Indian Journal of Chemical Technology
Vol. 31, March 2024, pp. 298-306
DOI: 10.56042/ijct.v31i2.9222

Pro-osteogenic activity of soybean derived bioactive peptides on the surface of titanium sheets

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Received 13 March 2023; accepted 21 November 2023

In this study, a group of active peptides named P3 isolated from soybean protein is used to modify the surface of titanium sheets. The modified titanium sheets are characterized by X-ray photoelectron spectroscopy and contact angle measurement. The effects of modified titanium sheets coated with P3 on the adhesion, proliferation, differentiation and mineralization of MC3T3-E1 cells are investigated. Amino acid analysis showed that the hydrophobic amino acids and aromatic amino acids are enriched in P3. After being coated with P3, the surface nitrogen content of titanium sheets is significantly increased in a dose-dependent manner. The contact angle and surface hydrophobicity of titanium sheets are significantly changed ($p < 0.05$). In addition, the adhesion and proliferation activity of MC3T3-E1 cells on titanium sheets are significantly enhanced by P3 coating ($p < 0.05$). Further studies indicated that P3 coating increased the expression of alkaline phosphatase, osteocalcin, consequently promoting the differentiation and mineralization of MC3T3-E1 cells on the surface of titanium sheets. In conclusion, P3 coating enhances the surface hydrophobicity, leading to a significant increase in the cell adhesion, consequently promoting the differentiation and mineralization of MC3T3-E1 cells on the surface titanium sheets, suggesting that P3 may serve as a coating material for titanium-based implants to facilitate fracture healing and other applications.

Keywords: Osteoblast adhesion, Pro-osteogenic activity, Soybean derived bioactive peptide, Surface modification, Titanium implant

Introduction

Bone diseases such as arthritis, osteoporosis, bone defects and fractures caused by diseases, trauma or aging have seriously affected human health. The common treatment for bone diseases is internal fixation using bone implants or bioactive scaffolds[1]. At present, the latest research progress is to make composite scaffolds using biological materials and active substances for their application in the field of bone engineering. Unfortunately, these materials are still far away from the clinical application. In a clinical standpoint, titanium and titanium alloy are currently the most ideal and widely used implants[2]. However, the bioinertness of titanium makes it difficult to integrate with the surrounding bone tissue in the early stage of implantation. Currently, the bone integration ability of titanium implants is primarily improved via modification[3], such as the functional modification of titanium materials by loading proteins, peptides and bioactive compounds[4-6], enhancing the proliferation and differentiation of osteoblasts around implantation, and promoting the combination of titanium implants with bone tissue[7;8]. The active peptides used for modification are mainly polypeptides or oligopeptides with the function for promoting cell adhesion, proliferation and differentiation, such as RGD peptide, fibronectin, etc. A study combining the active cyclopeptide (cRGDfK) with titanium sheets, resulted in a good bone integration performance of the titanium sheets.[9] This modification promoted the osteoblasts adhesion, proliferation and differentiation on the surface of implants. However, most of these bioactive components used in the surface modification of titanium sheets are synthetic polypeptides. The high cost of synthesis limits their application in the field of bone engineering.

Soybean protein is a vegetable protein with nutritional values equivalent to animal protein. Recent studies have shown that soybean protein has certain advantages in preventing bone diseases and treating osteoporosis. A soybean protein containing diet significantly improved the biomechanical properties of tibia and bone tissue in ovariectomized rats[10]. Soy protein has also been developed as bone adhesives, hydrogels and other materials for application in the

field of bone engineering. For example, soybean protein, as a raw material, was combined with sodium carboxymethyl cellulose dramatically enhanced bone adhesive strength.[11] Natural soybean active peptides are small molecular products after proteases hydrolysis. Compared to soybean protein, soybean active peptides have the advantages of low sensitization, high biological activity and easy digestion and absorption. One of our previous works identified P3, a group of bioactive peptides from soybean protein isolates by two-step enzymatic hydrolysis[12]. Subsequent activity studies demonstrated that P3 stimulated the proliferation rate of osteoblasts and promoted the differentiation and mineralization of osteoblasts.

