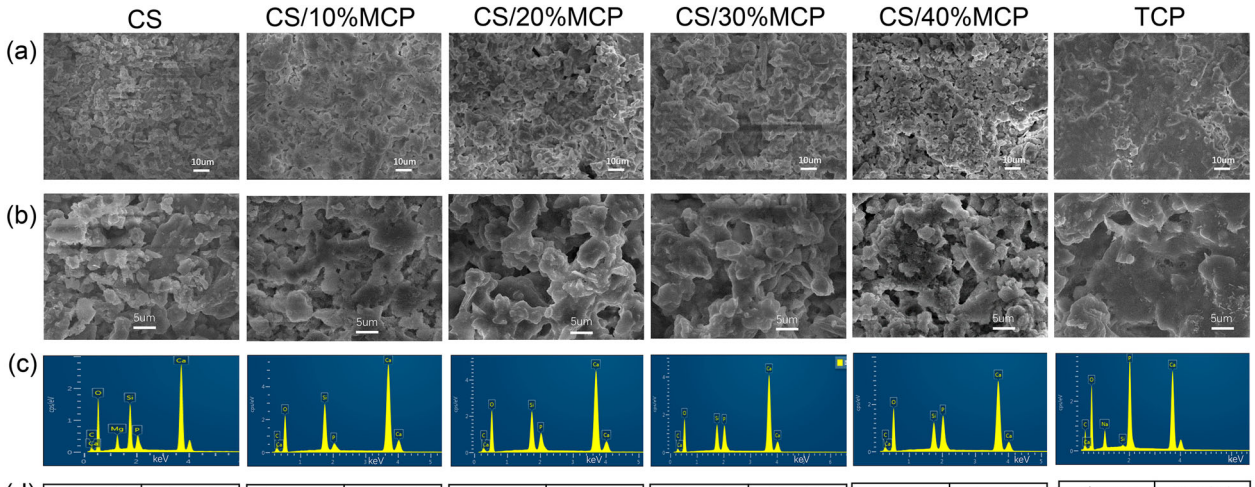
Statistical analysis

The experiments were performed twice to ensure reproducibility. IBM SPSS Statistical software v.19.0 (SPSS, Chicago, IL, USA.) was used to analyse our data. Data were analysed by two-way ANOVA and complemented by Least-Significant Difference test for observation of the significance differences between the study groups. A p-value less than 0.05 was considered significant.

Results

Compressive strength of cements

There was no statistical difference between the compressive strength values of CS cement and that of TCP cement ($p > 0.05$). However, the compressive



Element	Atomic %
C	8.51
O	61.06
Mg	2.52
Si	8.06
P	0.25
Ca	19.60
Total:	100.00

Element	Atomic%
C	11.39
O	56.69
Si	7.88
P	0.85
Ca	23.19
Totals	100.00

Element	Atomic%
C	8.11
O	60.78
Si	6.64
P	2.82
Ca	21.65
Totals	100.00

Element	Atomic%
C	6.52
O	59.24
Si	5.14
P	5.02
Ca	24.08
Totals	100.00

Element	Atomic%
C	10.25
O	60.31
Si	4.57
P	5.90
Ca	18.97
Totals	100.00

Element	Atomic%
C	8.66
O	65.52
Na	3.01
Si	0.12
P	9.46
Ca	13.22
Totals	100.00

Figure 1. Morphochemical characterization of cement. (a), (b) SEM view of the cements surface, magnified 1000 times and 3000 times respectively. (c) EDX spectra of the whole area. (d) The elemental X-ray microanalysis performed on the cement surface.

strength values of the composite cements decreased in response to increases in MCP content (Table 1). Thus, differences in the composition may play an important role in the mechanical properties of these cements.

Cement microstructure

The SEM image of cements observed by magnification of 1000 and 3000 (Figure 1(a,b)) showed a granular surface. The porosity of the materials increased with increasing MCP content. EDX analyses revealed the presence of calcium (Ca), Si and phosphorous (P) elements (Figure 1(c,d), Figure 2). It was indicated that with increasing MCP content, the content of Si decreased, the content of P increased and the content of Ca basically remained unchanged.

pH value measurement

Figure 3(a) shows that the pH value of CS/MCP (pH 9.5–11.5) was slightly lower than that of CS (pH 11.5–12) after soaking water 1 h. The pH value of the soaking water of TCP was lower (pH 8.5–9) than either of the CS and CS/MCP. Nevertheless, after 24 h immersion, the pH value of each groups approached that of α-MEM (pH $\approx$ 8).

