Colloids and Surfaces B: Biointerfaces 169 (2018) 233–241

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Micro-porous polyetheretherketone implants decorated with BMP-2 via phosphorylated gelatin coating for enhancing cell adhesion and osteogenic differentiation

Jiuping Wu[a,b,c], Linlong Li[a,e], Chuan Fu[b], Fan Yang[a], Zixue Jiao[a], Xincui Shi[a,e], Yoshihiro Ito[d], Zongliang Wang[a,e,*], Qinyi Liu[b,**], Peibiao Zhang[a,e,*]

[a] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China
[b] Department of Orthopaedics, The Second Hospital, Jilin University, Changchun 130041, PR China
[c] Department of Orthopaedics, First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, PR China
[d] Nano Medical Engineering Laboratory and Emergent Bioengineering Materials Research Team, RIKEN Center for Emergent Matter Science, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[e] University of Chinese Academy of Sciences, Beijing 100039, PR China

ARTICLE INFO

Keywords:
Polyetheretherketone (PEEK)
Phosphorylated gelatin
BMP-2
Cell adhesion
Osteogenesis

ABSTRACT

Polyetheretherketone (PEEK) is a high-performance semicrystalline thermoplastic polymer that is widely used in the orthopedics treatment. However, due to its biological inertness, the surface modification of PEEK using different methods to improve the biocompatibility remains a significant challenge. Herein, we attempted to use the covalently coating of phosphorylated gelatin loaded with bone morphogenetic protein 2 (BMP-2) on hydroxylated micro-porous PEEK films for enhancing the biological activity. Environmental scanning electron microscope (ESEM), fourier transform infrared spectroscopy (FT-IR), X-ray photoelectron spectroscopy (XPS) and water contact angle measurements were applied to characterize the surface of modified or untreated PEEK films. The influence on cell adhesion, proliferation and differentiation was evaluated by culturing of mouse pre-osteoblasts (MC3T3-E1) on different modified PEEK substrates *in vitro*. Surface characterization showed that the modification was successfully performed on PEEK films. The biological results indicated that surface modification of micro-porous PEEK using phosphorylated gelatin significantly promoted cell adhesion and proliferation. And the osteogenic differentiation was effectively improved while loading with different amounts of BMP-2. Findings from this study indicated that this novel biological modification on PEEK films might be helpful for altering its biological inertness and further expand its medical applications as a kind of orthopedic implants.

1. Introduction

Polyetheretherketone (PEEK) is a member of polyaryletherketone family, which has an aromatic backbone combining ketone and ether functional groups between the aryl rings [1]. Based on the excellent mechanical properties, broad chemical resistance, high temperature durability, non-toxicity, bone-like stiffness and radiolucency, PEEK has become an optimal thermoplastic candidate for replacing metal implants in orthopedic applications [2,3]. PEEK was first synthetized by British scientists through nucleophilic displacement in 1972 [4]. Since then, PEEK was widely used in industrial fields, such as aircraft, automobile, machine manufacturing and chemical products. By the late 1990s, as an implantable biomaterial approved by US Food and Drug Administration (FDA), PEEK had been applied in various orthopedic fields, especially for interverbral fusion cages [2]. Compared with conventional metal biomaterials, the elastic modulus of PEEK is extremely similar to human cortical bone, which could maximally avoid the stress-shielding effect and peri-implant bone resorption [5]. However, biological intertness of PEEK severely limits its applications for cell adhesion, growth and osteogenesis.

As a consequence, it is essential to modify its surface properties using different methods. Many physical or chemical methods were applied for functional modification of PEEK, such as electron deposition titanium, thermal plasma spray deposition hydroxyapatite and wet-chemistry method [6–8]. Combination of several different methods appears more attractive to induce tissue responses and provide a set of

* Corresponding authors at: Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China.
** Corresponding author.
E-mail addresses: wangzl@ciac.ac.cn (Z. Wang), 1226527208@qq.com (Q. Liu), zhangpb@ciac.ac.cn (P. Zhang).

https://doi.org/10.1016/j.colsurfb.2018.05.027
Received 13 September 2017; Received in revised form 20 March 2018; Accepted 13 May 2018
Available online 15 May 2018
0927-7765/ © 2018 Published by Elsevier B.V.

powerful signals for cell adhesion and differentiation. Unfortunately, in virtue of its stable chemical and mechanical properties, it seems to be difficult to render enough biological activity. Several studies had demonstrated that functionalization with some groups such as hydroxyls (−OH), carboxyls (−COOH) and amines ($-NH_2$) on the surface of PEEK films could improve cell attachment [9,10]. In addition, what is more meaningful, these functional groups provide a possibility for covalently bonding to different kinds of specific bioactive agents. The hydroxyl groups (−OH) were selectively generated from carbonyl groups on the PEEK surface via sodium borohydride assisted reduction in dimethylsulfoxide (DMSO) [11].

Gelatin is an irreversibly hydrolyzed form of collagen which presents mainly in bone and skin. As a kind of nonspecific degradable biomaterials, gelatin has shown excellent biocompatibility, adhesiveness and chemical stability [12,13]. Gelatin was identified as a safe and effective excipient by FDA, which was currently used as a constituent of various biomaterials in the biomedical applications [14]. Besides, phosphonated gelatin was synthesized by our collaborators to enhance cell adhesion and growth on titanium implants [15,16]. On the other hand, bone morphogenetic proteins (BMPs) are the most potent osteoinductive factors [17]. Among them, bone morphogenetic protein 2 (BMP-2) is currently being applied in tissue engineering to stimulate production of bone growth through increasing activation of the BMP signaling pathways [18]. Hence, as shown in Scheme 1, in this study, we attempted to enhance cell adhesion and osteogenic differentiation of mouse pre-osteoblasts (MC3T3-E1) through covalently modification of micro-porous PEEK films by phosphorylated gelatin loading with BMP-2.

2. Experimental section

2.1. Materials and methods

PEEK films (APTIV® 1000, 100 μm in thickness) were acquired from Victrex (England). Porcine gelatin was purchased from Sigma-Aldrich (USA). Recombinant human BMP-2 was obtained from BeiJingWishbiotechnology Co., LTD (China). Sodium borohydride ($NaBH_4$, 99%) was purchased from Beijing Chemical Works (China). The chemical reagents were used as received.

2.2. Preparation of micro-porous PEEK films

Before the sulfation treatment, all the PEEK films were rinsed by ultrasonic for twice with acetone, ethanol and distilled water, respectively. Subsequently, the films were dried under vacuum at 60 °C for 4 h. Then the undefiled PEEK films were immersed into concentrated sulfuric acid at room temperature for 30 s under ultrasonic condition and removed out quickly in order to create porous structure on the surface of PEEK. Then they were rinsed thoroughly with distilled water and methanol successively. Finally, the obtained micro-porous PEEK films were vacuum dried at room temperature and stored under nitrogen.