In the current study, titanium sheets were modified by coating with P3. The surface characterizations of P3-coated titanium were evaluated by contact angle measurements and X-ray photoelectron spectroscopy (XPS). In addition, we confirmed that P3 maintained its osteogenic activities after the coating process. Our finding provides a potential application for realizing high-value utilization of soybean protein and developing natural functional ingredients for biochemical modification of bone implants and related applications.

Experimental Section

Preparation and amino acid analysis of P3

Using the osteoblast proliferation assay as an indicator, peptides with pro-osteogenic activity from the soybean protein isolates were obtained by two-step enzymatic hydrolysis according to the protocol previously described[12]. Briefly, 5% (w/v) soybean protein isolates were hydrolyzed by papain (Beijing Solarbio Science & Technology Co., Ltd., China) (enzyme: substrate ratio at 3000 U/g) in phosphate buffer (pH 7.0) at 50 °C for 6 h. The enzymatic hydrolysates were fractionated using ultrafiltration membranes with different molecular weight cut-offs at 4 °C (Amicon, Cambridge, MA, USA). The crude <10 kDa fractions that induced high MC3T3-E1 cell proliferation were further hydrolyzed by alkaline protease (Beijing Solarbio Science & Technology Co., Ltd., China) (enzyme: substrate ratio of 3000 U/g) in phosphate buffer (pH 8.4) at 55 °C for 0.5 h. The secondary enzymatic hydrolysates were separated by gel filtration chromatography (Sephadex G-15; column size, 25 mm × 150 mm) into several fractions and the amino acid composition of P3 were analyzed by L8900 (Hitachi, Japan) equipped with a cation exchange column (4.6 mm × 60 mm, 3 μm) [13].

P3 coated titanium sheets preparation and characterization

Preparation of titanium sheets modified with P3

Titanium sheets (20 mm × 20 mm in size and 0.3 mm thick) were polished with metallographic sandpaper (80, 240, 800, 1200 mesh) and washed with acetone, 75% ethanol and double distilled water in an ultrasonic cleaner for 20 min, separately[14]. After sterilization by high temperature and pressure, the titanium sheets were placed in PBS with or without indicated concentrations (100 μg/mL, 300 μg/mL and 500 μg/mL) of P3 at 37 °C for 8 h. After coating with P3, the titanium sheets were rinsed with distilled water and dried at room temperature[15].

Determination of surface element composition of titanium sheets

The surface elemental composition was characterized by X-ray photoelectron spectroscopy (Thermo Fisher, ESCALAB 250XI, Waltham, MA, USA) and analyzed using XPS PEAK 4.1 software[16]. The parameters of the spectrometer were set as follows: (1) X-ray laser source was Al: K-Alpha radiation, 150 W; Vacuum pressure: 2×10^{-7} Pa; (3) Resolution: 0.7 eV / 104 CPS; Full scanning range: 0-1200 eV.

Determination the adsorption stability of P3 on the surface of titanium sheets

The P3 coated titanium sheets were placed in 6-well plates, and 2 mL PBS solution was added to each well and placed in a 37 ℃ incubator. The PBS solution was replaced in every 24 h. After 12, 24, 48, 72 and 96 h, the titanium sheets were taken out and washed with ultra-pure water. After air dried, the surface element composition was measured. The adsorption of active peptide was characterized by the content of N element.

Determination the contact angle on the surface of P3 coated titanium sheets

JY-82 contact angle measuring instrument (Dingsheng Testing Machine Testing Equipment Co., LTD., China) was used to measure the static contact angle on the surface of titanium sheets[16]. With deionized water as the test liquid, the contact diameter between the test droplet and the titanium sheet surface was controlled to be about 2 mm under the conditions of 20 ℃ and 40% RH. At least 6 macro-separated areas on the titanium sheets were selected for measurement, and the average value was obtained with the relative error less than 1%.

Cell adhesion, proliferation, differentiation and mineralization assays on P3-coated titanium sheets

Cell culture

The MC3T3-E1 cell line was purchased from the Cell Bank of Type Culture Collection (CBTCC, Chinese Academy of Sciences, Shanghai, China). MC3T3-E1 cells were cultured in growth medium (Minimum Essential Medium-α, MEM-α, with 10% FBS)[12]. All cells were cultured in a 5% CO_2 humidified incubator at 37 °C. For differentiation, the growth medium was replaced with differentiation medium (DM) (MEM-α supplemented with 10% FBS, 4 mM β-glycerophosphate, and 50 μg/mL ascorbic acid). Fresh DM was applied every 72 h with or without indicated concentrations of P3.

