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RESEARCH ARTICLE

In vitro and *in vivo* evaluation of bone morphogenetic protein-2 (BMP-2) immobilized collagen-coated polyetheretherketone (PEEK)

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ABSTRACT: Polyetheretherketone (PEEK) is regarded as one of the most potential candidates of biomaterials in spinal implant applications. However, as a bioinert material, PEEK plays a limited role in osteoconduction and osseointegration. In this study, recombinant human bone morphogenetic protein-2 (rhBMP-2) was immobilized onto the surface of collagen-coated PEEK in order to prepare a multi-functional material. After adsorbed onto the PEEK surface by hydrophobic interaction, collagen was cross-linked with N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). EDC/NHS system also contributed to the immobilization of rhBMP-2. Water contact angle tests, XPS and SEM clearly demonstrated the surface changes. ELISA tests quantified the amount of rhBMP-2 immobilized and the release over a period of 30 d. *In vitro* evaluation proved that the osteogenesis differentiation rate was higher when cells were cultured on modified PEEK discs than on regular ones. *In vivo* tests were conducted and positive changes of major parameters were presented. This report demonstrates that the rhBMP-2 immobilized method for PEEK modification increase bioactivity *in vitro* and *in vivo*, suggesting its practicability in orthopedic and spinal clinical applications.

KEYWORDS: polyetheretherketone (PEEK); spinal implantation; bone morphogenetic protein-2 (BMP-2); collagen; surface modification

Contents

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1 Introduction

Polyetheretherketone (PEEK) is a considerable suitable biomaterial for orthopedic applications and spinal implants with many great properties such as chemical stability, wear resistance, radiolucency, non-cytotoxicity, non-genotoxicity and non-immunogenesis [1–4]. Compared with conventional metal biomaterials used in orthopaedics, PEEK exhibits excellent elastic modulus closely matching cortical bone, which could prevent stress-shielding effect and consequently reduces the risks of bone resorption and implant loosening [5]. However, biological inertness makes PEEK relatively low osseointegration. For that, different strategies have been used to modify PEEK in order to enhance its bioactivity. Traditionally, modifications were mainly conducted by blending or coating some relatively bioactive materials such as hydroxyapatite and titanium [6–7]. Immobilization of bioactive molecule on polymer surfaces is another strategy and seems to be more efficient [8].

Collagen is an important constituent of extracellular matrix (ECM) consisted by 19 isotypes. Up to 96% of them is type-I isotype. Because of the dimensions and self-assembling properties, collagen could offer promising perspectives for preparing protein coatings on biomaterials [9]. Although some reports are conflicting about the effect of collagen-coating on the cell attachment and differentiation, collagen-coating has been widely used in the modification of titanium [10–13]. The hydrophobic surface of PEEK is also noticed with the contact angle of water reported more than 75° [14]. According to the report by Ying et al., hydrophilic surface is not suitable for the absorption of protein [15]. When the contact angle increased from 50° to 85°, much more adsorption amount of collagen would be adsorbed because of the strong hydrophobic interaction between collagen and hydrophobic surface. Poly(ethylene terephthalate) (PET) and polystyrene (PS) with similar molecular structure and hydrophobicity to PEEK were reported emerging irreversible collagen physical absorption on the surface [16–18].

To improve the resistance against enzymatic degradation, cross-linking is necessary for collagen used *in vivo* [19]. Many cross-link agents are optional such as glutaraldehyde, epoxy compound, genipin and carbodiimide. Among those, the non-cytotoxic water-soluble N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC), a kind of amidating agents, is well suitable for cross-linking proteins for biomaterials. Because of the intermediate is unstable in aqueous solutions, N-hydroxysuccinimide (NHS) is required in the reaction [20]. EDC/NHS is also suitable to immobilize bioactive molecule that containing amino and carboxyl functional groups like heparin and bone morphogenetic protein-2 (BMP-2) to biomaterials surface [21].