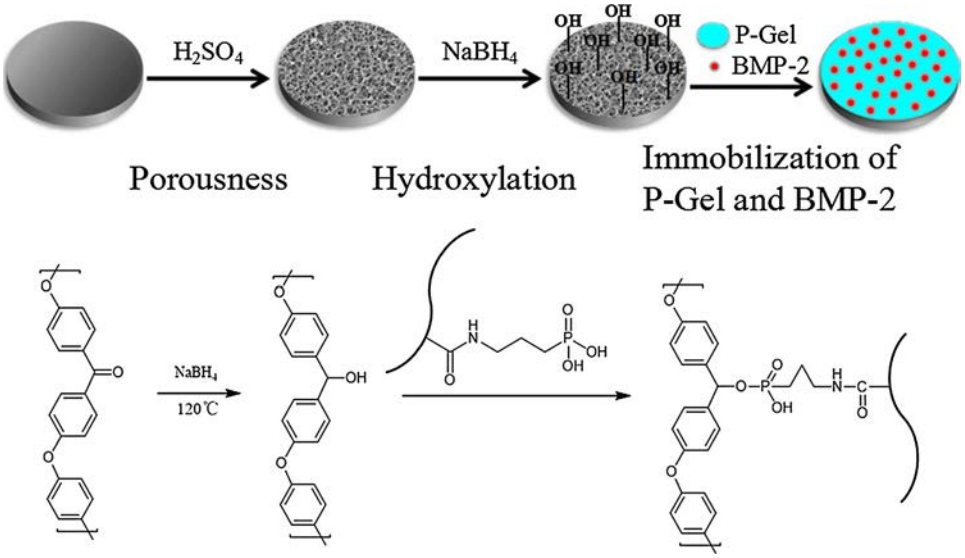
2.3. Hydroxylation of micro-porous PEEK films

The hydroxylation of PEEK films was achieved through the reduction reaction of the carbonyl groups according to the literature [8]. DMSO had been distilled to remove water and oxygen prior to use. Then $NaBH_4$ (100 mg) and DMSO (50 mL) were added into a dried reactor and heated at 120 °C under stirring. Micro-porous PEEK films were immersed in the $NaBH_4$ solution at 120 °C for 24 h under the protection of nitrogen. Subsequently, PEEK films were rinsed with methanol (15 min), distilled water (10 min), 0.5 M HCl (10 min), distilled water (10 min) and ethanol (10 min), respectively. Then the hydroxylated PEEK substrates (PEEK-OH) were dried under vacuum at 60 °C for 3 h and stored in the dark under nitrogen.

2.4. Immobilization of phosphorylated gelatin loading with BMP-2

Phosphorylated gelatin (P-Gel) was synthesized according to a similar procedure reported in the literatures [15,16]. Briefly, 20 mg/mL gelatin solution was dissolved and stirred at 40 °C for 1 h. Then 3-aminopropylphosphonic acid (12.5 mM) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride coupling reagent (4 mM) were added. The solution was stirred at 40 °C for 48 h and dialyzed using a seamless cellulose tube (cutoff molecular weight of 10 kD). After freezing dried in a vacuum, a white solid was obtained as phosphorylated gelatin.

Then, 1 mL P-Gel with different concentrations of 0.1%, 1%, 2% and 4% was added to 24-well plate containing a micro-porous PEEK film, respectively. After incubated at room temperature overnight, the substrates were washed three times with phosphate buffered saline (PBS, pH = 7.4) and left for air-drying in a sterile environment. For ESEM observation, various substrates were abbreviated as follows: untreated PEEK film (un-PEEK), micro-porous PEEK (PEEK), PEEK-OH (hydroxylated micro-porous PEEK), PEEK-OH@P-Gel with 0.1% P-Gel (0.1%



Scheme 1. Schematic diagram illustrating the processes of modification on PEEK films.

P-Gel coating on micro-porous PEEK-OH), PEEK-OH@P-Gel with 1% P-Gel (1% P-Gel coating on micro-porous PEEK-OH), PEEK-OH@P-Gel with 2% P-Gel (2% P-Gel coating on micro-porous PEEK-OH), PEEK-OH@P-Gel with 4% P-Gel (4% P-Gel coating on micro-porous PEEK-OH), and PEEK@P-Gel (2% P-Gel coating on micro-porous PEEK).

The immobilization of BMP-2 was carried out as described above for P-Gel coating, 2% P-Gel and various amounts of BMP-2 were mixed in PBS, and the final concentrations of BMP-2 were 5, 20 and 100 ng/mL, respectively. For subsequent surface characterization and cell experiments, various substrates were abbreviated as follows: PEEK-OH@P-Gel (2% P-Gel coating on micro-porous PEEK-OH), PEEK-OH@P-Gel/BMP-2 (5 ng) (2% P-Gel and 5 ng/mL BMP-2 coating on micro-porous PEEK-OH), PEEK-OH@P-Gel/BMP-2 (20 ng) (2% P-Gel and 20 ng/mL BMP-2 coating on micro-porous PEEK-OH), PEEK-OH@P-Gel/BMP-2 (100 ng) (2% P-Gel and 100 ng/mL BMP-2 coating on micro-porous PEEK-OH), and PEEK@P-Gel (2% P-Gel coating on micro-porous PEEK).

2.5. Surface characterization

The surface micromorphology of different PEEK substrates was examined by ESEM (XL30 FEG, Philips). All the substrates were sputter-coated with gold in advance. The chemical composition of the surface was analyzed by XPS (AHIS-HS, Kratos, Manchester, UK). Each substrate was performed by high-resolution XPS measurement for quantitative determination of the binding energy and atomic concentration. FT-IR (Bio-Rad Win-IR spectrometer, UK) was used to detect chemical groups. The FT-IR spectra were carried out in the wavelength range between 600 and 4000 cm^{-1} with a resolution of 2 cm^{-1}. The static water contact angles of the different substrates were measured using the sessile drop method on a contact angle system (Kruss, DSA100, German). Each different substrate was measured at three separate points and the final values were an average of these measurements with the standard deviation.

2.6. Cell culture

MC3T3-E1 was used to investigate the biological responses *in vitro*, which was purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Sigma), 63 mg/L penicillin (Sigma) and 100 mg/L streptomycin (Sigma) at 37 °C in 5% CO_2. The medium was refreshed every two days.

2.7. Cell adhesion

To investigate the effect of surface modification of various PEEK substrates on cell adhesion and spreading, MC3T3-E1 cells were incubated for 6 and 12 h, respectively. Double staining methods with Alexa Fluor conjugate-phalloidin (Invitrogen) for actin filaments staining (red) and 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) for cell nucleus staining (blue) were performed. When specified time point reached, the medium was removed and cells were washed three times with PBS. Then the cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min, followed by washing in PBS for three times. After incubated with phalloidin for 15 min and DAPI for 3 min, the substrates were observed and imaged under fluorescent microscopy (Zeiss, Axio Imager A2m, German).

2.8. Cell proliferation

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay was adopted to observe the cell proliferation on different substrates. Briefly, MC3T3-E1 cells were cultured for 1, 4 and 7 d, respectively. When each culture cycle was reached, 100 μL MTT (5 mg/mL in PBS) was added to each well and cells were continued to culture for 4 h. Subsequently, the medium was removed, 400 μL DMSO was added to dissolve the dyeing crystal substance. After low-speed oscillation to mix thoroughly, the mixed solution (200 μL) was transferred to a 96-well plate and the absorbance value at 490 nm wavelength was measured on a Full Wavelength Microplate Reader (Tecan Infinite M200).