Cell adhesion assay

P3-coated titanium sheets were placed in a 6-well plate and MC3T3-E1 cells (2×10^5 cells per well) were seeded in corresponding wells. After incubation for 30, 60, 120 and 180 min, cells were digested from P3-coated titanium sheets using trypsin-EDTA (0.05%) and quantified using a blood cell counting chamber. Cell adherence was calculated using the following formula: cell adherence rate (%) = number of adherent cells/number of cells inoculated × 100%.

Cell proliferation assay

After cultured for 24 and 48 h, MTT and Acridine orange staining assays were used to determine MC3T3-E1 cell proliferation on P3-coated titanium sheets.[12] After removing the medium, MC3T3-E1 cells on P3-coated titanium sheets were fixed with 0.1% Acridine orange staining solution and then washed thrice with distilled water. Cells were photographed using an inverted fluorescence microscope under an excitation light of 520 nm.[17]

Cell differentiation and mineralization assays

Alkaline phosphatase (ALP) (ALP colorimetric assay kit, Beyotime Biotech, Beijing, China) activities were determined at 7 days after exposed to DM. Osteocalcin (OC) (OC ELISA kit, Nanjing Jiancheng Biological Technology, Nanjing, China) activities were determined after 14 and 21 days exposed to DM, respectively, for estimation of differentiation of MC3T3-E1 cells grown on P3-coated titanium sheets[18].

Cells cultured on days 14 and 21 were assayed for Alizarin Red S staining to estimate the number of calcium deposits. After washing thrice with PBS, cells were fixed with 75% ethyl alcohol, treated with 2% Alizarin Red S staining solution and then washed thrice with distilled water. To quantify calcium deposits, the stained cells were solubilized in 10% cetylpyridinium chloride in 10 mM sodium phosphate buffer (pH 7.0). The absorbance was measured at a wavelength of 562 nm[19].

Statistical analysis

All experiments were conducted at least three times, and the data are presented as mean ± SEM (n=3). Student t-test was conducted to examine the difference between two groups/treatments. One-way ANOVA with post hoc Tukey's test was applied when multiply groups/treatments were compared. $P < 0.05$ was considered to indicate a statistically significant result.

Results and Discussion

Amino acid compositions characteristics of P3

The amino acid compositions of P3 were shown in Table 1. Compared to the two-step enzymatic hydrolysates, hydrophobic amino acids and aromatic amino acids in P3 were increased 1.49 ± 0.05 fold and 2.22 ± 0.07 fold, respectively. Previous studies have shown that the hydrophobic interaction is the main driving force for the adsorption of protein and active peptide on titanic-based materials[20]. Therefore, enriched hydrophobic amino acids and aromatic amino acids in P3 may facilitate its being adsorbed on the surface of titanium sheets.

Characterization of P3 coated titanium sheets

Changes of element compositions on the surface of P3 coated titanium sheets

The nitrogen (N) content on the surface of titanium sheets reflects the adsorption of soybean active peptides on the surface of titanium sheets[15]. The compositions of elements on the surface of titanium sheets modified with P3 peptides were shown in Table 2. Titanium (Ti) (20.53 ± 0.13 %), oxygen (O) (48.72 ± 0.23 %), carbon (C) (29.04 ± 0.27 %) and a small amount of N (0.17 ± 0.04 %), sulfur (S) (1.44 ± 0.08 %) were detected on the surface of titanium sheets without any coating, serving as a control. Compared to the control group, the surface N contents of titanium sheet after 100 μg/mL, 300 μg/mL and 500 μg/mL P3 modification were significantly increased up to 5.63 ± 0.03 %, 6.97 ± 0.13 % and 7.76 ± 0.09 %, respectively ($P < 0.05$), indicating that P3 peptides were successfully adsorbed on the surface of titanium sheets. Similarly, relative C contents were increased whereas relative Ti contents were decreased in a P3 dose dependent manner.

