Fabrication and Characterization of Polyphosphazene/Calcium Phosphate Scaffolds Containing Chitosan Microspheres for Sustained Release of Bone Morphogenetic Protein 2 in Bone Tissue Engineering

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Abstract Bone morphogenetic protein 2 has a major role in promoting bone regeneration in tissue engineering scaffolds. Growth factor release rate is a remaining crucial problem in these systems. The aim of this study was to fabricate and characterize a novel calcium phosphate/polyphosphazenes porous scaffold for the sustained release of bone morphogenetic protein 2 in bone tissue engineering. Polyphosphazenes were substituted with 2-dimethylaminoethanol and evaluated by GPC, NMR, and *in vitro* degradation. Calcium phosphate porous samples were prepared from hydroxyapatite nanoparticles and naphthalene using the sintering method at 1250 °C before being composited with poly(dimethylaminoethanol)phosphazenes containing chitosan microspheres loaded with bone morphogenetic protein 2. The characteristics and biodegradability of the product were evaluated by SEM, XRD, and *in vitro* degradation. Moreover, the release rate and mechanical properties of the scaffolds were investigated. The release behavior was found to be sustained since the scaffolds had been fabricated from polyphosphazenes with a low degradation rate. The release rates of the scaffolds were observed to increase with increasing chitosan microspheres content from 10 to 30%. The bioactivity of the scaffolds depended on the release rate of growth factor while bone morphogenetic protein 2 was able to induce an osteoblast proliferation. The results of cell adhesion and cell viability tests showed that scaffolds displayed a non-toxic behavior and western blot analyses confirmed that the scaffolds loaded with growth factor increased the osteogenic differentiation potential of cells when compared with scaffolds alone. These results demonstrate that these scaffolds can be successfully utilized in bone tissue engineering.

Keywords Bone tissue engineering · Controlled biodegradation · Sustained release · Polyphosphazenes · Bone morphogenetic proteins

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1 Introduction

Conventional treatments used for the repair of segmental bone defects such as autograft and allograft are plagued with such considerable limitations as transmitting infections [1], significant costs, lack of available donors, and morbidity of donor sites [2]. These restrictions have resulted in much attention to bone tissue engineering as an alternative approach to the design of scaffolds with appropriate properties that are capable of controlling the regeneration process. For a successful application of bone

tissue engineering, it is necessary to utilize the scaffold with optimally controlled degradation, desirable mechanical properties, interconnected pore network, biocompatibility, osteoconductive properties, and delivery of specific bioactive factors to enhance and guide the regeneration process [3, 4].

Hydroxyapatite (HA) and β -tricalcium phosphate (β -TCP) have been extensively used in biomedical applications [5, 6] due to the bioactivity of HA and the bioabsorption properties of β -TCP [7, 8]. However, they are too brittle to be used as porous scaffolds under loaded conditions. Recent studies directed efforts to enhancing the mechanical properties of calcium phosphates by fabrication of biopolymer/bioceramic composites as the scaffold; examples include HA-chitosan/chitin [9], Polycaprolactone/HA [10], and β -TCP-Chitosan-Fucoidan [11].

The studies have revealed that infusion by releasing such inductive bioactive factors as bone morphogenetic proteins (BMPs) can be efficient in promoting bone regeneration [12, 13]. Growth factors (GFs) have also shown their capability in enhancing bone healing [14], cell growth, and differentiation of cells in bone tissue engineering [15]. However, GFs are limited by high cost, short half-life [16], and fast toxicity [17]. Different methods may be used to regulate the degradation rate of the scaffolds in order to enhance GFs retention and GF release into the scaffolds [18, 19]. Calcium phosphate/biopolymer scaffolds containing chitosan microspheres (CMs) loaded with GFs with adjustable biodegradation properties are promising approaches that can be employed in controlling bone morphogenetic protein-2 (BMP-2) release rate in tissue engineering.

Nowadays, biodegradable polymers like poly lactic acid (PLA), poly glycolic acid (PGA), and their copolymers (PLGA) are widely used to control scaffold degradation [20] since they have excellent biodegradability and undergo hydrolytic degradation into natural metabolites [21, 22]. However, their degradation products (LA, GA) create a local acidic environment and can have adverse effects when used in long-term implantable systems for bone tissue engineering [23, 24]. Polyphosphazenes (PPhos) can be an alternative candidate in these systems. These polymers have been developed as tissue engineering scaffolds [25, 26] due to their excellent biocompatibility, lack of acidic degradation products, and the possibility they offer for regulating their properties by the incorporation of different side group substitutes [27]. Consequently, incorporation of BMP-2 loaded with CMs within the porous structure of calcium phosphate/polyphosphazene can serve as a model for controlling the degradation rate of scaffolds and to sustain the release of BMP-2 in bone tissue engineering.