2.9. Alkaline phosphatase (ALP) activity assay

ALP activity was determined by quantitation of the *p*-nitrophenol using *p*-nitrophenol phosphate substrate (*p*NPP) solution (Sigma) after being incubated for 7 and 14 d. The medium of each well was carefully removed and cells were washed three times with PBS. After incubated in a RIPA lysis buffer (Sangon) at 37 °C for 30 min, cells were freezed at −80 °C for 30 min and thawed at 37 °C to ensure that cells were lysed thoroughly. The cell lysis was centrifuged at 12,000 × *g* for 10 min at 4 °C to get the supernatant. Then 50 μL *p*NPP solution and 150 μL cell supernatant were added to a 96-well plate. After mixed and reacted fully, the absorbance value at 405 nm was measured by Full Wavelength Microplate Reader (Tecan Infinite M200). The OD values represented the level of ALP activity.

2.10. Calcium deposition

After 14 and 21 d of culture, the capacity of calcium deposition was evaluated by Alizarin Red staining (ARS). MC3T3-E1 cells were washed with PBS and fixed with 4% PFA for 10 min. After removed and rinsed adequately, 0.1% ARS was added and incubated for 30 min at room temperature. Then, the samples were washed three times in distilled water for 5 min each and dissolved by 10% (w/v) cetylpyridinium chloride for 15 min at room temperature. The absorbance value at 540 nm was recorded by Full Wavelength Microplate Reader (Tecan Infinite M200).

2.11. Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) technique was used to quantitatively assess the mRNA expression of osteogenesis-related genes. After cultured for 7 and 14 d, total RNA was extracted by TRIzol Reagent (Invitrogen) according to the protocol of manufacture. The concentration and purity of RNA were measured using Nanodrop Plates (Tecan Infinite M200) and the RNA was reverse transcribed using PrimeScript RT Reagent Kit. Real-time PCR was performed by Mx3005P (Stratagene, USA) and osteogenesis-related genes including runt-related transcription factor 2 (Runx2) and osteopontin (OPN) were assessed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for the evaluation of expression of target genes (Table 1). The standard ΔΔCt (threshold cycles) method was used for the calculation of relative transcript quantities.

2.12. Statistical analysis

Triplicate samples were analyzed in each experiment. All independent experiments were performed at least three times and representative data were shown. All the data were analyzed using Origin 8.0 and are expressed as the mean ± standard deviation (SD). Statistical difference was evaluated by variance analysis (ANOVA one-

Table 1
Sequences of relative primers for qRT-PCR.

Genes	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
Runx2	GCCGGGAATGATGAGAACTA	GGACCGTCCACTGTCACTTT
OPN	TCAGGACAACAACGGAAAGGG	GGAACTTGCTTGACTATCGATCAC

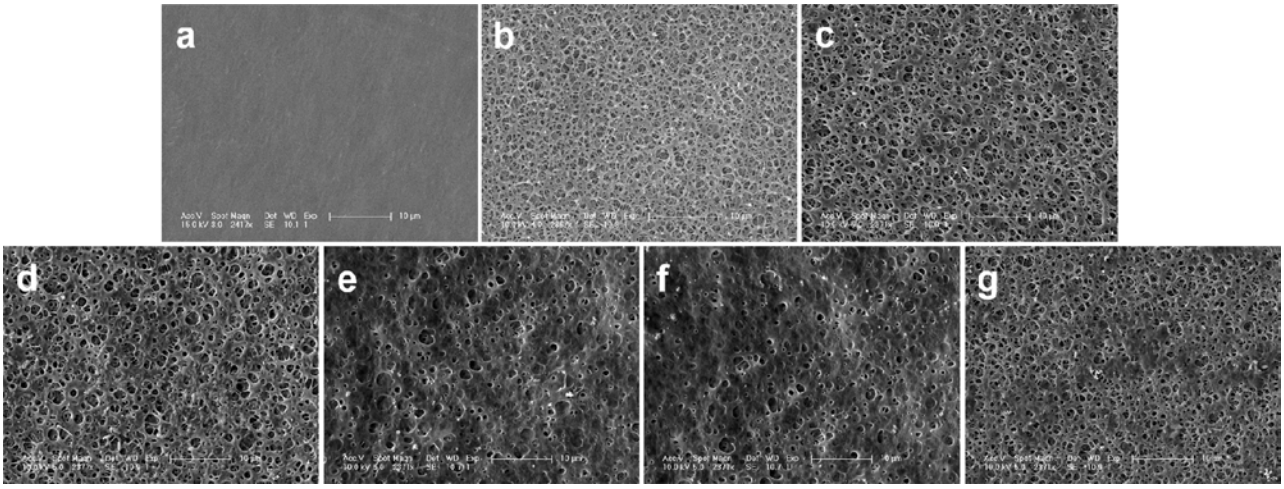


Fig. 1. ESEM images of un-PEEK (a), PEEK (b), PEEK-OH with 0.1% P-Gel (c), PEEK-OH with 1% P-Gel (d), PEEK-OH with 2% P-Gel (e), PEEK-OH with 4% P-Gel (f), and PEEK with 2% P-Gel (g). All scale bar length are 10 μm.

way, Origin 8.0). A value of $P < 0.05$ was regarded as statistically significant.

3. Results and discussion

3.1. Preparation and characterization of biofunctional PEEK

The preparation of biofunctional micro-porous PEEK substrates was performed by phosphorylated gelatin coating decorated with BMP-2. The surface morphologies of different PEEK substrates examined by ESEM were shown in Fig. 1. Compared with untreated PEEK film with smooth surface (Fig. 1a), homogeneous and interconnected micro-porous structure was observed on the surface of PEEK substrate etched with concentrated sulfuric acid (Fig. 1b). The immobilization of P-Gel was dramatically improved after PEEK hydroxylation and it could be increased along with higher P-Gel concentration (Fig. 1c–f). Viornery et al. [19] also demonstrated that the existence of a chemical link was persistent and stable between the hydroxyl groups and the phosphonic acid molecule. However, the immobilization of P-Gel did not present significant difference between 2% P-Gel and 4% P-Gel groups. It was speculated that sufficient P-Gel might be adsorbed on the PEEK surface with the concentration of 2%. These observations suggested that the P-Gel concentration of 2% might be an optimal concentration for the modification of PEEK surface.

The FT-IR spectra was performed to monitor the changes of chemical groups on different PEEK films (Fig. 2). After hydroxylation, the band at around 1650 cm^{-1} which is contributed to the stretching vibration of the carbonyl group of the benzophenone segment reduced significantly compared to the untreated PEEK. On the other hand, the new band at around 3400 cm^{-1} which presented the stretching vibration of alcoholic hydroxyl group was appeared on the hydroxylated PEEK-OH [20]. These variations on the FT-IR spectra confirmed that the hydroxyl groups was successful formed on the PEEK surface and the hydroxyl groups were derived from a reduction of the carbonyl groups.

The surface elemental compositions of PEEK, PEEK@P-Gel, PEEK-OH@P-Gel and PEEK-OH@P-Gel/BMP-2(100 ng) samples from XPS analysis were presented in Fig. 3a and Table 2. The elements of C1 s (284.6 eV), N1 s (400.0 eV), O1 s (533.0 eV) and P2p (133.4 eV) were observed. Compared with PEEK substrates, two kinds of new elements (N and P) could be seen on the surface of P-Gel immobilized PEEK or PEEK-OH, indicating successfully immobilization of P-Gel on both the substrates. Furthermore, the hydroxylation was contributed to adsorb more P-Gel and it was proved by an increase of the N and P contents as shown in Table 2. Compared with the group of PEEK-OH@P-Gel, a small increase in the N content and corresponding slight decrease in the P content demonstrated the successful immobilization of BMP-2 on the PEEK surface. The results from XPS spectra were a vital evidence to confirm the success of the biological modification on the PEEK surface via P-Gel and BMP-2 which will play a key role in the enhancement of cell adhesion and osteogenic differentiation.