Table 1 — Main factional amino acid compositions of soybean active peptides

Amino acids		Amino acid content (g/100 g)	
		Two-step enzymatic hydrolyates	P3
Hydrophobic amino acids	Tyr Phe Val Leu Ile Ala Pro Met	36.64±1.05[a]	54.44±1.84[b]
Aromatic amino acids	Phe Tyr	12.02±0.51[a]	26.63±0.78[b]

Data were presented as Mean ± SEM (n = 3 in each group). Different letters in the same row represented significant differences based on Tukey's post-test ($p < 0.05$)

Table 2 Surface element composition of titanium sheets

Element (%)	P3			
	0 μg/mL	100 μg/mL	300 μg/mL	500 μg/mL
C	29.04 ± 0.27[a]	30.41 ± 0.63[b]	34.48 ± 0.13[c]	42.12 ± 0.72[d]
N	0.17 ± 0.04[a]	5.63 ± 0.03[b]	6.97 ± 0.13[c]	7.76 ± 0.09[d]
O	48.72 ± 0.23[c]	44.54 ± 0.65[b]	45.89 ± 0.18[b]	38.20 ± 0.64[a]
S	1.44 ± 0.08[b]	1.34 ± 0.07[a]	1.41 ± 0.17[ab]	1.58 ± 0.04[c]
Ti	20.53 ± 0.13[a]	18.08 ± 0.35[b]	11.26 ± 0.33[c]	10.33 ± 0.05[d]

Data were presented as Mean ± SEM (n = 3 in each group). Different letters in the same row represented significant differences based on Tukey's post-test ($p < 0.05$)

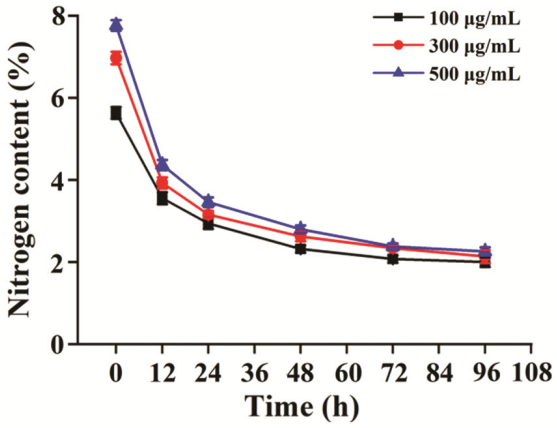

Fig. 1 — Nitrogen content on the surface of titanium sheets coated with indicated concentrations of P3 within 96 h

Stability of adsorbed P3 on the surface of titanium sheets

To investigate the stability of adsorbed P3 on the surface of titanium sheet, P3 coated titanium sheets were incubated in PBS solution with different time (from 12 to 96 h). As shown in Fig. 1, after the P3 coated titanium sheets were incubated in PBS solution for 12 h, the nitrogen content decreased from 5.63 ± 0.03 % to 3.55 ± 0.14 % at 100 μg/mL, from 6.97 ± 0.13 % to 3.92 ± 0.13 % at 300 μg/mL, and from 7.76 ± 0.09 %, to 4.36 ± 0.11 % at 500 μg/mL, indicating that the active peptides adsorbed on the surface of titanium sheets were released. During incubation for 24-48 h, a decrease in the nitrogen content slowed down. After 48 h, the nitrogen content on the surface of titanium sheets was stabilized, and the nitrogen contents on the surface of titanium sheets remained above 2.22% at 96 h, indicating that the certain amount of P3 peptides could be stably adsorbed on the surface of titanium sheets.

Surface hydrophobicity of titanium sheets after P3 coating

The contact angle of the material surface is related to its hydrophilicity[16]. To investigate whether P3 coating enhanced surface hydrophobicity of titanium sheets, the P3 coated titanium surface contact angle was measured. As shown in Table 3, for the control group, the average contact angle (θ) of titanium surface was 51.48 ± 0.93°. After P3 modification with different concentration, the average contact angle increased up to 55.01 ± 0.55°, 57.47 ± 0.40° and 58.15 ± 0.48°, respectively. The surface hydrophobicity of titanium sheets increased significantly ($p < 0.05$), which presumably was caused by the adsorption of polypeptides containing hydrophobic amino acids on the surface of titanium sheets.[9]