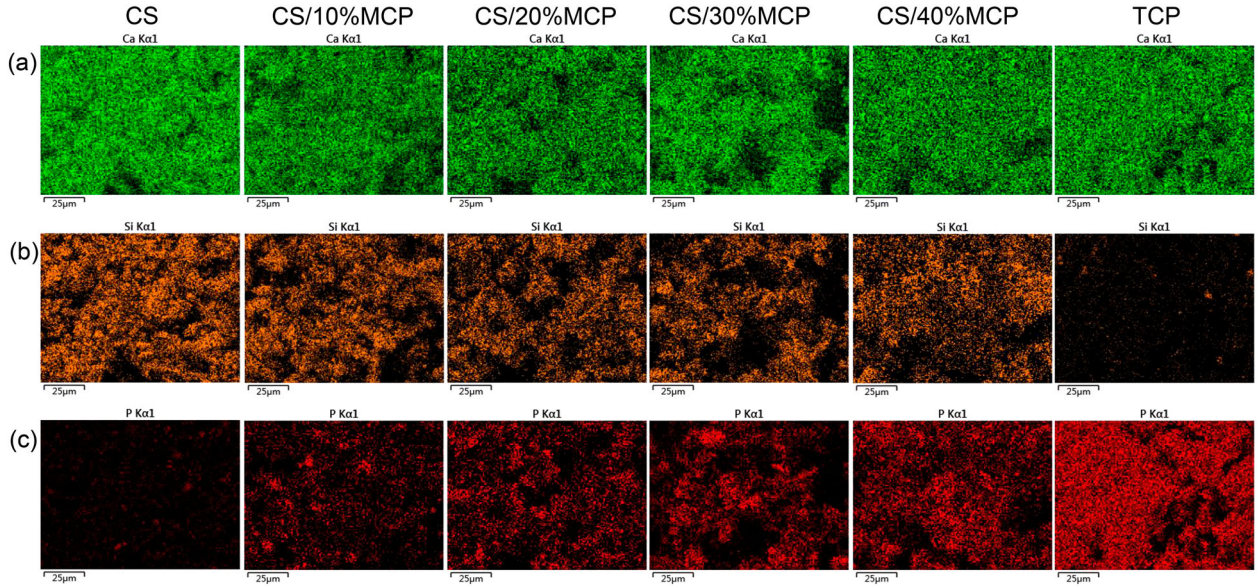


Figure 2. SEM-EDS elemental maps for (a) Ca, (b) Si and (c) P (magnified 1000 times) showed that with increasing MCP content, the content of Si decreased, the content of P increased and the content of Ca basically remained unchanged.