The BMPs are a group of growth factors which could induce the differentiation of various types of cells including osteoblasts, chondrocytes and osteoclasts [22–23]. Among them, BMP-2 has been widely used in bone regeneration and repair with a strong osteoinductive activity [24]. However, the clinical application of BMPs is limited by its short half-life, rapid degradation and large doses required. Therefore a series of methods such as surface immobilization or preparation of controlled release systems have been utilized for making BMPs more efficient in the application [25–28].

In this study, the bioactive modification of PEEK via immobilization of recombinant human bone morphogenetic protein-2 (rhBMP-2) was investigated and its application in bone regeneration was evaluated. Firstly, acid soluble type-I collagen was chosen as the coating protein because of its excellent bioactivity. Strong hydrophobic interactions between PEEK surface and acid soluble type-I collagen fibers would result in an irreversible absorption layer of collagen. Coupling reaction between amine groups and carboxyl groups facilitated the immobilization of rhBMP-2 into the collagen coatings. The bioactivity of modified PEEK was investigated *in vitro* by measuring cell proliferation and differentiation ability of MC3T3-E1 cells and *in vivo* by implanting these PEEK prostheses into a rabbit lumbar vertebrae model. MicroCT

and mechanical tests were used to analyze the difference between the modified and unmodified PEEK disc prostheses.

2 Materials and methods

2.1 Materials

The PEEK raw materials (VESTAPEEP@ i4 R 400×8 mm and 1000×18 mm) were acquired from Evonik Specialty Chemicals (Shanghai) Co., Ltd. For *in vitro* tests, the unmodified PEEK samples were machined to thin discs and then underwent polishing processing. For *in vivo* tests, simplified intervertebral disc prostheses were manufactured according to the dimension measured in advance. The type-I collagen was purchased from Gibco (Collagen I, Rat Tail Cat. # A10483-01), and the rhBMP-2 was purchased from Prospec (Cat. # cyt-261).

2.2 Coating of type-I collagen

The type-I collagen solution (3 mg/mL) was dissolved in phosphate buffer saline (PBS; Gibco, USA) to a final concentration of 1 mg/mL. The samples with diameters of 8 and 18 mm were deposited in 48-well and 12-well plates, respectively. Then 500 μL and 2 mL of collagen solution were added to 12-well and 48-well cell culture plates respectively for 24 h before the samples were rinsed 6 times and dried overnight.

2.3 Collagen cross-linking and grafting of rhBMP-2

The type-I collagen coating were cross-linked using EDC and NHS (Aladdin, China). First 50 mL PBS solution containing 1.15 g EDC and 0.28 g NHS was prepared, and the samples were immersed in the buffer. After 1 h, the samples were carried out carefully by tweezers and transferred to another wells. Then 100 and 20 μL rhBMP-2 solution (1 μg/mL) was added to the surface of the samples and let the solution spread over the entire surface of the PEEK samples. After 1 h, when the reaction was completed, the samples were washed with 0.1 mol/L Na_2HPO_4 solution for 2 h and deionized water 4 times and dried overnight. Finally, all the samples were sterilized with ethylene oxide for following experiments.

The collagen-coated PEEK and BMP-2 immobilized collagen-coated PEEK were hereafter abbreviated as CCPEEK and BIPEEK, respectively.

2.4 Characterization of PEEK discs

The surface phases were observed by a field emission scanning electron microscope (FEI Nova NanoSEM 450, USA) at an acceleration voltage of 5 kV after sputter-coating with gold/palladium. Surface elements of different samples were analyzed using X-ray photoelectron spectroscopy (XPS; Thermo Scientific ESCALAB 250XI, USA) with an Al anode (15 kV, 150 W) and a quartz monochromator. The order of peak analysis was: survey scan, C 1s, O 1s and N 1s. A drop-shaped contact angle measurement instrument (Solon SL200B, China) was used to compare the wettability of PEEK, CCPEEK and BIPEEK samples. Discs were put on a flat plate, and a drop of distilled water was applied. The contact angle was then calculated from a charge-coupled device camera image.