MC3T3-E1 cells adhesion and proliferation on the surface of P3 coated titanium sheets

MC3T3-E1 cell adhesion

Cell adhesion to materials is a fundamental prerequisite for cell growth and differentiation.[21;22] Previous studies have shown that bone implants with certain hydrophobicity promote the osteoblast adhesion and bone formation[23]. To assess MC3T3-E1 cell adherence to P3-coated titanium sheets, adhesion assays were performed within 180 min. Table 4 listed the adhesion rate of MC3T3-E1 cells on the surface of P3 coated titanium sheets within 30-180 min of

Table 3 — Contact angle of the P3-coated titanium sheets

	P3			
	0 μg/mL	100 μg/mL	300 μg/mL	500 μg/mL
Contact Angle (°)	51.48 ± 0.93[a]	55.01 ± 0.55[b]	57.47 ± 0.40[c]	58.15 ± 0.48[c]

Data were presented as Mean ± SEM (n = 3 in each group). Different letters in the same row represented significant differences based on Tukey's post-test ($p < 0.05$)

Table 4 — Rate of MC3T3-E1 cell adherence on P3-coated titanium sheets

Group	Cell adhesion rate(%) at different culture time (min)			
	30	60	120	180
0 μg/mL	17.43 ± 0.74[a]	28.73 ± 0.66[a]	32.38 ± 0.90[a]	48.75 ± 0.93[a]
100 μg/mL	19.65 ± 0.46[b]	30.84 ± 1.21[b]	34.34 ± 0.49[b]	50.59 ± 1.95[a]
300 μg/mL	26.20 ± 1.13[c]	34.28 ± 0.35[c]	38.43 ± 0.66[c]	53.71 ± 1.80[b]
500 μg/mL	27.68 ± 1.70[c]	35.68 ± 0.99[c]	38.74 ± 1.05[c]	53.86 ± 2.18[b]

Data were presented as Mean ± SEM (n = 3 in each group). Different letters in the same row represented significant differences based on Tukey's post-test ($p < 0.05$)