In this work, the controlled degradation approach was used to sustain the release of BMP-2 loaded with CMs

from porous scaffolds of calcium phosphate/poly (dimethylaminoethanol)phosphazenes (CaP/PDMAEP) for bone tissue engineering applications. The characteristics and performance of these scaffolds were studied. Biodegradability, BMP-2 release rate, mechanical properties, bioactivity, and cell attachment of the scaffolds were examined in terms of biodegradation of the polymers and different CM contents. Osteogenic activity of the scaffolds was also evaluated by checking the expression of bone differentiation markers (osteonectin and osteopontin) on bone marrow stromal cells (BMSCs) seeded in the presence of the scaffolds.

2 Experimental

2.1 Synthesis and characterization of PDMAEP, CMs, and HA

Poly(organo)phosphazenes were synthesized in a two-step reaction process: (1) polydichlorophosphazene (PDCP) was made from hexachlorocyclotriphosphazene (HCCTP) using the thermal ring-opening polymerization method (Fig. 1A) [28], and (2) chlorine atoms were replaced with 2-dimethylaminoethanol (DMAE) (Fig. 1B) [29]. In the first step, 12.5, 25, 50, and 100 mg of sulfamic acid (Merck, Germany) used as the catalyst and 40 mg of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (Merck, Germany) used as the promoter were added to 6 g of HCCTP (sigma Aldrich, USA) dissolved in 5 ml of 1,2,4-trichlorobenzene (Merck, Germany). The solution was then heated to 210 °C until a viscous mixture was obtained for different times (Table 1) while it was being stirred in a nitrogen atmosphere. The mixture thus produced was added to 150 ml 1,4-dioxane (Merck, Germany).

In the second step, 15 ml of DMAE (Merck, Germany) was added to a PDCP solution and stirred at 55 °C for different times (Table 1). Since the synthetic polymer dissolves in tetrahydrofuran, the polymers obtained were purified by dialysis against tetrahydrofuran (THF, Merck, Germany). For this purpose, the polymer was dissolved in THF (200 ml) and the solution was poured in a dialysis bag (cellulose acetate with a molecular weight cut-off of 12,000–14,000 Da). After closing the ends of the bags, they were placed in a beaker containing 1 l of THF. For more purification, THF was continuously replaced. Small molecules and the non-reactive chains passed through the membrane bag. Large chain polymers remained inside the bag and the purification process was carried out. THF was then removed under reduced pressure and the polymers obtained were dissolved in water and freeze-dried at –60 °C for 8 h. Finally, the samples were stored in vacuum to avoid exposure to moisture.

Fig. 1 Synthesis of poly(2-dimethylaminoethanol)phosphazenes: **A** Synthesis of polydichlorophosphazene from hexachlorocyclotriphosphazene using the thermal ring opening polymerization method and **B** Substitution of chlorine atoms with DMAE

Table 1 Polymer designations and characteristics

Polymer	Hexachlorocyclotriphosphazene (g)	Sulfamic acid (mg)	Polymerization time (h)	Substitution time (days)
P-1	6	12.5	5.2	6
P-2	6	25	3.1	4
P-3	6	50	1.7	3
P-4	6	100	0.75	1

The chemical structure of PDMAEP was determined by nuclear magnetic resonance (NMR, AVANCE 400, Bruker) spectrometer. Gel permeation chromatography (GPC, 1100, Agilent) was used to determinate both the number-average molecular weight (M_n) and the weight-average molecular weight (M_w).

To investigate the degradation rate of polyphosphazenes, the cylindrical samples (10 mm in diameter and 5 mm in height) were soaked in phosphate buffered saline (6 ml, PBS, pH 7.4) and kept for 30 days in an incubator (37 °C, at 40 rpm). Finally, the samples were removed from the phosphate buffered saline at different times, washed with distilled water, and dried [30]. The weight loss was calculated using Eq. 1:

$$\text{Weight loss (\%)} = (W_0 - W_e)/W_0 \times 100 \quad (1)$$

where W_e and W_0 represent the weights of the polymers after and before degradation, respectively. The weight loss was reported as the average obtained for three samples.