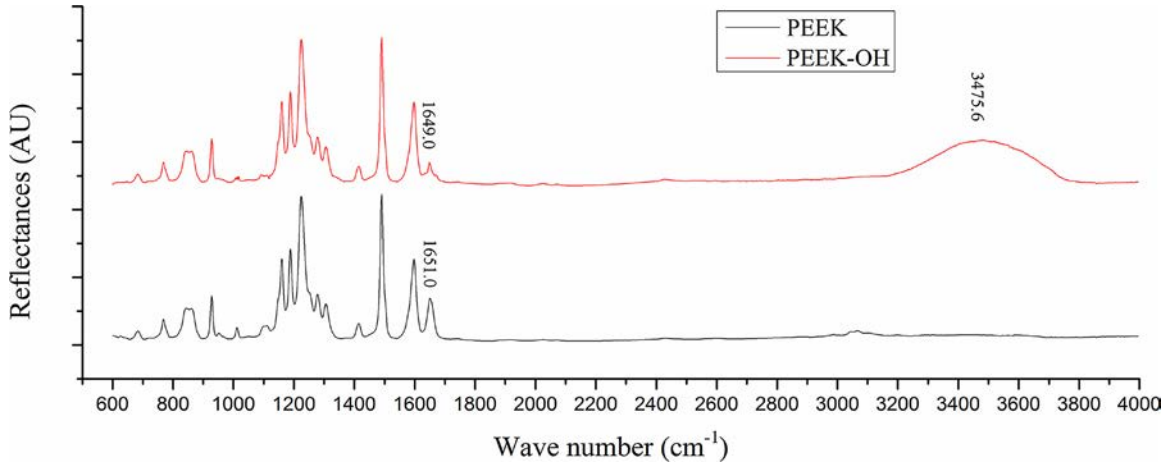


Fig. 2. FT-IR spectra of PEEK (black) and PEEK-OH films (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

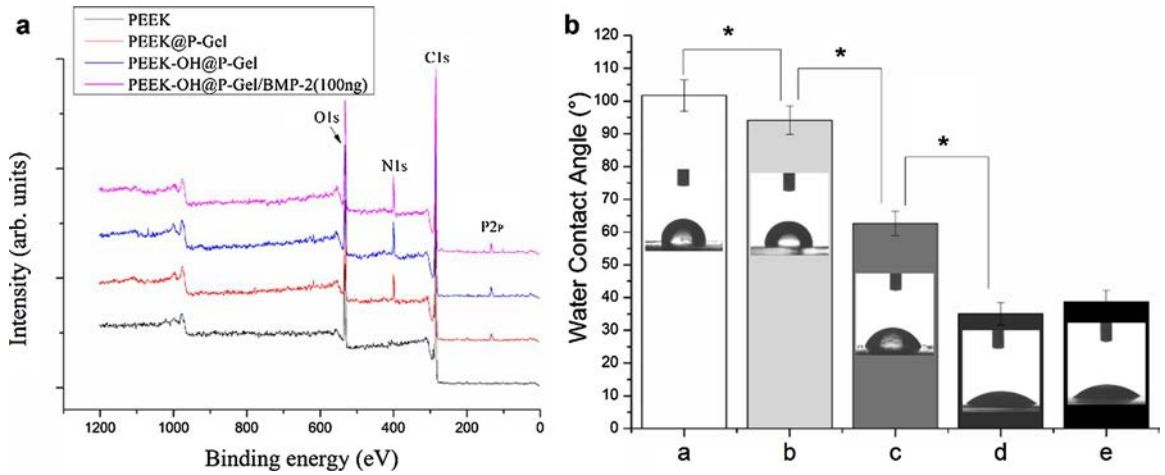


Fig. 3. (a) XPS spectra of PEEK (black), PEEK@P-Gel (red), PEEK-OH@P-Gel (blue), PEEK-OH@P-Gel/BMP-2 (100 ng) (purple). (b) Water contact angle of un-PEEK (a), PEEK (b), PEEK-OH (c), PEEK-OH@P-Gel (d), PEEK-OH@P-Gel/BMP-2 (100 ng) (e). * indicates significant differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Elemental composition on different PEEK surface as determined by XPS.

Substrate	C%	N%	O%	P%
PEEK	83.41	–	15.13	–
PEEK@P-Gel	75.69	5.05	17.79	1.02
PEEK-OH@P-Gel	71.40	8.38	18.11	2.11
PEEK-OH@P-Gel/BMP-2 (100 ng)	71.42	9.01	18.04	1.53

The static water contact angle measurement could also provide supporting evidence that the PEEK surface had been successfully modified (Fig. 3b). For the untreated PEEK, the water contact angle was (101.67 ± 4.79)°, whereas it decreased to (94.10 ± 4.33)° after the formation of micro-porous structure on PEEK and further to (62.67 ± 3.69)° after hydroxylation (PEEK-OH). Besides, the contact angle was reduced to (35.03 ± 3.42)° with the immobilization of 2% P-Gel. However, the immobilization of BMP-2 did not significantly change the surface wettability. On the basis of these results, it was concluded that all the modifications of porousness, hydroxylation and the immobilization of P-Gel could obviously improve the surface hydrophilic property and further enhance the biological activity of PEEK.

The surface properties of implants might have a direct effect on the cell adhesion, proliferation and differentiation [21,22]. In summary, the surface characterization such as ESEM, FT-IR, XPS and water contact angle indicated that P-Gel loading with BMP-2 had been successfully coated on the surface of hydroxylated micro-porous PEEK and the properties including hydrophilicity and biological inertness were changed by these modifications.

3.2. Cell adhesion and proliferation

Cellular biological responses to osteoblastic functions include cell adhesion, spreading, proliferation, relevant enzyme (ALP) activity, calcium deposition and expression of osteogenesis-related genes [23]. Cell adhesion is a critical factor to reflect the biocompatibility of implants and a prerequisite for cell proliferation and differentiation [24,25]. Furthermore, surface modification plays a most important role in determining the initial success of a bioengineered implant. In this study, double staining method was chosen to evaluate the adhesion of MC3T3-E1 cells on different PEEK substrates under fluorescent microscopy.

As shown in Fig. 4, after cultured for 6 and 12 h, micro-porous PEEK with the immobilization of 2% P-Gel (Fig. 4c–f) showed significantly higher surface cell density compared to the PEEK without P-Gel immobilization (Fig. 4a and b). Besides, as shown in Fig. 4b, the micro-porous structure on PEEK surface also improved cell adhesion to some extent. However, there was no significant difference in the cell adhesion incorporating various concentrations of BMP-2 (Fig. 4d–f). In addition, more better cell spreading was observed on the micro-porous PEEK substrates with immobilization of P-Gel instead of that on the untreated PEEK which remained as isolated single cells. Thus, these results suggested that the immobilization of P-Gel on the micro-porous PEEK films would be more favorable for cell adhesion and spreading. As an important physiological event, chemotactic migration of bone-forming cells is a critical process during bone formation. A number of materials and methods have been investigated to accelerate the chemotactic response of osteoblasts including gelatin. Lim at al [26] and Hou et al. [27] reported that conjugation of gelatin could obviously enhance cell adhesion, spreading and proliferation. In our study, the enhancement of cell adhesion and spreading indicated that modifications of porousness and immobilization of P-Gel could effectively improve the surface bioactivity of PEEK.