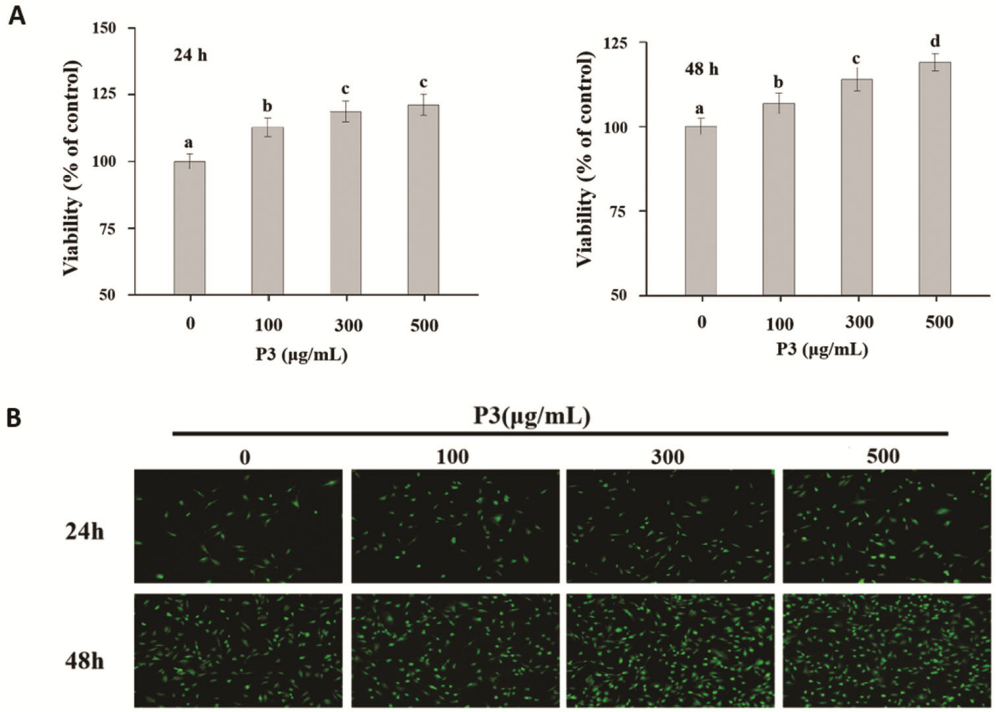

Fig. 2 — P3 coating increased MC3T3-E1 cell proliferation on the surface of titantium sheets . (A) Cell viability of MC3T3-E1 cells cultured with indicated concentrations of P3 coated on titantium sheets for 24 and 48 h. (B) Fluorescent acridine orange staining of MC3T3-E1 cells cultured with indicated concentrations of P3 coated on titantium sheets for 24 and 48h, the magnification is 40. The figures were representative of three independent experiments. Graph bars marked with different letters represented significant differences based on Tukey's post-test ($p < 0.05$)

culture. Coating P3 on the surface of titanium sheets significantly increased the adherence rate of MC3T3-E1 cells over time in a P3 dose-dependent manner ($p < 0.05$) and reached a plateau at 300 μg/mL of P3. This indicates that the modification of P3 promotes the adhesion of MC3T3-E1 cell on the surface of titanium sheets.

MC3T3-E1 cell proliferation

MTT assay was used to study the effect of active peptide modification on osteoblast proliferation on the surface of titanium sheets[24]. The results of MTT assay were shown in Fig. 2A. Compared to the control group, P3 coating significantly improved the MC3T3-E1 cell proliferation on the surface of titanium sheets

in a concentration-dependent manner ($p < 0.05$). After 24 h and 48 h of culture, the MC3T3-E1 cell proliferation on 500 μg/mL of P3 coated titanium surface were increased to 121.19 ± 3.79 % and 119.04 ± 2.52%, respectively. To confirm this result, cells on the surface of the P3 coated titanium sheets were analyzed under a fluorescence microscope after acridine orange staining. After 24 h and 48 h of culture, the number of cells on the surface of titanium sheets were significantly increased (Fig. 2B) in a P3 concentration dependent manner. From the results of MTT assay and acridine orange staining it may be concluded that P3 coating promotes the proliferation of osteoblasts on the surface of titanium sheets.

MC3T3-E1 cells differentiation and mineralization on the surface of P3-coated titanium sheets

MC3T3-E1 cells differentiation

Alkaline phosphatase (ALP) is one of the indicators of predifferentiation of osteoblasts[25;26], whose main function is to hydrolyze organophosphates and release inorganic phosphates[22]. The ALP activity of MC3T3-E1 cells on the surface of titanium sheets was evaluated on day 7 after exposure to DM.[27] Compared to control group, the ALP activity levels of cells on the surface of P3 coated titanium sheets significantly increased in a P3 dose-dependent manner (Fig. 3A). These results indicated that the modification the surface of titanium sheets with P3 significantly increased the early stage differentiation of osteoblasts.

Osteocalcin (OC) is a late osteoblastic differentiation marker[26;28]. To assess the expression of OC in MC3T3-E1 cells on P3-coated titanium sheets, the content of OC was determined by ELISA on days 14 and 21 after DM exposure.[29] As shown in Fig. 3B, P3 coating significantly increased the expression level of OC in MC3T3-E1 cells in a P3 dose-dependent manner ($p < 0.05$) on the surface of titanium sheets, indicating that the modification with P3 promoted the late differentiation of osteoblasts.

MC3T3-E1 cell mineralization

The bone extracellular matrix maturates at the end of differentiation and is subsequently mineralized to bone tissue. Alizarin red binds specifically to the nodules that is then redissolved by surfactants such as Cetylpyridine chloride (CPC) and released into solution[30]. Therefore, mineralization of MC3T3-E1 cells on titanium sheet surface was observed based on Alizarin Red S staining on day 21 after exposed to DM.[31] As shown in Fig. 4, compared to the control group, numerous mineralized calcium nodules were detected on the surface of titanium coated with P3 in a dose-dependent manner ($p < 0.05$). Therefore, P3 coating accelerated the mineralization of MC3T3-E1 cells on the surface of titanium sheet.