In order to prepare CMs, the emulsion-ionic cross-linking method was used [31]. Briefly, a transparent solution of chitosan (150 mg, Merck, Germany) and aqueous acetic acid [2% (v/v), 10 ml, Merck, Germany] was prepared by mechanical stirring for 3 h at room temperature. BMP-2 (15 mg) was added and agitated for another 1 h. Then, the solution containing BMP-2 was added drop-by-drop to liquid paraffin [100 ml, 2% (w/v) of surfactant span 80] and disturbed for 3 h. Tripolyphosphate [20 ml, 5% (w/v)] was then added to the above emulsion and stirred for 2 h. Finally, the microspheres were washed with isopropyl alcohol and then freeze-dried.

HA nanoparticles were prepared using a precipitation technique [32]. The morphological characteristics and average particle size of the microspheres and HA particles were examined by scanning electron microscopy (SEM, TESCAN VEGA//XMU). The crystalline phases of HA particles were identified by the X-ray diffraction (XRD, JDX-8030) analysis.

2.2 CaP/PDMAP/CMs scaffold fabrication and characterization

The calcium phosphate/poly(DMAE)phosphazene/chitosan microspheres (CaP/PDMAP/CMs) scaffolds were fabricated in two stages. In the first stage, the three-dimensional (3-D) porous scaffolds of CaP ceramic were prepared by sintering. The details may be found elsewhere [33]. In the second stage, PDMAP/CMs solutions were prepared by adding 5 mg of PDMAP and 10, 20, and 30% on PDMAP weight basis of CMs to 1 ml of 1,4-dioxane and stirred gently at 25 °C for 5 h. The CaP porous scaffolds prepared in the first stage were then immersed in the PDMAP/CMs solution for 24 h. The samples were placed in a freezer at −70 °C for 24 h. Finally, the frozen samples were immediately freeze-dried at −80 °C for 12 h (Table 2).

Scanning electron microscopy (SEM, TESCAN VEGA//XMU) was used to examine the morphology and the average size of the scaffold pores. Average pore size was evaluated using the Image J software. Type crystalline phases of the porous scaffolds were determined before immersing into the polyphosphazene solution by X-ray diffraction (XRD, JDX-8030).

The method described by Wei et al. [34] was used to determine the porosity of the scaffolds using Eq. 2:

$$\varepsilon = (1 - D_a/D_s) \times 100\% \quad (2)$$

where ε is the porosity of the scaffolds, D_a and D_s are the measured apparent density and the skeletal density of the scaffolds, respectively.

The scaffolds were tested according to ASTM D5024-95a for their mechanical properties [35]. The degradation rate of the scaffolds was evaluated using the immersion method and Eq. 3 below [30]:

$$\text{Weight loss (\%)} = (W_0 - W_e)/W_0 \times 100 \quad (3)$$

where W_e and W_0 are the weights of the samples after and before degradation, respectively. Weight loss was reported as average of three samples.

2.3 In vitro release determination

The rate of BMP-2 release from the scaffolds was quantified using a BMP-2 ELISA kit (PeproTech, Rocky Hill, NJ). The composite scaffolds were immersed in glass vials containing 20 ml of PBS with pH 7.4 and incubated at 37 °C under orbital shaking at 40 rpm for various time periods up to 30 days. The cumulative release values of the growth factor were plotted versus time (each data point is the mean of 3 values).

2.4 Cytotoxicity, bioactivity and in vitro cell attachment

The indirect MTT assay was used to investigate the cytotoxicity of the scaffolds according to ISO/EN 10993.5 [36]. Firstly, the dissolution extracts of scaffolds were prepared by soaking the samples in the DMEM/HAMS medium for 7, 14, 21, and 28 days at 37 °C. Then, rat osteosarcoma ROS17/2.8 cells were trypsinized, pelleted, resuspended in a known amount of DMEM/HAMS medium, counted, adjusted to a density of 1×10^4 cells/ml, and incubated at 37 °C, 5% CO₂ for 24 h. Then, 80 μ l of the diluted extracts, 10 μ l DMEM and 10 μ l FBS were added to each well of the plate for 24 h. The negative control was the cells seeded into the wells with the regular DMEM/HAMS medium and 10% FBS without any dissolution extract. The positive control consisted of the DMEM/HAMS medium and 10% FBS supplemented with 0.1% Triton X-100. Finally, the media were removed from the wells and 50 μ l of the MTT solution (0.05%) was added to each well and incubated for 4 h. The MTT solution was removed, 100 μ l of isopropanol was added to each well, and the plates were swayed for 15 min. The ELISA reader was used at 570 nm to determine the optical densities (OD).