Cell proliferation at 1, 4 and 7 d measured by the MTT assay was shown in Fig. 5a. Cell proliferation on different PEEK substrates progressed gradually for up to 7 d. After incubated for 1 d, no significant difference was observed between different groups ($P > 0.05$). After 4 and 7 d of culture, cell proliferation on micro-porous PEEK was found to be slightly better than that on the untreated PEEK, whereas cells on the PEEK with immobilization of P-Gel showed apparently higher proliferation rates compared to that of the untreated PEEK and micro-porous PEEK ($P < 0.05$). The results suggested that cell proliferation was obviously enhanced by the coating of P-Gel. In addition, it was interesting that the incorporation of BMP-2 was seemed to exhibit a limited capacity for promoting cell proliferation. Even when the concentration of BMP-2 exceed a critical value (20 ng/mL), the capacity for promoting cell proliferation would be obviously decreased. A similar conclusion has been observed by Hu et al. [24] and Shen et al. [28] with the BMP-2 immobilization on titanium and polymer scaffolds. As one of the most potentially effective osteoinductive substance for enhancing bone formation, BMP-2 had significant effect on stimulating early osteogenic differentiation and matrix mineralization instead of cell proliferation [29].

It has been reported that surface wettability, roughness and biological properties would intensively affect cellular responses to biomaterials [30,31]. According to the above results, the increase of surface wettability and roughness as well as the tight immobilization of biological materials including P-Gel and BMP-2 could remarkably improve the biological activity of PEEK films and enhance the cellular responses

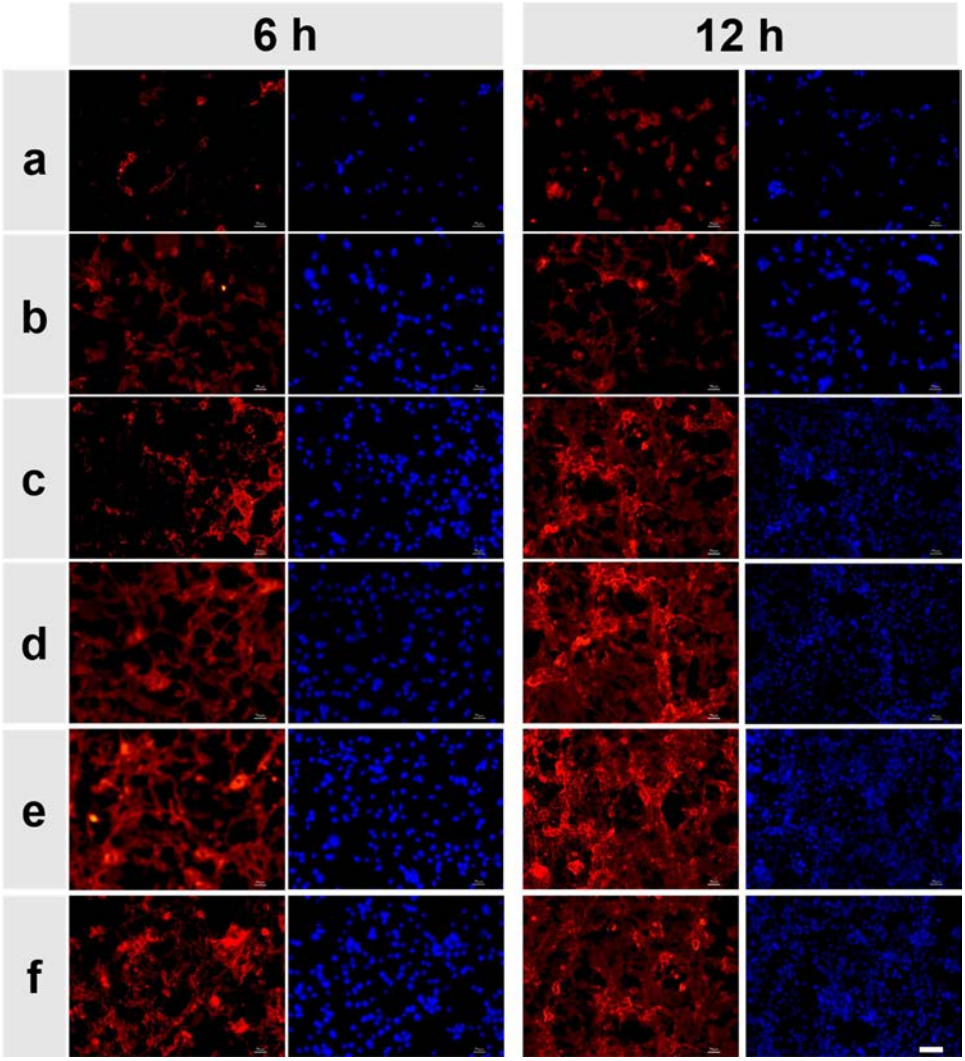


Fig. 4. Fluorescent micrographs of MC3T3-E1 cells cultured for 6 and 12 h on un-PEEK (a), PEEK (b), PEEK-OH@P-Gel (c), PEEK-OH@P-Gel/BMP-2 (5 ng) (d), PEEK-OH@P-Gel/BMP-2 (20 ng) (e), PEEK-OH@P-Gel/BMP-2 (100 ng) (f). Actin filaments (red) and cell nucleus (blue) were shown. All scale bar lengths are 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in vitro.

3.3. ALP activity and calcium deposition

To further investigate the early and late osteogenic differentiation of MC3T3-E1 cells, the ALP activity and calcium deposition were investigated successively. ALP was one of the important early-stage osteoblastic markers [32] and it's activity was evaluated after the cells were incubated for 7 and 14 d. As shown in Fig. 5b, the ALP activity was not obviously different from that of the untreated PEEK substrate at 7 and 14 d ($P > 0.05$). On the other hand, a significant enhancement of ALP activity was observed at both 7 and 14 d with the immobilization of BMP-2 ($P < 0.05$). Furthermore, the ALP activity increased slightly accompanying by the concentration increase of BMP-2 after 7 d of culture ($P > 0.05$). After 14 d of culture, the enhancement of ALP activity was significantly strengthened as BMP-2 concentration increased from 5 ng/mL to 20 ng/mL. However, ALP activity was slightly decreased as the concentrations of BMP-2 up to 100 ng/mL at 14 d ($P > 0.05$). These results indicated that the immobilization of BMP-2 via P-Gel coating for the surface modification of PEEK films played a critical role on the enhancement of osteogenic differentiation. As consistent with the previous literature [33], the ALP activity was also significantly increased on different surfaces such as titanium with the immobilization of BMP-2.

On the other hand, as similar to the ALP activity results, the amount of calcium deposited on the modified PEEK substrates with BMP-2 immobilization was significantly greater compared to the others at both 14 and 21 d (Fig. 5c). However, no significant difference was observed among the untreated PEEK and micro-porous PEEK analyzed from quantitative results ($P > 0.05$). At 21 d of cell cultivation, cellular calcium deposition was moderately reinforced with P-Gel coating ($P < 0.05$). Calcium deposition is a marker of late-stage osteoblastic differentiation which indicates that osteoblasts enter into the mineralization phase to deposit organic materials for bone formation [34]. Combining the results of ALP activity and calcium deposition, the surface immobilization of BMP-2 via P-Gel coating appeared to play a more important role to stimulate osteogenic differentiation which was in accordance with the previous studies [35,36].