2.5 Determination of binding rhBMP-2

The rhBMP-2 immobilized PEEK (BIPEEK) discs with a diameter of 18 mm were placed at the bottom of a 12-well plate (n = 7). The total rhBMP-2 amount immobilized onto the discs and the released amounts at different 8 time points (1 h and 1, 3, 5, 7, 13, 16 and 30 d) were measured by the enzyme-linked immunosorbent assay (ELISA) method. At the specific time points, 1 mL PBS was added to each well and rinsed the samples 3 times, then the supernatant was transferred to a 2 mL centrifuge tube and reserved under −80°C until concentration assay. Human BMP-2 ELISA Development Kit (PeroTech, USA) and related materials and reagents including ELISA microplates (Corning, USA), Tween-20 (Sigma, USA), BSA (Sigma, USA), ABTS Liquid Substrate Solution (Sigma, USA) were used to determine the contents of rhBMP-2 in PBS following the manufacturer's instructions. The absorbance of the reaction products at 405 nm with correction set at 650 nm was determined by microplate reader (BioTek Synergy 4, USA).

2.6 Osteoblast cell culture

Mouse preosteoblast cells (MC3T3-E1 Subclone 14) were used to investigate the cell behavior on the surface of PEEK, CCPEEK and BIPEEK. The proliferation medium for MC3T3-E1 cell is α-minimum essential medium (α-MEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Bioind, Israel) and 1% penicillin-streptomycin (Gibco, USA). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.7 Cell proliferation assay

The PEEK, CCPEEK and BIPEEK discs with a diameter of 8 mm were placed into a 48-well plate and 250 μL of MC3T3-E1 cell suspension with a cell density of 5×10^4 cells/mL was seeded on each substrate. The culture medium was changed every 2 d. The cell proliferation on different materials was assessed at days 1, 3, 5 and 7.

At each time point, after the culture medium was aspirated, 200 μL of CCK-8 solution (10% v/v in α-MEM) was added to each well. After incubation at 37°C for 1 h, 100 μL of incubated CCK-8 solution from each well was transferred from 48-well plate to 96-well plate after enough mixing by pipette. The absorbance of solution in each well was measured at a wavelength of 450 nm by microplate reader (BioTek Synergy 4, USA).

2.8 Alkaline phosphate (ALP) activity assay

PEEK, CCPEEK and BIPEEK discs with the diameter of 18 mm were placed into a 12-well plate and 1 mL of MC3T3-E1 cell suspension with the cell density of 1×10^5 cells/mL was seeded on the facilitated ach substrate. After 1 h, proliferation medium was aspirated, and osteogenic differentiation medium (proliferation medium supplemented with 50 μg/mL ascorbic acid and 10 mmol/L β-glycerophosphate) was introduced. The culture medium was changed every 2 d, and the ALP activity was assessed at days 7 and 14.

At each time point, after the culture medium was aspirated, the materials discs were wash with PBS and 200 μL cell lysates (Zomanbio, China) supplemented with protease inhibitors (1% v/v) was added in each well. After incubation on ice for 30 min, the solution was collected into centrifuge tubes and centrifuged for reserve. Alkaline Phosphatase Assay Kit (Beyotime, China) and Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, USA) were used to measure the ALP activity of the intracellular protein according to manufacturer's instruction. For an absolute quantitative analysis, the unit of ALP activity converted uniformly into μmol (para-nitrophenyl phosphate)/min (incubation time)/μg protein (intracellular total protein).