Table 2 Sample abbreviations and compositions

Samples	Type of polymers	Naphthalene/HA	CMs contents %
CaP	...	1:1	0
CaP/P-1	P-1	1:1	0
CaP/P-2	P-2	1:1	0
CaP/P-1/CMs 10	P-1	1:1	10
CaP/P-1/CMs 20	P-1	1:1	20
CaP/P-1/CMs 30	P-1	1:1	30
CaP/P-2/CMs 30	P-2	1:1	30

The bioactivity of the scaffolds was determined by measuring alkaline phosphatase (ALP) activity [37]. Briefly, the mesenchymal stem cells (MSCs) isolated, seeded with adjustment at 2×10^4 cells onto the samples, and were incubated (in 5% CO₂ at 37 °C) in the DMEM medium (100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum) for 3, 7, 14, and 21 days. The negative control was the CaP/P-1 scaffold. Then, the media were removed, 300 µl of the *p*-nitrophenyl phosphate disodium salt (PNPP) solution was added to each sample for 45 min at 25 °C before NaOH (150 µl, 2 M) was added to terminate the reaction. Finally, the release of *p*-nitrophenol from *p*-nitrophenyl phosphate was assessed as ALP activity with a spectrophotometer at 405 nm and the ALP concentrations produced by MSCs were calculated using the ALP standard.

To evaluate *in vitro* cell attachment, rat osteosarcoma ROS17/2.8 cells were isolated by soaking the bone in DMEM containing antibiotics (penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were cultured together with the scaffolds in DMEM and Ham's F12 with 10% fetal bovine serum and incubated in 5% CO₂ at 37 °C. On the third, seventh, and fourteenth days of seeding, ROS17/2.8 cells together with the scaffolds were rinsed twice with PBS and fixed with glutaraldehyde (4%) for 2 h. Then, the fixed cells together with the scaffolds were dehydrated by graded ethanol series of 10, 20, 40, 50, 70, 80, 90, 95, and 100% (v/v), each for 10 min. Finally, the dried specimens were sputter-coated with gold and imaged using a scanning electron microscope (SEM, S360, Cambridge) to investigate the cell morphology of the samples.

2.5 Western blot study

To investigate protein expression, Western blot analysis was performed. Bone marrow stromal cells (BMSCs) were seeded on CaP/P-1, CaP/P-1/CMs10, and CaP/P-1/CMs30 scaffolds for 14 and 28 days. Then, the cells were washed with cold phosphate-buffered saline (PBS), harvested with the ice-cold radio-immunoprecipitation assay (RIPA) buffer (Thermo Scientific), and centrifuged at 12,000 rpm for 10 min at 4 °C. The proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose

membranes (Bio-Rad, USA). Blots were blocked in 5% non-fat milk in TBS-0.05% Tween 20 (TBST) for 1 h. The membranes were further incubated for 1 h at room temperature with the primary antibodies of polyclonal rabbit-anti-human beta-actin (Delta Biolabs), osteonectin (ON), and osteopontin (OPN) (Abcam). Subsequently, the membrane was washed three times with TBST buffer and incubated with rabbit anti-goat horseradish peroxidase-conjugated secondary IgG (Zymed) at 1:10,000 for 1 h at RT. After washing thrice with TBST, the immunocomplex bands were detected using an enhanced chemiluminescence solution (ECL, Bio-Rad, USA) and a VersaDoc Imaging System (Bio-Rad).

2.6 Statistical analysis

All the data were averaged in triplicate samples and expressed as mean standard deviations ($\pm$ SD). Statistical analysis was performed using analysis of variance (ANOVA) by Duncan post hoc test and statistically significant values were attained as *p* values <0.05.

3 Results

3.1 Characterization of poly(DMAE)phosphazenes

The M_w , M_n , and polydispersity of poly(DMAE)phosphazenes with different catalyst concentrations are shown in Table 3. It is seen that polymerization reaction times and molecular weights of the polymers decreased with increasing catalyst concentrations (Tables 1, 3).