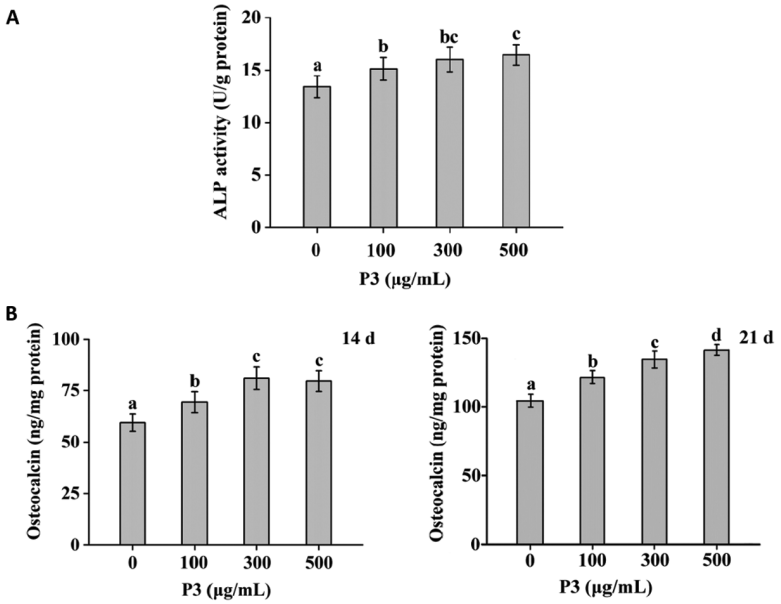

Fig. 3 — P3 coating stimulated MC3T3-E1 cell differentiation on the surface of titantium sheets. (A) ALP activity of MC3T3-E1 cells cultured on the surface of titantium sheets coated with indicated concentrations of P3 after 7 days exposed to DM. (B) Osteocalcin of MC3T3-E1 cells cultured on the surface of titantium sheets coated with indicated concentrations of P3 for 14 and 21 days after exposed to DM, respectively. Graph bars marked with different letters represented significant differences based on Tukey's post-test ($p < 0.05$)

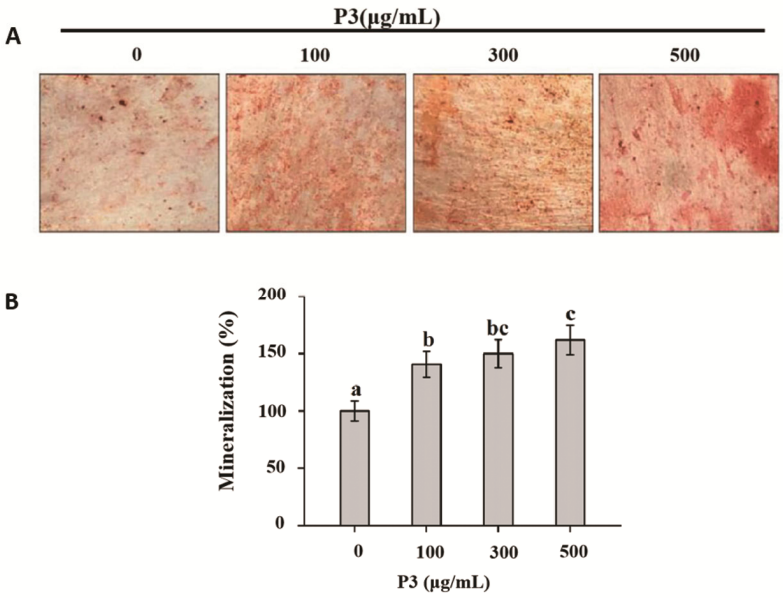

Fig. 4 — P3 coating stimulated MC3T3-E1 cell mineralization on the surface of titantium sheets. (A) Alizarin Red S staining of MC3T3-E1 cells cultured on the surface of titantium sheets coated with indicated concentrations of P3 after 21 days exposed to DM. (B) Quantitative analysis of Alizarin Red S staining of MC3T3-E1 cells cultured as panel A. The images were representative of three independent experiments. Graph bars marked with different letters represented significant differences based on Tukey's post-test ($p < 0.05$).