2.9 Alizarin red S staining

The sample for the assessment of calcifying nodules was prepared as described in the section on ALP activity assay. Alizarin red S (Sigma, USA) was used to stain calcifying nodules at day 14. Briefly, after the culture medium was aspirated, the samples was washed twice by PBS, then the cells were fixed with 10% paraformaldehyde in PBS for 30 min and washed by deionized water 3 times. Then 500 μL 0.1% alizarin red S solution (pH = 8.3) was added to each well and incubated for 30 min. The stained samples were washed by deionized water several times. For semi-quantitative analysis, 500 μL (1-hexadecyl)pyridinium chloride monohydrate (Alfa Aesar, USA) aqueous solution (100 mmol/L) was added to each sample and incubated over night. The absorbance of solution was measured at a wavelength of 562 nm by microplate reader (BioTek Synergy 4, USA).

2.10 *In vivo* study

The *in vivo* study was approved by Animal Ethics Committee in Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS) (No. SIAT-IRB-131111-YGS-SPENG-A0029). The animals were maintained on a normal, solid lab diet and regular tap water. For the *in vivo* tests, simplified PEEK intervertebral disc prostheses were manufactured according to the metrical data measured before (Fig. 1). One half of these prostheses were modified following the previous modification method of BIPEEK. For all the studies, 28 adult female New Zealand White rabbits (3.2–3.7 kg) were used. The animals were randomly divided into two groups: 14 implanted with rhBMP-2 immobilized PEEK prostheses (experimental group) and 14 implanted with non-modified PEEK prostheses (control group).

An anterior extraperitoneal approach was adopted. Firstly, the animals were anesthetized with 3% pentobarbital sodium by ear marginal vein injection. Shaved skin of hypogastrium was prepared with betadine, and then the L5, L6 vertebra was exposed by pushing fascia aside through about 5 cm abdominal midline incision. The L5/6 intervertebral discs were taken out with small detacher and micro spatula. Prostheses were implanted into L5/6 intervertebral disc area. Finally, both the skin and fascial layers were sutured with resorbable sutures. The same surgeon inserted all implants. After the surgery, penicillin sodium (800 000 units per day) was given to the animals by intramuscular injection for 3 d. After surgery for 4 and 8 weeks, the animals were sacrificed by injecting overdose pentobarbital sodium.

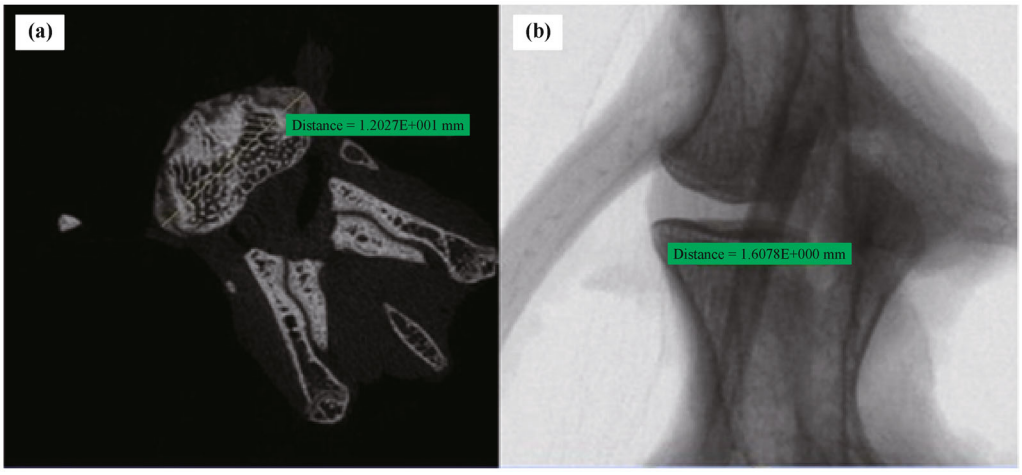

Fig. 1 The dimension of intervertebral disc space was measured by microCT **(a)** cross-section image and **(b)** side image.