Figure 2A shows the ³¹P-NMR spectrum of PDMAEP in which one peak is observed at −8.7 ppm, indicating the successful replacement of chlorine with the DMAE group. The peaks for ¹H-NMR were as 2.27 [N(CH₃)₂], 2.52 (CH₂N), and 3.83 (POCH₂), and those for ¹³C-NMR were 44.8, 57.1 and 62.9. The results indicate that the necessary time for the completion of the substitution depends on polymer type (Table 1) since the other peaks are observed only with incomplete substitution (Fig. 2B) and solely one signal was anticipated at −8.7 ppm for ³¹P-NMR. The percentage of weight loss versus the maintenance time is showed in Fig. 2C. As can be seen, the weight losses of P-1 and P-2 proceed slowly during the whole degradation

Table 3 Molecular weight and polydispersity of poly(DMAE)phosphazene for different catalyst concentrations

Polymer	Catalyst concentration (mg)	M_n (kDa)	M_w (kDa)	M_w/M_n
P-1	12.5	24	420	16.8
P-2	25	17	290	17.05
P-3	50	12	130	10.83
P-4	100	7	38	5.42

Fig. 2 ^{31}P -NMR spectra of poly(2-dimethylaminoethanol)phosphazenes: **A** complete substitution, **B** incomplete substitution, and **C** percent of original mass of polyphosphazenes during hydrolytic degradation in PBS solution (pH 7.4) at 37 °C

Fig. 3 SEM micrograph of chitosan microspheres loaded with BMP-2

period while 50% of the weights of P-1, P-2, P-3, and P-4 degraded in 17, 11, 6.5, and 2 days, respectively.

3.2 Characterization of CMs and HA particles

Figure 3 shows SEM images of CMs morphology. As can be seen, CMs have a spherical shape with identical

morphology. Quantitative analysis of the evidence provided by the image J software that the CMs' size distribution was in the range of 15–45 μm and the average diameter of the microspheres was 37.4 μm .

Figure 4A, B, respectively, present the XRD patterns and the scanning electron micrographs of HA nanoparticles. All the peaks correspond to the HA ICDD card (ICDD File Card No. 9-432). The diffraction pattern corresponds to the peaks of HA phase without any impurities. SEM observation indicated that the size range of HA particles is less than 100 nm. All the particles have edges and irregular shapes. Based on the results obtained, for HA particles to be synthesized in the nano-scale, it is necessary to control process parameters; for instance, reaction temperature at 100 °C and reaction mixture pH at about 10–11.

3.3 Characterization of CaP/PDMAP/CMs scaffolds

Figure 5 shows the X-ray diffraction (XRD) patterns of the porous samples before immersion into the polyphosphazene solution. Both TCP peaks (ICDD File Card No. 9-169) and HA peaks (ICDD File Card No. 9-432) were observed at 1250 °C.

Figure 6 shows the morphology of scaffolds before and after soaking in the solution of polyphosphazene/chitosan

Fig. 4 **A** X-ray diffraction (XRD) and **B** scanning electron microscopy (SEM) of HA powders prepared by the precipitation technique at 100 °C

microspheres. It can be seen that the diameter of the porous calcium phosphate scaffolds ranged from 100 to 300 μm and that it decreased to the range from 100 to 200 μm when they were composited (or internally coated) with polyphosphazene. The average porosity of the scaffolds

with and without polyphosphazene was 77.4 and 86.7%, respectively.

The compressive properties were affected based on both polyphosphazene coating and microspheres' content. The compressive strengths of the scaffolds without (CaP) and with the polyphosphazene coating (CaP/P-1) were 1.27 and 2.83 MPa and their compressive moduli were 7.2 and 31.2 MPa, respectively.

Figure 7A, B show the *in vitro* degradation of scaffolds with changes in polymer type and different chitosan microsphere content. The weight loss of the CaP/P-1 scaffold proceeds slowly during the whole degradation period (Fig. 7A). The degradation rates of the samples increased when CMs contents increased from 10 to 30% (Fig. 7B).

3.4 Protein release

The cumulative release of BMP-2 against the maintenance time of the scaffolds with changes in polymer type and CM content are summarily presented in Fig. 8. Figure 8A shows P-1 with a lower degradability that can decrease the BMP-2 release kinetics in CaP/P-1/CMs 30 scaffolds. The release profile of BMP-2 from CaP/P-1/CMs 30 exhibits the release of the growth factor in a sustained manner without any initial higher release so that it released gradually and steadily during the whole delivery period. The effect of CMs' content on the release BMP-2 kinetics was evaluated. Figure 8B shows the relationship between the cumulative releases of BMP-2 from the CaP/P-1 scaffolds with enhancements in CMs' content from 10 to 30%. It can be seen in Fig. 8B that BMP-2 was released in a sustained manner without any burst release in all the samples. However, the slopes of the release profiles increased with enhancements in CMs' content from 10 to 30% and the cumulative release in CaP/P-1 with CMs 10, 20 and 30% were 20.8, 32.4, and 55.4%, respectively, in 15 days.