3.4. Quantitative real-time PCR analysis for gene expression

Osteogenesis-related gene expression was investigated for cells cultured on the various modified PEEK substrates using qRT-PCR and the results were presented in Fig. 6. Some key cytokines and functional proteins such as Runx2 and OPN will be expressed regularly during the osteoblastic differentiation. Runx2 is an early differentiation marker

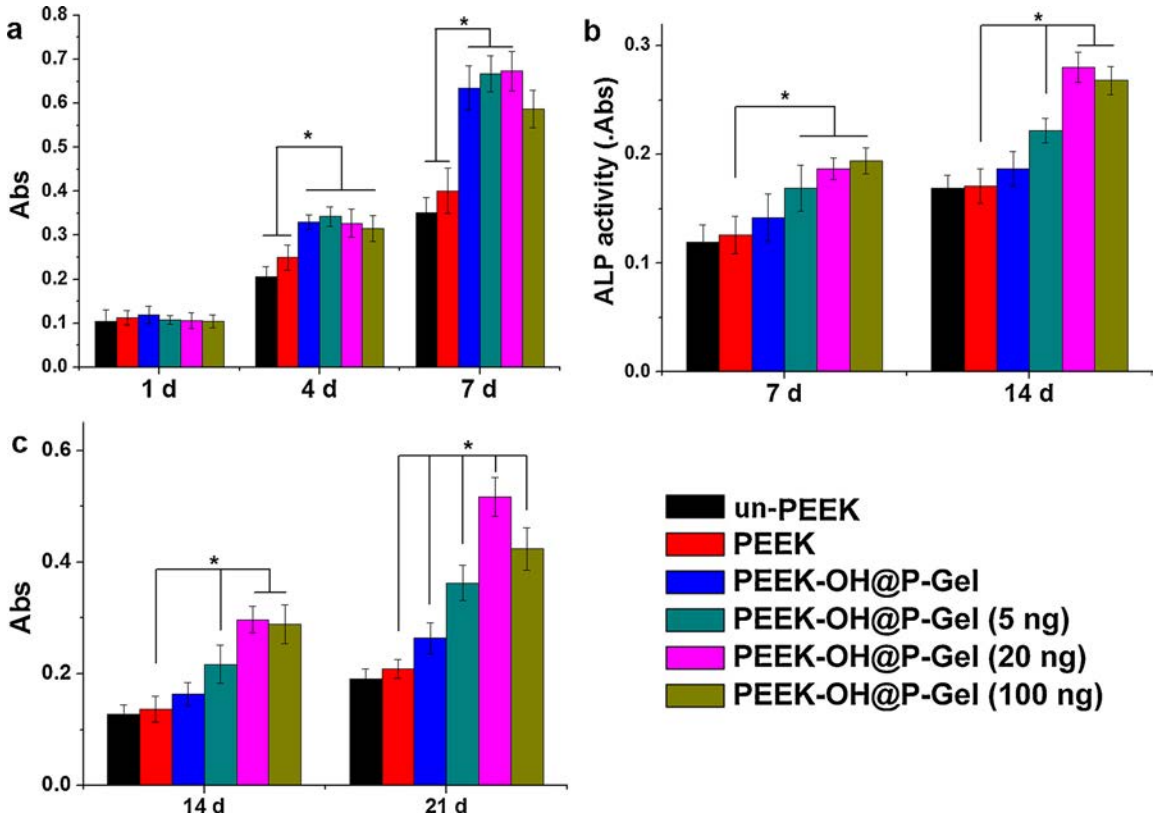


Fig. 5. (a) Cell proliferation performed by MTT assay cultured on different PEEK substrates for 1, 4 and 7d. (b) ALP activity of MC3T3-E1 cells cultured on different PEEK substrates for 7 and 14 d. (c) Calcium deposition of MC3T3-E1 on different PEEK substrates for 14d and 21d. * indicates significant differences ($P < 0.05$).

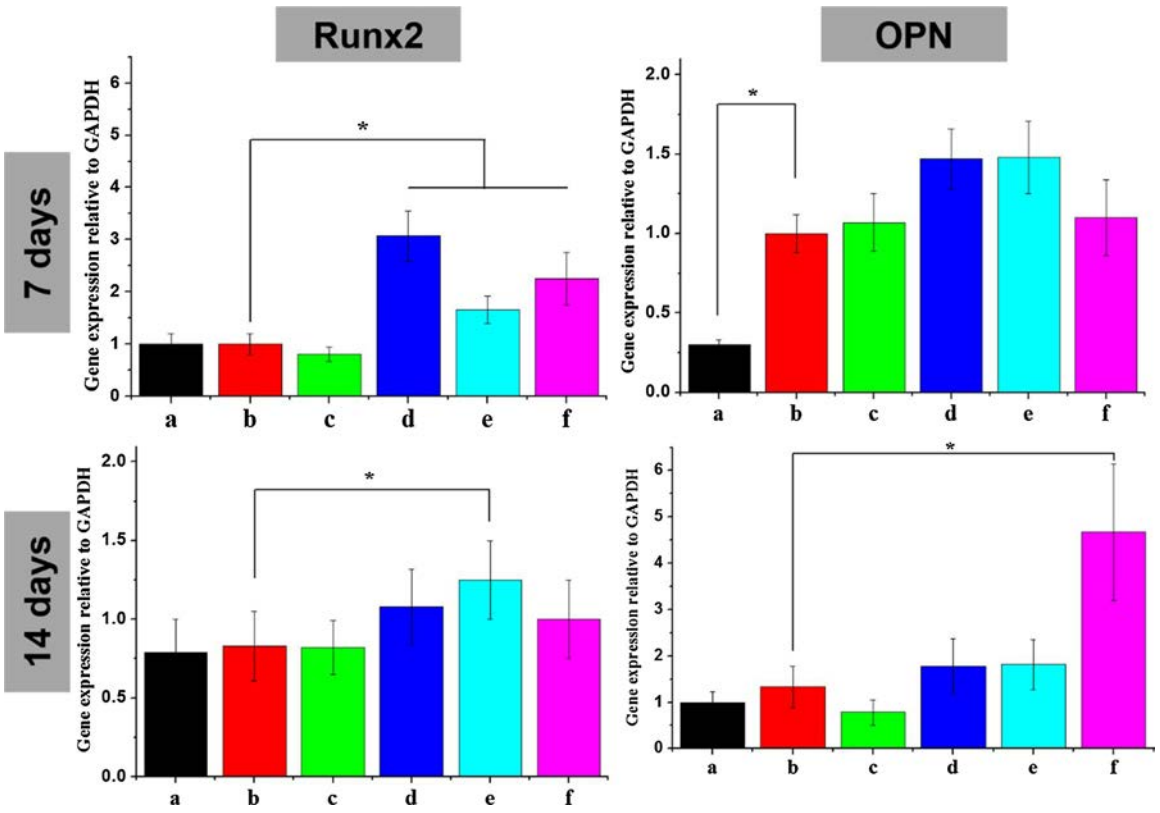


Fig. 6. The qRT-PCR analysis for Runx2 and OPN expression by MC3T3-E1 cells cultured on un-PEEK (a), PEEK (b), PEEK-OH@P-Gel (c), PEEK-OH@P-Gel/BMP-2 (5 ng) (d), PEEK-OH@P-Gel/BMP-2 (20 ng) (e), PEEK-OH@P-Gel/BMP-2 (100 ng) (f) for 7 and 14 d. * Indicates significant differences ($P < 0.05$).

occurred at the early osteogenesis, whereas OPN is usually expressed at the middle/late stage of differentiation [37,38].