2.11 MicroCT analysis

High-resolution images of all the specimens were obtained from a MicroCT scanner (Bruker SkyScan 1173, Belgium) selecting Cu + Al filter and running at 80 kV and 313 μA with the scanning resolution of 18 μm. Software NRecon, CTAn, CTVol and CTVox were used for reconstruction and analysis. In the reconstruction process, the dynamic range image log value was set from 0 to 0.02. After reconstruction, horizontal line of the highest point of L6 vertebra was set as the center mark (the red midline in Fig. 2), and 100 cross-section images area above and below center mark were conducted as the analyzed area (the non-fluorescent area in Fig. 2). In the analyzed area, region of interest (ROI) circled carefully (Fig. 3) with the gray threshold value was set from 100 to 250. The selected bone area was analyzed by bone mineral density (BMD), the percent of bone volume (bone volume/total volume, BV/TV), the bone surface/bone volume ratio (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), and trabecular bone pattern factor (Tb.Pf).

2.12 Tensile strength test

There were six specimens per group (8 weeks groups) were used for tensile testing. Tensile testing was performed with a wire was glued to the polymer prostheses and stretched in horizontal direction. The tensile test was conducted using a self-making tensile machine based on electricity measure principle, with a tensile speed of 1 mm/s (Fig. 4(b)).

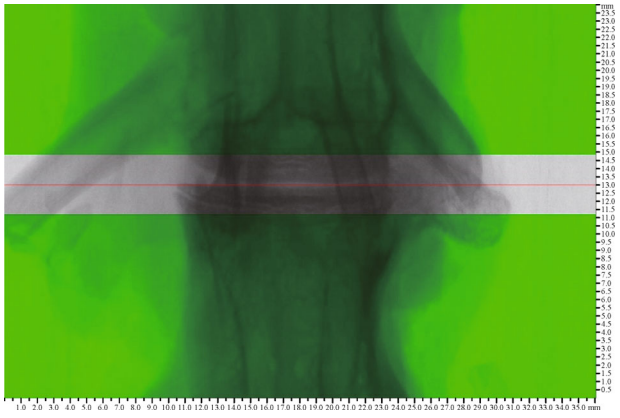

Fig. 2 The red midline was the center mark which was set according to the highest point of L6 vertebra and the non-fluorescent area was the scanning area containing 100 cross-section images above and below the red line.

2.13 Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using Student's *t*-test. Results were considered statistically significant with $P < 0.05$.

3 Results

3.1 Surface characterization

The scanning electron microscopy (SEM) images clearly showed the surface condition of unmodified PEEK (Fig. 5(a)) and CCPEEK (Figs. 5(b)–5(d)). Limited by the

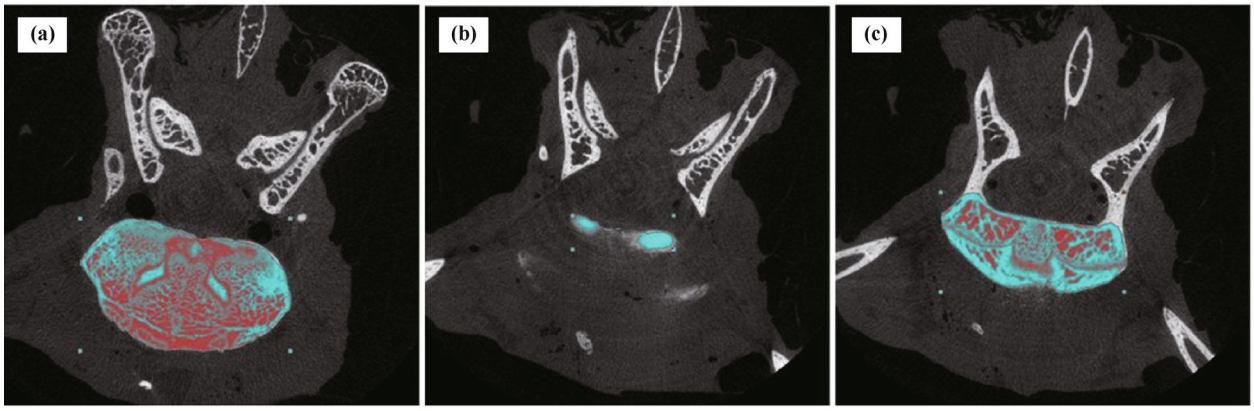

Fig. 3 The ROI was circled carefully with the gray threshold value set from 100 to 250: **(a)** the cross-section image above the center mark; **(b)** the cross-section image on the center mark; **(c)** the cross-section image below the center mark.