Fig. 5 The X-ray diffraction pattern of the porous ceramics before immersion into the polyphosphazene solution

Fig. 6 SEM micrographs of porous scaffolds prepared via sintering at 1250 °C: **A** without coating, **B** the red box in **A**, **C** coated with polyphosphazene, **D** the red box in **C** and **E** polymer/microsphere deposition on calcium phosphate body (microsphere shown by the red arrow). (Color figure online)

3.5 Cell viability and *in vitro* bioactivity

Figure 9A depicts the results of MTT assay of dissolution extracts from CaP/P-1/CMs 30 scaffolds containing BMP-2, CaP/P-1/CMs 30 scaffolds without BMP-2, positive control, and negative control after different extraction times. The results did not exhibit reduced cell viability when they were incubated with higher concentrations of the extracts. Moreover, the optical density values for the dissolution extracts of the scaffolds in 21 and 28 days were significantly higher (p value <0.05) than those in 7 and 14 days.

ALP activity of the cultured cells on porous scaffolds and the negative controls after different incubation periods

(3, 7, 14, and 28 days) are shown in Fig. 9B. It is seen that increased incubation periods resulted in enhanced ALP activity in all the test groups.

3.6 Cell adhesion on CaP/PDMAP/CMs scaffold

To evaluate cell adhesion on the CaP/PDMAP/CMs scaffolds, the cells were cultured together with the scaffolds and imaged by SEM. Figure 10 shows the attachment of cells to the surface of the scaffold (CaP/P-1/CMs 30) after (A) 3 days, (B) 7 days, and (C) 14 days. Clearly, the cells exhibit a normal morphology. The cells were adhered, spread with a flat shape, and extended in a polygonal shape. The results of this study indicate that CaP/PDMAP/CMS

Fig. 7 Degradability behavior of scaffolds with **A** different polymers (P-1 and P-2) and **B** different CMs (10, 20 and 30%) contents during hydrolytic degradation in PBS solution

Fig. 8 Cumulative release of BMP-2 for scaffolds with **A** different polymers (P-1 and P-2) and **B** different levels of CMs content (10, 20, and 30%)

scaffolds can be utilized in bone tissue engineering applications due to their good cell biocompatibility.

3.7 Western blot analysis

Western immunoblotting was used to analyze protein expression by BMSCs cultured on scaffold constructs after 14 and 28 days; the results are shown in Fig. 11A, B, respectively. Blots were re-probed with anti-beta-actin as the loading control. After 14 days, the band intensity at 66 kDa (OPN) is 1.3 and 2.1 times higher on CaP/P-1/CMs10 and CaP/P-1/CMs30 samples compared with that on CaP/P-1 samples, respectively. After 30 days, the band intensity at 66 kDa (OPN) is 3.2 and 4.1 times higher on CaP/P-1/CMs10 and CaP/P-1/CMs30 samples compared with that on CaP/P-1 samples, respectively.

After 14 days, the cells cultured on CaP/P-1/CMs10 and CaP/P-1/CMs30 scaffolds, respectively, showed about 1.2 and 1.7 times higher expressions of ON than those cultured on CaP/P-1 samples. After 28 days, the cells cultured on CaP/P-1/CMs10 and CaP/P-1/CMs30 scaffolds, respectively, showed about 1.8 and 2.3 times higher expressions of ON than those cultured on CaP/P-1 samples. Compared to the results in Fig. 11A, B for CaP/P-1/CMs30 scaffolds, OPN significantly (p value <0.05) increased from 2.1 to 4.1 times higher, while the ON increased from 1.7 to 2.3 times higher from days 14 to 28, respectively.

4 Discussion

Study has shown that BMPs enhance new bone regeneration in bone tissue engineering [12–14] and that BMPs release should be accomplished in a sustainable way. In this study, a controlled degradation strategy was presented to achieve sustained release of BMP-2 in CaP/PDMAEP/CMs porous scaffolds.