Previous studies have shown that titanium coated with compounds exhibits good biocompatibility and may represent an ideal implant material for bone integration in patients with osteoporotic fractures[14;32;33]. To explore the possibility of using natural small molecule P3 isolated from soybean as a coating material for titanium-based implants, we investigated the biological function of P3 on titanium sheets. We have successfully obtained P3-coated titanium sheets and confirmed that P3 has high potential as a coating material for surface modification of titanium implants to improve their osteogenic properties, which facilitates their application to treat bone diseases.

The adsorption behaviour of active peptides on the solid surface is related to the hydrophilicity/ hydrophobicity of the amino acids that compose the peptide. This is mainly because the hydrophobic interaction is the main driving force for the adsorption of active peptides on the surface of titanium sheets[34]. Under normal atmospheric environment, a layer of oxide (titanium dioxide (TiO_2)) film will be formed on the surface of titanium sheets[35]. The oxide film contains hydroxyl complex, which provides acidic ($-O^-$) or alkaline ($-OH_2^+$) surface that has a strong adsorption effect on proteins and other biological molecules[36]. In this study, titanium sheets were modified by soybean active peptides those were obtained by two-step enzymatic hydrolysis. The results of XPS and contact angle measurement confirmed that soybean active peptides were successfully loaded on the surface of titanium sheets, which were mainly attributed to the high contents of hydrophobic amino acids in the active peptides. The adsorption of hydrophobic amino acids improves the hydrophobicity of titanium sheets. Previous studies have shown that the application of high hydrophobic functional groups coating on titanium implants significantly improved the activity of osteoblasts, promoted cell differentiation and accelerated the formation of new bone in osteoporotic rats[23]. Consistently, the current study demonstrated that P3 coating promoted the adhesion of osteoblasts and early bone formation on the surface of titanium implants. Moreover, P3 contains more aromatic amino acids, which makes it have good antibacterial and anti-inflammatory activities and maintains biohomeostasis. Bacterial infection and inflammatory responses caused by implants are another major factors limiting the effect of osseointegration on bone implants[37;38]. Therefore, subsequent studies can further improve the success rate of soybean active peptides-loaded biomaterials implantation from the perspective of anti-inflammatory and antibacterial.

Bone homeostasis is achieved through the regulation of osteoblasts and osteoclasts to exert the function of

new bone formation and local bone resorption.[39] Osteoblasts are the main body of bone formation, whose main function is to synthesize and secrete alkaline phosphatase and synthesize non-collagen proteins, such as osteocalcin. As such, enhancing the activity of osteoblasts can directly promote bone formation. Osteoblasts undergo four stages, *i.e.*, proliferation, early differentiation, late differentiation and mineralization, during the process of body osteogenesis.[40] Accordingly, this study characterized the activity of osteoblasts on the surface of titanium sheets at four different stages by MTT assay, ALP activity assay, OC content assay and mineralized nodules assay respectively. The results confirmed that compared to unmodified titanium sheets, the proliferation rate, differentiation and mineralization degree of osteoblasts on titanium sheets loaded with P3 soybean active peptides were significantly increased. One of our previous studies demonstrated that ERK1/2 and p38 MAP kinase signalling pathways of cells were activated in cell medium with P3 soybean active peptide components, which stimulated the proliferation, differentiation and mineralization of MC3T3-E1 cells, respectively.[12]

Unfortunately, this study has not examined the molecular mechanism of soybean active peptide-induced changes in the activity of osteoblasts on the surface of titanium implants. In addition, the influence of soybean active peptide-modified titanium implants on the proliferation and differentiation of surrounding osteoblasts and the status of bone integration will need to be further explored in vivo in the future study.

Conclusion

In summary, a group of soybean active peptides named P3 enriched in hydrophobic amino acids and aromatic amino acids were successfully coated on the surface of titanium sheets. P3 coating enhanced the surface hydrophobicity, leading to a significant increase in the MC3T3-E1 cell adhesion on titanium sheet. In addition, P3 coating promoted the MC3T3-E1 cell proliferation, differentiation and mineralization on the surface of titanium sheets. Therefore, this study provides a novel coating material for bone implants to treat a variety of bone diseases.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (32172203, 32172139, the Excellent Postdoctoral Program of Jiangsu Province, the Natural Science Foundation of Jiangsu Province of China (BK20211292), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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