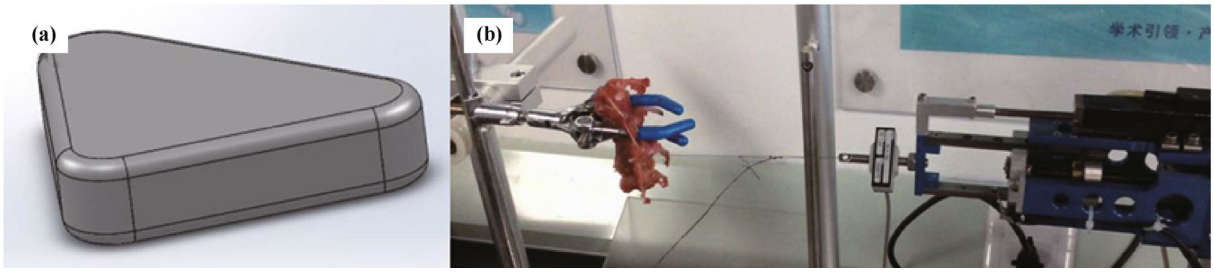

Fig. 4 **(a)** The geometry design of simplified PEEK intervertebral disc prostheses by SolidWorks 2013 software. **(b)** A wire was glued to the polymer prostheses and elongated in horizontal direction.

resolution of SEM, the structure of small triple helix could not be seen clearly, but collagen fibrils were obvious with the diameter of about 50–200 nm (Fig. 5(d)). The contact angles of different samples are tabulated in Table 1, showing a significant difference of hydrophilicity between PEEK and modified PEEKs. The contact angle of unmodified PEEK was measured to be (82.48±0.82)° with water. With the coating of cross-linked type-I collagen, CCPEEK was more hydrophilic with the water contact angle significantly decreased to (38.26±3.07)°. The contact angle of BIPEEK seemed to have no obvious difference compared to that of CCPEEK, which was (38.58±2.38)°.

The appearance of N 1s absorption peak in the survey XPS spectrum at 398 eV of CCPEEK and BIPEEK indicated that collagen was successfully coated onto the surface of PEEK (Fig. 6). No obvious difference in survey scan and narrow scan between CCPEEK and BIPEEK was observed because only a trace amount of rhBMP-2 was employed and it has a similar chemical constitution with collagen. Higher oxygen contents were detected on the surface of CCPEEK and BIPEEK compared with the unmodified PEEK. The C/O ratio of the unmodified PEEK was 5.76, which was different to the theoretical value of 6.33 calculated based on the PEEK repeat unit. The reason should be contributed to unavoidable oxidation reactions happened during the processing. With collagen coating, the C/O ratios of CCPEEK and BIPEEK were decreased to 3.46 and 3.33, respectively.

The C 1s spectra of unmodified PEEK surface could be fitted by two peak components attributable to the aliphatic carbon (C–C/C–H) and ether bond (C–O–C) (Fig. 7(a)). The main difference between BIPEEK and unmodified PEEK in C 1s spectra was the presence of carbonyl carbon (C = O), which is the characteristic group of protein (Fig. 7 (b)). The O 1s peak was attributed to ether bond (C–O–C) and carbonyl carbon (C = O) (Fig. 7(c)). More contribution of carbonyl carbon (C = O) to the O 1s peak was confirmed on the surface of BIPEEK compared to the unmodified PEEK (Fig. 7(d)). The results of narrow scans of XPS analysis of three discs confirmed the protein-coating was successful on the surface of PEEK.