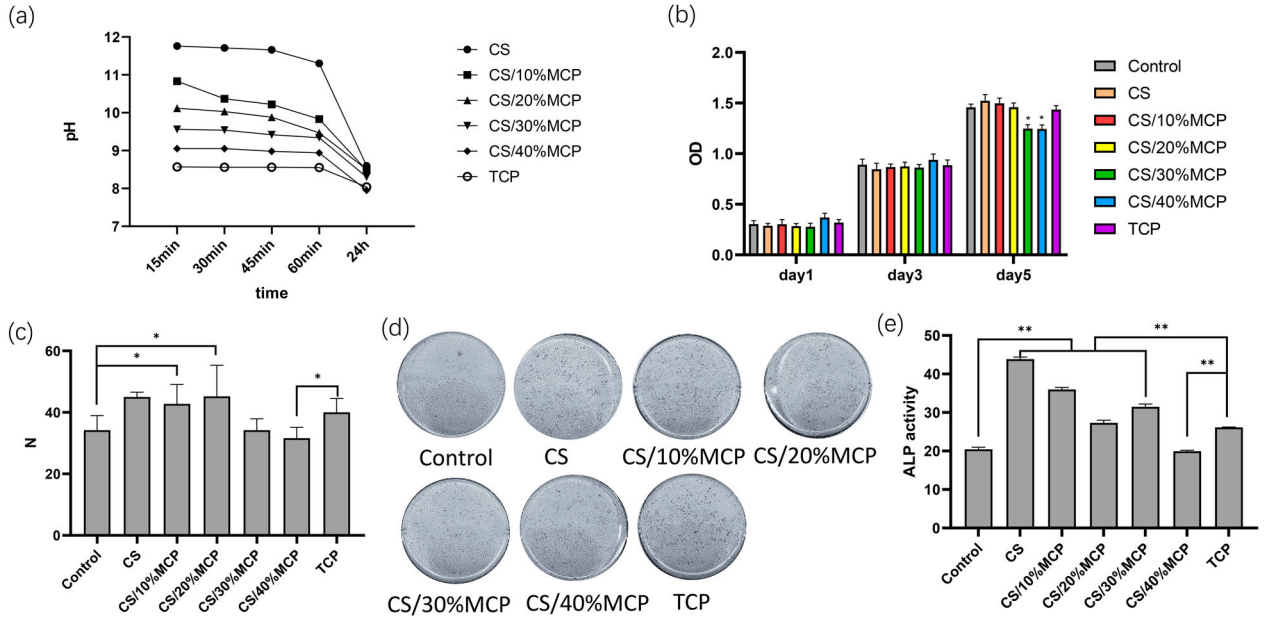


Figure 3. (a) pH of soaking water of different cements for 24 h. (b) Cell viability of hBMSCs cultured in six kinds of extracts and in *α*-MEM. (c) The number of migratory hBMSCs cultured in six kinds of extracts and in *α*-MEM. (d) ALP staining of hBMSCs cultured in six kinds of extracts and in *α*-MEM. (e) ALP activity of hBMSCs cultured in six kinds of extracts and in *α*-MEM. (*$p < 0.05$, **$p < 0.01$).

Cell proliferation

The effects of different extracts on the proliferation of hBMSCs are shown in Figure 3(b). On the first and third day, there were no significant differences in the optical density (OD) of cells cultured in each extract. On the fifth day, the OD values of cells cultured in CS/30%MCP and CS/40%MCP extracts were lower than those of the other groups. Moreover, the OD values of cells cultured in CS and CS/10%MCP were slightly higher than those of cells cultured in TCP extracts, although the difference was not significant.

Effects on the migration motility of hBMSCs

Each kind of cements induced migration of hBMSCs through the transwell membrane. Figure 3(c) shows the effects of different cements on the migration motility of hBMSCs. Compared to the negative control group, CS/10%MCP extracts and CS/20%MCP extracts significantly enhanced the migration of hBMSCs ($p < 0.05$), although this effect was not significantly different with the TCP group ($p > 0.05$).

ALP staining and activity

Figure 3(d,e) shows ALP staining and activity of hBMSCs in different groups. The ALP activity of CS, CS/10%MCP, CS/20%MCP and CS/30%MCP groups was higher than that of TCP group, while the ALP activity of CS/40%MCP extract was lower than that of TCP ($p < 0.05$).

Alizarin red S staining

Alizarin red staining indicated that CS and CS/10%MCP had a stronger mineralisation ability than TCP group ($p < 0.05$). CS/20%MCP also promoted mineralisation compared with TCP group ($p < 0.05$), but the effect was somewhat weaker than that of CS and CS/10%MCP. However, CS/30%MCP and CS/40%MCP had similar mineralisation ability to the TCP group ($p > 0.05$), as seen in Figure 4(a,b).

Gene expression

The results of semi-quantitative PCR of hBMSCs cultured in different extracts are shown in Figure 4(c–e). Runx2 mRNA levels were tested after cells cultured for 7 days and OCN and COL-1 mRNA levels were tested after cells cultured for 14 days. These results indicated that the CS group and the CS/10%MCP group featured the highest mRNA gene expression of Runx2 ($p < 0.05$). Meanwhile, CS, CS/10%MCP and CS/20%MCP group showed higher mRNA gene expression of OCN and COL-1 than TCP group ($p < 0.05$).

Discussion

The use of synthetic bone substitutes is highly recommended in case that the reconstruction of bone defects [20]. Calcium silicate cement is considered to be a candidate bone-grafting material because of its excellent bioactivity and osteostimulatory property [21]. In addition, the mircoenvironment of around the CS cement could be more facilitates osteogenesis and the mechanical properites of the material maybe improved by adding MCP [19]. The present study provides insightful explanations regarding to how CS/MCP composite cement mechanical, chemical and physical properties changes with different weight