Polydichlorophosphazenes were synthesized with different amounts of the catalyst while chlorine atoms were replaced with 2-dimethylaminoethanol and characterized by GPC, NMR, and *in vitro* degradation. It was observed that catalyst concentration affects polymer molecular weight. While the catalyst may be present as a chain end, increasing catalyst concentrations increases the number of chain ends [38]. If true, then the growth time of polymers will be short and the average molecular weight decreases. By changing catalyst concentrations, it is possible to synthesize polyphosphazenes with different chain lengths and different molecular weights. The degradability results of polyphosphazenes were in agreement with the molecular weight results. This observation can be attributed to the number of DMAE side groups since the high percentage of altered DMAE side groups decreases the mobility of

Fig. 9 **A** MTT assay of dissolution extracts of scaffolds, negative control and positive control after different extraction times, and **B** alkaline phosphatase (ALP) activity of cultured cells on porous scaffolds with different levels of CMs content (10 and 30%) with different polymers (P-1 and P-2) and negative control after different times

Fig. 10 Cell adhesion and spreading on CaP/PPhos/CMs scaffold after **A** 3 days, **B** 7 days, and **C** 14 days of cell culture observed by SEM

polymer chains. P-1 and P-2 can be utilized to control the degradation of scaffolds and the release of growth factors due to their continual degradation. Hence, they were chosen as appropriate materials for fabricating scaffolds.

CaP/PDMAP/CMs scaffolds were prepared from naphthalene/HA by sintering at 1250 °C and freeze-drying methods. Based on XRD results, both TCP and HA peaks were observed at 1250 °C due to the instability of the HA phase at high temperatures. β -TCP peaks might appear through the HA transformation reaction to β -TCP [39]. The scaffolds should have interconnected pores with diameters of at least 100 μm for cell behavior [40] since cell viability is affected by the microstructure of the scaffolds [41]. The microstructure and the average pore size of the scaffolds were uniformly constructed from distributed pores with diameters of at least 100 μm (Fig. 6). Average porosity is an important property of scaffolds in tissue engineering applications because it determines the possibility for the insertion of nutrients, escape of waste materials, and exchange of different gases [42]. It was observed that pore size, number of interconnected pores, and average porosity decreased when the porous calcium phosphates were composited with polyphosphazene. This was because some pores were filled with polyphosphazene internal coating. The presence of CMs was not, however, observed to affect

Fig. 11 Western blot analysis protein expression on scaffolds with BMSCs cultured on the scaffold constructs after **A** 14 and **B** 28 days

the average porosity of the scaffolds due to the small average size of CMs.

The CaP/PDMP/CMs scaffolds exhibited better compressive properties than the CaP scaffolds alone. The enhanced compressive properties might be due to the reduced porosity from 86.7 to 77.4% and the reduced average pore size in the scaffolds coated with polyphosphazene. These results are in agreement with those reported elsewhere [43, 44], showing that the mechanical properties of CaP porous scaffolds can be enhanced by internal coating of polyphosphazenes and adding CMs.

An ideal tissue engineering scaffold should have a suitable degradation rate [45] for controlling GFs release and creation of space for bone regeneration. Figure 7A shows that the degradation rate of CaP/P-1 scaffold is low due to the low degradation rate in P-1 (Fig. 2C). Compared with CMs 10 and 20%, samples with CMs 30% exhibited higher weight losses (Fig. 7B), and this is most likely due to the hydrophilic behavior of chitosan [46].

The amounts of BMP-2 released from the scaffolds were measured using a BMP-2 ELISA kit. By soaking the porous scaffolds in a phosphate buffer solution (PBS), the solvent (PBS) enters into the samples through the pores and BMP-2 release takes place via a degradation mechanism. Studies have reported that the degradation properties of scaffolds play an important role in the GFs' release kinetics [18, 19]. Hence, BMP-2 release was tested in porous scaffolds prepared with different types of polymer (P-1 and P-2) and varying levels of CMs content (10, 20, and 30%). The initial higher release rate of CaP/P-2/CMs 30 was due to the higher degradation rate in P-2 and the slower release at the later stage was due to the gradual depletion of BMP-2 in the samples (Fig. 8A).

In order to evaluate the effect of CMs' content on the release behavior of BMP-2, CaP/P-1 scaffolds were prepared with different levels of CMs content ranging from 10 to 30%. Comparison of the release profiles of CaP/P-1 scaffolds containing chitosan microspheres loaded with

BMP-2 revealed that CMs content during the release phase played an important role in determining the amount of BMP-2 release. In fact, the CMs present in the scaffolds affected the degradation rates of the samples. Degradation rates of the samples increased with increasing CMs' content (Fig. 7B) due to the hydrophilic properties of chitosan [46]. Thus, the slopes of BMP-2 release profiles increased with enhancements in CMs content (Fig. 8B). Comparison of the results reported in Fig. 8B and the degradation profile of P-1 in Fig. 2C shows that the slopes of the release profiles are approximately constant due to the slope of the approximately constant degradation profile of P-1 while BMP-2 release is a sustained one with no abrupt releases. It would be possible to control the degradation rate and the GFs' release rate of these scaffolds by optimizing the CMs content and choosing the right polymer type.