The measured content of rhBMP-2 tested by ELISA was 1420 pg/well. After 1 h, approximately 1323.62 pg of

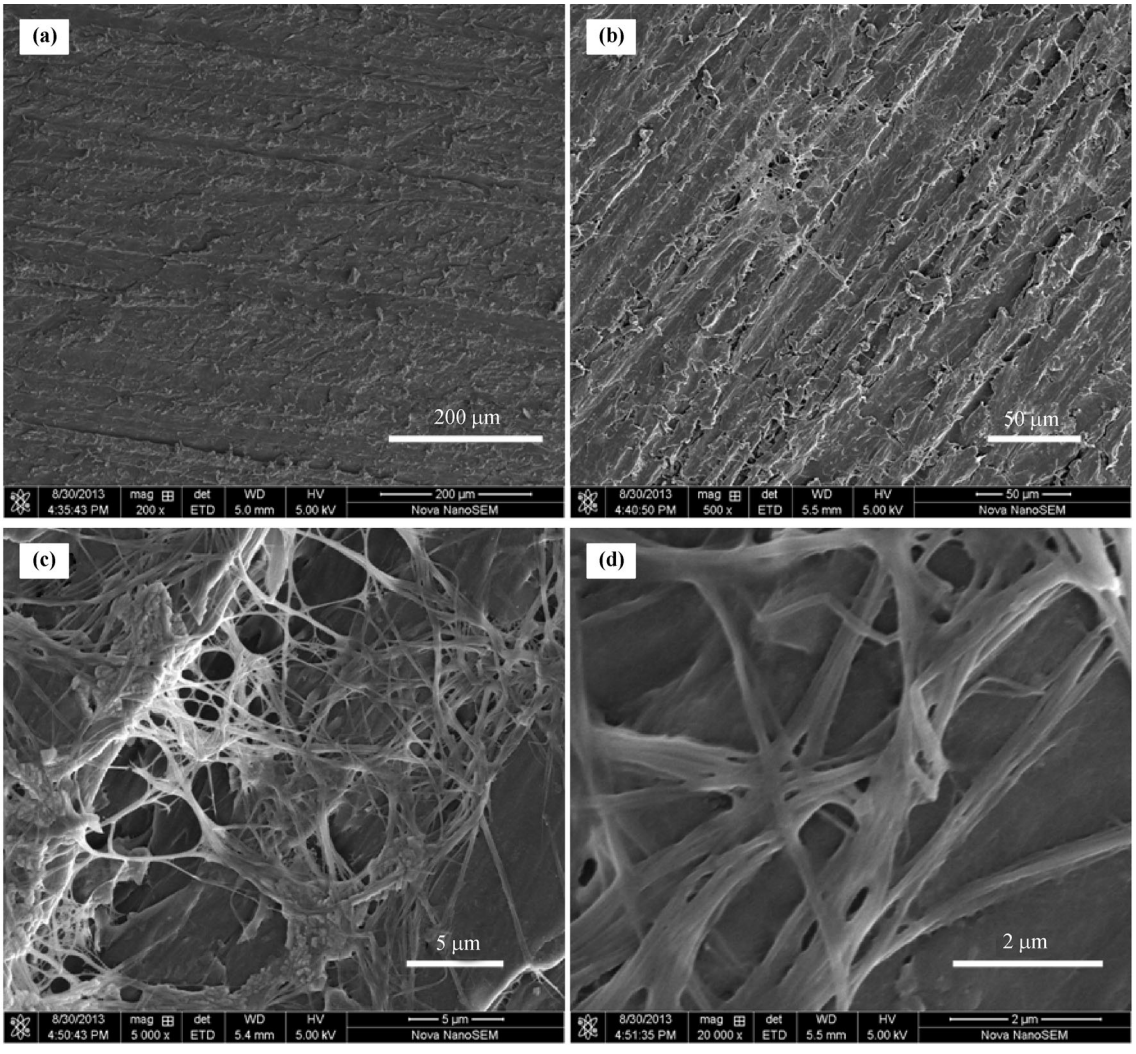

Fig. 5 SEM images of **(a)** unmodified PEEK and **(b)(c)(d)** BIPEEK. Collagen fibrils were obvious with the diameter of about 50–200 nm in (b)–(d).