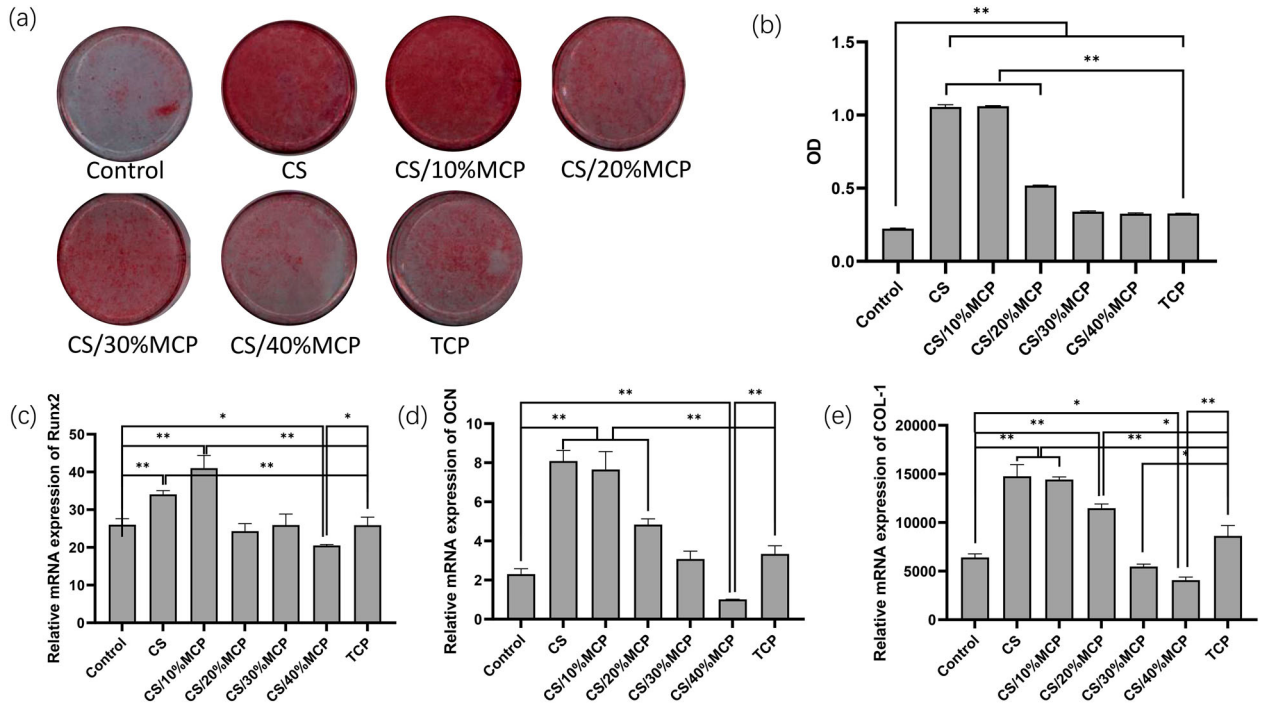


Figure 4. Photographs (a) and quantification (b) of calcium mineral deposits by Alizarin Red S assay of hBMSCs cultured in different extracts. Effects of extracts of different cements on Runx2 (c), OCN (d) and COL-1 (e) mRNA levels in hBMSCs were determined using qRT-PCR. (*$p < 0.05$, **$p < 0.01$).

percentage of MCP contents and how it affects the biological properties of bone marrow stroma cells.

In the present study, it has been demonstrated that adding MCP could reduce the pH value of composite cement during the cement setting. It was widely accepted that, for most human cells, long-term exposure to either an acidic (pH < 6.0) or basic (pH > 9.0) environment has adverse effects on cell growth and proliferation [22]. Previous studies have shown that hydration of CS, which is accompanied by the formation of $Ca(OH)_2$, causes a significant increase in the pH value [23] according to the following equation:

$$3CaO \cdot SiO_2 + nH_2O \rightarrow xCaO \cdot CaO_2 \cdot aH_2O(CSH) + (3 - x)Ca(OH)_2$$

$$2CaO \cdot SiO_2 + nH_2O \rightarrow xCaO \cdot CaO_2 \cdot aH_2O + (2 - x)Ca(OH)_2$$

The pH variation of tested materials in α-MEM was monitored in the present study. Our results indicated that the addition of acidic MCP suppressed the pH increase, which induced during the hydration of CS with the $Ca(OH)_2$ production [24]. MCP is believed to react with $Ca(OH)_2$ during hydration according to the following equation [19]:

$$3Ca(H_2PO_4)_2 \cdot H_2O + 3Ca(OH)_2 \rightarrow 6CaHPO_4 \cdot 2H_2O + 3H_2O$$

$$6CaHPO_4 \cdot 2H_2O + 4Ca(OH)_2 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O$$

The process can be summarised as follows: CS reacts with water to form CSH gel and $Ca(OH)_2$, and MCP reacts with $Ca(OH)_2$ to form HA, thereby shifting the reaction to the formation of CSH gel. HA and CSH then generate a composite structure during cement hydration. On the other hand, the formation of bone-like apatite on the surface of an artificial material could promote bonding to living bone, thus improving the biological properties of the composite cements [25].