The cytotoxic effects of the scaffold components and their degradation products might restrict their use in bone tissue engineering applications. Thus, the cytotoxicity potential of the dissolution extracts from the scaffolds was evaluated on osteoblast cells. The absorbance values might be proportional to the number of living cells and the indication of cell proliferation. The osteoblast proliferation increased due to the higher concentrations of BMP-2 in dissolution extracts after 21 and 28 days because BMP-2 can induce osteoblast proliferation [47]. To evaluate the effect of BMP-2 on cell growth, the dissolution extract of CaP/P-1/CMs 30 was used without BMP-2. Cell proliferation in their dissolution extracts increased at a slow trend. As a result, the CaP/PDMAP/CMs scaffolds did not show toxicity to osteoblast cells.

Bioactivity of scaffolds plays an important role in bone regeneration by tissue engineering scaffolds [48]. Studies have shown that the osteoinduction property of scaffolds enhances bone regeneration after 2–4 weeks [49, 50]. Based on the results of ALP activity, the ALP activity on CaP/P-2/CMs 30 was higher than those on CaP/P-1/CMs 30 after 3 and 7 days due to the higher BMP-2 concentration (Fig. 8A) and the higher P-2 degradation rate (Fig. 2C). The ALP activity on CaP/P-2/CMs 30 decreased after 14 and 28 days and was lower than that of CaP/P-1/CMs 30 because the BMP-2 release rate with CaP/P-2/CMs 30 decreased at later stages (Fig. 8A). ALP activity on CaP/P-1/CMs 30 was higher than that on CaP/P-1/CMs 10 at all times because BMP-2 concentration was higher in CaP/P-1/CMs 30 than in CaP/P-1/CMs 10 and BMP-2 was gradually released from both samples at all times (Fig. 8B). The present study indicated that ALP activity results were closely related to the scaffold degradation and BMP-2 release rates.

The interaction between cells and the surface of the scaffolds determines cell behavior in biomedical applications [51]. The interaction between cells and scaffold surface took place during the initial days; the cells grew to and

adhered on the scaffolds after 3 days as a multipolar extension (Fig. 10A). After 7 days, the cells exhibited a high proliferation and attached tightly to the surface of the scaffolds (Fig. 10B). After 14 days, the cells extended toward and completely covered the surface of all the scaffolds with further cell proliferation (Fig. 10C).

Osteopontin (OPN) is an important mineral-binding protein secreted by osteoblasts which are the cells responsible for bone remodeling [52]. OPN is indicative of osteogenic maturation and is associated with bone mineralization and proliferation as well as cell attachment. Osteonectin (ON) is another bone-specific protein secreted by osteoblasts, which binds to both hydroxyapatite and collagen [53]. Western blot analysis revealed that OPN and ON were expressed by seeded BMSCs on the scaffolds after 14 and 28 days, which are suitable indicators of BMSC cell differentiation toward the osteogenic pathway. OPN and ON bands were seen in all the lanes. Comparison of Fig. 11A, B (i.e., the intensity profile of protein expression) revealed that the intensities of OPN and ON bands increased in all the scaffolds over time from days 14 to 28. The Western blot results indicated a correlation between the expression of proteins (OPN and ON) and the presence of BMP-2 in the samples, because BMP-2 is a key factor in promoting bone regeneration [12].

Considering the benefits of sustainable GFs release in tissue engineering scaffolds, a novel porous scaffold was fabricated in this study from calcium phosphate and poly(DMAE)phosphazene with controlled biodegradation for the sustained release of BMP-2. Polyorganophosphazenes form a large number of polymers due to the wide variety of side groups such as alcohols, phenols, and amines. The polyphosphazenes have been employed in previous studies to manufacture tissue engineering scaffolds and drug delivery systems in the form of nanoparticles, substitution with different side groups, and complexed with other polymers [54, 55]. In this study, polyphosphazene with DMAE side groups was used to control the degradation rate and BMP-2 release in tissue engineering scaffolds. It was found that calcium phosphate/polyphosphazenes scaffolds promote cell proliferation and enhance ALP activity of osteoblast cells. Moreover, the results indicate that these systems have a potential application in controlled growth factor release and could serve as an improved alternative to calcium phosphate scaffolds for bone tissue engineering applications.

Compliance with ethical standards

Conflicts of interest The authors have no financial conflicts of interest.

Ethical statement There are no animal experiments carried out for this article.

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