After 7 d of incubation, the expression of Runx2 was significantly higher on the PEEK substrates with immobilization of different concentrations of BMP-2 ($P < 0.05$). However, Runx2 expression was obviously decreased for all the samples at 14 d and significant difference was only observed among the samples of micro-porous PEEK and PEEK-OH@P-Gel/BMP-2 (20 ng) ($P < 0.05$). For the expression of OPN, there was no significant difference for all the groups after 7 d of incubation. Much higher expression levels of OPN were observed in the groups with BMP-2 immobilization until 14 d. Previous studies demonstrated that BMP-2 directly induced osteogenesis-related gene expression such as Runx2, OPN, OCN and ALP [39], which were consistent with the strong osteoinduction by BMP-2 from the results in our study. More interestingly, the higher expression level of Runx2 was occurred in lower concentration of BMP-2 (5 ng/mL), whereas that of OPN expression was found in the higher concentration of BMP-2 (100 ng/mL). It was deduced that the low/medium concentration of BMP-2 was more favorable for the osteoblastic differentiation at the early stage, while high concentration of BMP-2 might be able to accelerate the further differentiation at the middle/late stage. Combined with the results of ALP activity, calcium deposition and osteogenesis-related gene expression, the immobilization of BMP-2 via P-Gel coating on the PEEK substrates seemed to be an optimal strategy for the osteogenic differentiation *in vitro*.

Although many conventional techniques has been reported to increase the bioactivity of PEEK materials to improve the bone-implant interface, various drawbacks still existed such as weak bonding, complex operation, strict operation condition, single effect and time-consuming post-treatment. In the present study, we designed a biomimetic interfacial system via a simple modification method using phosphorylated gelatin (P-Gel) layer loading with different amounts of BMP-2 through covalently bonding to the micro-porous hydroxylated PEEK films. Cell adhesion and osteogenic differentiation were significantly enhanced by the coating of gelatin and immobilization of BMP-2 on the PEEK substrates. This novel method seems to be relatively simple, stable, effective and mild for the modification conditions compared to the previous methods for surface modification of PEEK. Due to its excellent thermal and mechanical properties and broad chemical resistance, PEEK has been an attractive material for extensive clinical applications. In the future, medical PEEK with desirable surface biological activity achieved in our study will hold great practical potential for further scale-up its clinical application, in particular as a kind of orthopedic implants. However, more further studies seem to be carried out to overcome the current drawbacks and promote clinical applications of this novel technical. We believe that this work can provide a valuable approach for surface modification of PEEK materials and further expands its medical application as a kind of orthopedic implants.

4. Conclusions

In our study, a novel surface modification method was successfully applied to overcome the biological inertness and improve the biological activity of PPEK substrates. This surface modification was carried out by several steps, including surface porousness, hydroxylation and immobilization of P-Gel and BMP-2. The characterization results including ESEM, FT-IR, XPS and water contact angle demonstrated that the biological modification of PEEK films was achieved successfully. The hydroxylation process was helpful for efficiently coating of P-Gel and then significantly enhance cell adhesion, spreading and proliferation. What is more, the immobilization of BMP-2 via P-Gel coating obviously enhanced the osteogenic differentiation of mouse pre-osteoblasts. The combination of P-Gel and BMP-2 might be contributed to induce higher osteoblasts growth and osteoinduction activity during all the process of bone formation. In future, *in vivo* study are necessary for providing further evidences of the osteogenesis functions using this novel modification method on PEEK substrates. The combination of these studies *in vitro* and *in vivo* will provide a more convincing support to demonstrate the effectiveness to improve the surface bioactivity via this novel surface modification method and promote the clinical application of this technology.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Projects 51673186, 51473164 and 51403197), the Program of Scientific Development of Jilin Province (20170520121JH and 20170520141JH), the Joint Funded Program of Chinese Academy of Sciences and Japan Society for the Promotion of Science (GJHZ1519), and the Special Fund for Industrialization of Science and Technology Cooperation between Jilin Province and Chinese Academy of Sciences (2017SYHZ0021).

References

[1] D. Williams, Polyetheretherketone for long-term implantable devices, Med. Device Technol. 19 (2008) 10–11.
[2] S.M. Kurtz, J.N. Devine, PEEK biomaterials in trauma, orthopedic, and spinal implants, Biomaterials 28 (2007) 4845–4869.
[3] M.S. Abu Bakar, M.H. Cheng, S.M. Tang, S.C. Yu, K. Liao, C.T. Tan, K.A. Khor, P. Cheang, Tensile properties, tension-tension fatigue and biological response of polyetheretherketone-hydroxyapatite composites for load-bearing orthopedic implants, Biomaterials 24 (2003) 2245–2250.
[4] L. Eschbach, Nonresorbable polymers in bone surgery, Injury 31 (2000) S22–S27.
[5] C. Morrison, R. Macnair, C. MacDonald, A. Wykman, I. Goldie, M.H. Grant, In vitro biocompatibility testing of polymers for orthopaedic implants using cultured fibroblasts and osteoblasts, Biomaterials 16 (1995) 987–992.
[6] C.M. Han, E.J. Lee, H.E. Kim, Y.H. Koh, K.N. Kim, Y. Ha, S.U. Kuh, The electron beam deposition of titanium on polyetheretherketone (PEEK) and the resulting enhanced biological properties, Biomaterials 31 (2010) 3465–3470.
[7] P. Robotti, G. Zappini, Thermal plasma spray deposition of titanium and hydroxyapatite on polyaryletheretherketone, PEEK Biomater. Handb. 1 (2012) 119–143.
[8] B. Yameen, M. Alvarez, O. Azzaroni, U. Jonas, W. Knoll, Tailoring of poly(ether ether ketone) surface properties via surface-initiated atom transfer radical polymerization, Langmuir 25 (2009) 6214–6220.
[9] R. Daw, S. Candan, A.J. Beck, A.J. Devlin, I.M. Brook, S. MacNeil, R.A. Dawson, R.D. Short, Plasma copolymer surfaces of acrylic acid/1,7 octadiene: surface characterisation and the attachment of ROS 17/2.8 osteoblast-like cells, Biomaterials 19 (1998) 1717–1725.
[10] R. Nakaoka, Y. Yamakoshi, K. Isama, T. Tsuchiya, Effects of surface chemistry prepared by self-assembled monolayers on osteoblast behavior, J. Biomed. Mater. Res. A 94 (2010) 524–532.
[11] B.D. Hahn, D.S. Park, J.J. Choi, J. Ryu, W.H. Yoon, J.H. Choi, J.W. Kim, C.W. Ahn, H.E. Kim, B.H. Yoon, I.K. Jung, Osteoconductive hydroxyapatite coated PEEK for spinal fusion surgery, Appl. Surf. Sci. 283 (2013) 6–11.
[12] C. Mu, J. Guo, X. Li, W. Lin, D. Li, Preparation and properties of dialdehyde carboxymethyl cellulose crosslinked gelatin edible films, Food Hydrocoll. 27 (2012) 22–29.
[13] P. Hiwale, S. Lampis, G. Conti, C. Caddeo, S. Murgia, A.M. Fadda, M. Monduzzi, In vitro release of lysozyme from gelatin microspheres: effect of cross-linking agents and thermoreversible gel as suspending medium, Biomacromolecules 12 (2011) 3186–3193.
[14] K. Zwiorek, C. Bourquin, J. Battiany, G. Winter, S. Endres, G. Hartmann, C. Coester, Delivery by cationic gelatin nanoparticles strongly increases the immunostimulatory effects of CpG oligonucleotides, Pharm. Res. 25 (2008) 551–562.
[15] X. Zhou, S.H. Park, H. Mao, T. Isoshima, Y. Wang, Y. Ito, Nanolayer formation on titanium by phosphonated gelatin for cell adhesion and growth enhancement, Int. J. Nanomed. 10 (2015) 5597–5607.
[16] S.H. Park, L. Zhu, S. Tada, S. Obuse, Y. Yoshida, M. Nakamura, T.I. Son, S. Tsuneda, Y. Ito, Phosphorylated gelatin to enhance cell adhesion to titanium, Polym. Int. 63 (2014) 1616–1619.
[17] D. Chen, M. Zhao, G.R. Mundy, Bone morphogenetic proteins, Growth Factors 22 (2004) 233–241.
[18] S.N. Lissenberg-Thunnissen, D.J. de Gorter, C.F. Sier, I.B. Schipper, Use and efficacy of bone morphogenetic proteins in fracture healing, Int. Orthop. 35 (2011) 1271–1280.
[19] C. Viornery, H.L. Guenther, B.O. Aronsson, P. Pechy, P. Descouts, M. Gratzel, Osteoblast culture on polished titanium disks modified with phosphonic acids, J. Biomed. Mater. Res. 62 (2002) 149–155.
[20] A.M. Díez-Pascual, G. Martínez, M.A. Gómez, Synthesis and characterization of poly (ether ether ketone) derivatives obtained by carbonyl reduction, Macromolecules 42 (2009) 6885–6892.
[21] M. Jayaraman, U. Meyer, M. Buhner, U. Joos, H.P. Wiesmann, Influence of titanium surfaces on attachment of osteoblast-like cells in vitro, Biomaterials 25 (2004) 625–631.
[22] C.G. Spiteri, R.M. Pilliar, R.A. Kandel, Substrate porosity enhances chondrocyte