However, the CS/MCP silicon phosphate composite cement showed a slightly lower compression strength compared with that of the pure CS cement after setting. It is believed that *in situ* water release upon reaction of MCP with $Ca(OH)_2$ during cement setting increases the final porosity of set cement and consequently decreases the cement compressive strength [18]. The increased porosity of CS/MCP silicon phosphate composite cement along with the MCP adding, which maybe a good way to regulate the degradation rate *in vivo*. However, for clinical applications, bone-grafting materials are required to provide adequate mechanical support for the defect site [26]. Although the compressive strength of the composite cements showed a slightly decrease, it was similar to that of cancellous bone (2–20 MPa) [27] and therefore still showed suitable for clinical applications. The present study provides understanding of how the compressive strength of composite cement changes with MCP content and it could help researchers to optimise compressive strength in the future. More research is needed to evaluate the porosity of the CS/MCP composite cements and its more influences.

To evaluate the CS/MCP composite cements' biological effects on hBMSCs, proliferation, migration mineralisation and osteogenic differentiation of cells were assessed. CS/10%MCP and CS/20%MCP were thought to be of excellent biological performance. The CCK-8 assay confirmed that MCP incorporation into CS cement (up to 20%) had no cytotoxic effects on osteoblast cells, agreeing with previous studies [18,28]. Although the difference was not statistically significant, adding a 10% weight ratio of MCP promoted cell viability and proliferation. Only the CS/30%MCP and CS/40%MCP cements showed decreases in cell viability. Since the extracts have similar pH values with α-MEM after 24 h immersion, their effect on cell proliferation may be attributed to the cellular effect of Ca, Si and P concentrations [19]. Previous studies have shown that excess P ions may induce cell apoptosis and necrosis [29]. However, further *in vivo* studies are required to confirm the cytotoxicity of these cements. Regulation of cell motility, such as cell migration, is essential for normal development [30]. In the present study, hBMSCs migration was enhanced by CS, CS/10%MCP and CS/20%MCP, which supported the biocompatibility of these cements.

In order to further evaluate *in vitro* osteogenesis, cell mineralisation and differentiation were determined by ALP staining and activity, alizarin red staining and expressions of three commonly used osteoblast-specific genes. As a cell surface glycoprotein that is involved in mineralisation [31], ALP is the most commonly recognised marker of osteoblastic differentiation [32]. Alizarin red chelates with calcium salt to form an orange–red complex, which denotes calcium deposits. These calcium deposits indicate late-stage osteogenic differentiation [33]. Both ALP activity and Alizarin red S staining demonstrated the excellent osteogenic effect of CS/10%MCP and CS/20%MCP composite cements, which is assumed to attribute to the effect of Si ions [34]. Si ions are known to play an important role in bone metabolism and could increase ALP expression in human osteoblast-like cells, which is essential for the mineralisation process [35].

Osteogenic differentiation of osteoblasts is one of the key steps for determining the success of bone regeneration. As an activator, the transcription factor Runx2 plays an important role in osteogenic differentiation and is an early marker of osteogenic [36]. In the present study, the expression of Runx2 was enhanced by CS and CS/10%MCP cement extracts. OCN and COL-1 are later markers of osteoblasts [37,38]. OCN is the most abundant non-collagenous bone-matrix protein and is typically synthesised by mature osteoblast-like cells in the ECM of bone tissue [37]. COL-1 is a fundamental ECM protein secreted by odontoblasts before matrix mineralisation [38]. In the present study, it showed that the expression levels of OCN and COL-1 were enhanced by CS, CS/10% MCP, and CS/20%MCP composite ceramics, and this enhancement was dependent on the concentration of Si ions released from the materials. The mechanism by which these cement extracts affect collagen type I synthesis is likely associated with silicon regulation of prolyl-hydroxylase activity, an enzyme required for collagen cross-linking [39]. Silicon is a ubiquitous environmental element that binds to glycosaminoglycans, playing a role in cross-linking of this molecule with collagen and preventing enzymatic degradation, and promoting collagen stabilisation [40]. However, further studies are required to explore the exact mechanism. The formation of osteogenic markers such as Runx2, OCN and COL-1 suggested that CS, CS/10% MCP and CS/20%MCP cements played an important role in promoting osteogenic differentiation.

Conclusions

In present study, the addition of MCP can regulate the preliminary pH, porosity, mechanical strength and biological properties of CS cement. CS Cement containing 10–20% MCP showed appropriate mechanical properties and promoted the proliferation, migration and mineralisation and osteogenic differentiation of BMSCs, which was considered as a promising candidate material for bone defect restoration and bone tissue engineering.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Ethical approval

The experimental protocol was approved by the Ethical Committee of Peking University School of Stomatology and was performed in accordance with the institutional guidelines of the Biomedical Ethics Committee of Peking University School of Stomatology (protocol number: PKUSSIRB-201734036).

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