attachment spreading, and cartilage tissue formation in vitro, J. Biomed. Mater. Res. A 78 (2006) 676–683.
[23] C.A. Scotchford, M. Ball, M. Winkelmann, J. Voros, C. Csucs, D.M. Brunette, G. Danuser, M. Textor, Chemically patterned, metal-oxide-based surfaces produced by photolithographic techniques for studying protein- and cell-interactions. II: Protein adsorption and early cell interactions, Biomaterials 24 (2003) 1147–1158.
[24] X. Hu, K.G. Neoh, Z. Shi, E.T. Kang, C. Poh, W. Wang, An in vitro assessment of titanium functionalized with polysaccharides conjugated with vascular endothelial growth factor for enhanced osseointegration and inhibition of bacterial adhesion, Biomaterials 31 (2010) 8854–8863.
[25] A.M. DeLise, L. Fischer, R.S. Tuan, Cellular interactions and signaling in cartilage development, Osteoarth. Cartil. 8 (2000) 309–334.
[26] K.S. Lim, M.H. Alves, L.A. Poole-Warren, P.J. Martens, Covalent incorporation of non-chemically modified gelatin into degradable PVA-tyramine hydrogels, Biomaterials 34 (2013) 7097–7105.
[27] L. Hou, Y. Peck, X. Wang, D. Wang, Surface patterning and modification of polyurethane biomaterials using silsesquioxane-gelatin additives for improved endothelial affinity, Sci. China Chem. 57 (2013) 596–604.
[28] H. Shen, X. Hu, F. Yang, J. Bei, S. Wang, The bioactivity of rhBMP-2 immobilized poly(lactide-co-glycolide) scaffolds, Biomaterials 30 (2009) 3150–3157.
[29] Z. Shi, K.G. Neoh, E.T. Kang, C.K. Poh, W. Wang, Surface functionalization of titanium with carboxymethyl chitosan and immobilized bone morphogenetic protein-2 for enhanced osseointegration, Biomacromolecules 10 (2009) 1603–1611.
[30] L. Ponsonnet, K. Reybier, N. Jaffrezic, V. Comte, C. Lagneau, M. Lissac, C. Martelet, Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behavior, Mater. Sci. Eng. C 23 (2003) 551–560.
[31] T. Groth, G. Altankov, Studies on cell-biomaterial interaction: role of tyrosine phosphorylation during fibroblast spreading on surfaces varying in wettability, Biomaterials 17 (1996) 1227–1234.
[32] H. Cui, Y. Wang, L. Cui, P. Zhang, X. Wang, Y. Wei, X. Chen, In vitro studies on regulation of osteogenic activities by electrical stimulus on biodegradable electroactive polyelectrolyte multilayers, Biomacromolecules 15 (2014) 3146–3157.
[33] C.Y. Chien, W.B. Tsai, Poly(dopamine)-assisted immobilization of Arg-Gly-Asp peptides, hydroxyapatite, and bone morphogenic protein-2 on titanium to improve the osteogenesis of bone marrow stem cells, ACS Appl. Mater. Interfaces 5 (2013) 6975–6983.
[34] Q. Xie, Z. Wang, H. Zhou, Z. Yu, Y. Huang, H. Sun, X. Bi, Y. Wang, W. Shi, P. Gu, X. Fan, The role of miR-135-modified adipose-derived mesenchymal stem cells in bone regeneration, Biomaterials 75 (2016) 279–294.
[35] H. Lin, Y. Zhao, W. Sun, B. Chen, J. Zhang, W. Zhao, Z. Xiao, J. Dai, The effect of crosslinking heparin to demineralized bone matrix on mechanical strength and specific binding to human bone morphogenetic protein-2, Biomaterials 29 (2008) 1189–1197.
[36] O. Jeon, S.J. Song, S.W. Kang, A.J. Putnam, B.S. Kim, Enhancement of ectopic bone formation by bone morphogenetic protein-2 released from a heparin-conjugated poly(L-lactic-co-glycolic acid) scaffold, Biomaterials 28 (2007) 2763–2771.
[37] K. Atluri, D. Seabold, L. Hong, S. Elangovan, A.K. Salem, Nanoplex-mediated co-delivery of fibroblast growth factor and bone morphogenetic protein genes promotes osteogenesis in human adipocyte-derived mesenchymal stem cells, Mol. Pharm. 12 (2015) 3032–3042.
[38] B. Setzer, M. Bachle, M.C. Metzger, R.J. Kohal, The gene-expression and phenotypic response of hFOB 1.19 osteoblasts to surface-modified titanium and zirconia, Biomaterials 30 (2009) 979–990.
[39] F. Lecanda, L.V. Avioli, S.L. Cheng, Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2, J. Cell. Biochem. 67 (1997) 386–396.