Table 1 Contact angles of different samples

Sample (n = 3)	Contact angle /(°)
PEEK	82.48±0.82
CCPEEK	38.26±3.07
BIPEEK	38.58±2.38

BMP-2 retained on the surface of BIPEEK in each well, and the residual rate was about 93.23% (Fig. 8). After 1 d, the residual rate declined to 86.86%, which was the most significant decrease besides the initial burst. Then the release of rhBMP-2 slowed down in a period of 30 d. After 30 d, the residual rate of rhBMP-2 on the surface of BIPEEK remained higher than 80%. The amide bond formed by EDC/NHS would be stable in PBS, so the release of rhBMP-2 may be mainly contributed to desorption. Therefore, rhBMP-2 was immobilized onto the collagen-coating by both covalent bonding and physical absorption.

3.2 Cell proliferation and differentiation

Cell proliferation on PEEK, CCPEEK and BIPEEK discs was determined by the CCK-8 assay (Fig. 9). Cells were more proliferative on surfaces of CCPEEK and BIPEEK in the early stage (1–3 d). On day 1, a relatively higher level of cell proliferation on CCPEEK and BIPEEK was observed compared with PEEK, which may be contributed to the collagen-coating. Early attachment of cells on the collagen-coatings was reported in Ref. [28]. On day 3, the differences seemed more obvious, especially for the difference between BIPEEK and PEEK ($P < 0.05$). On

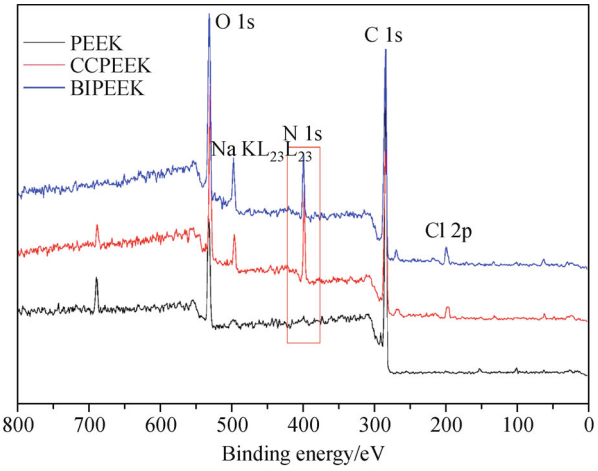

Fig. 6 The survey XPS spectra of PEEK, CCPEEK and BIPEEK.

days 5 and 7, three kinds of discs showed similar proliferation rates.

The ALP activity was conducted to investigate the differentiation of osteoblast on different materials (Fig. 10). The ALP activities of cells on all three kinds of materials increased with time. Cells on BIPEEK expressed the highest ALP activities and remarkable differences were observed on the both time points compared with unmodified PEEK ($P < 0.05$). On day 7, ALP activity of cells on CCPEEK was also higher than that of cells on unmodified PEEK significantly ($P < 0.05$). Although a much higher numerical value was measured on BIPEEK than that on CCPEEK, there was no significant difference between them ($P > 0.05$). On day 14, ALP activities of the cells cultured on PEEK and CCPEEK presented similar values, while the ALP activity with BIPEEK was significantly higher than those with PEEK and CCPEEK.

Alizarin red S staining showed positive results of three materials at day 14 (Fig. 11). Visible calcifying nodule staining emerged on all the samples. Semi-quantitative analysis (Fig. 12) proved that CCPEEK and BIPEEK showed similar values, which were significantly higher than those with PEEK ($P < 0.05$).

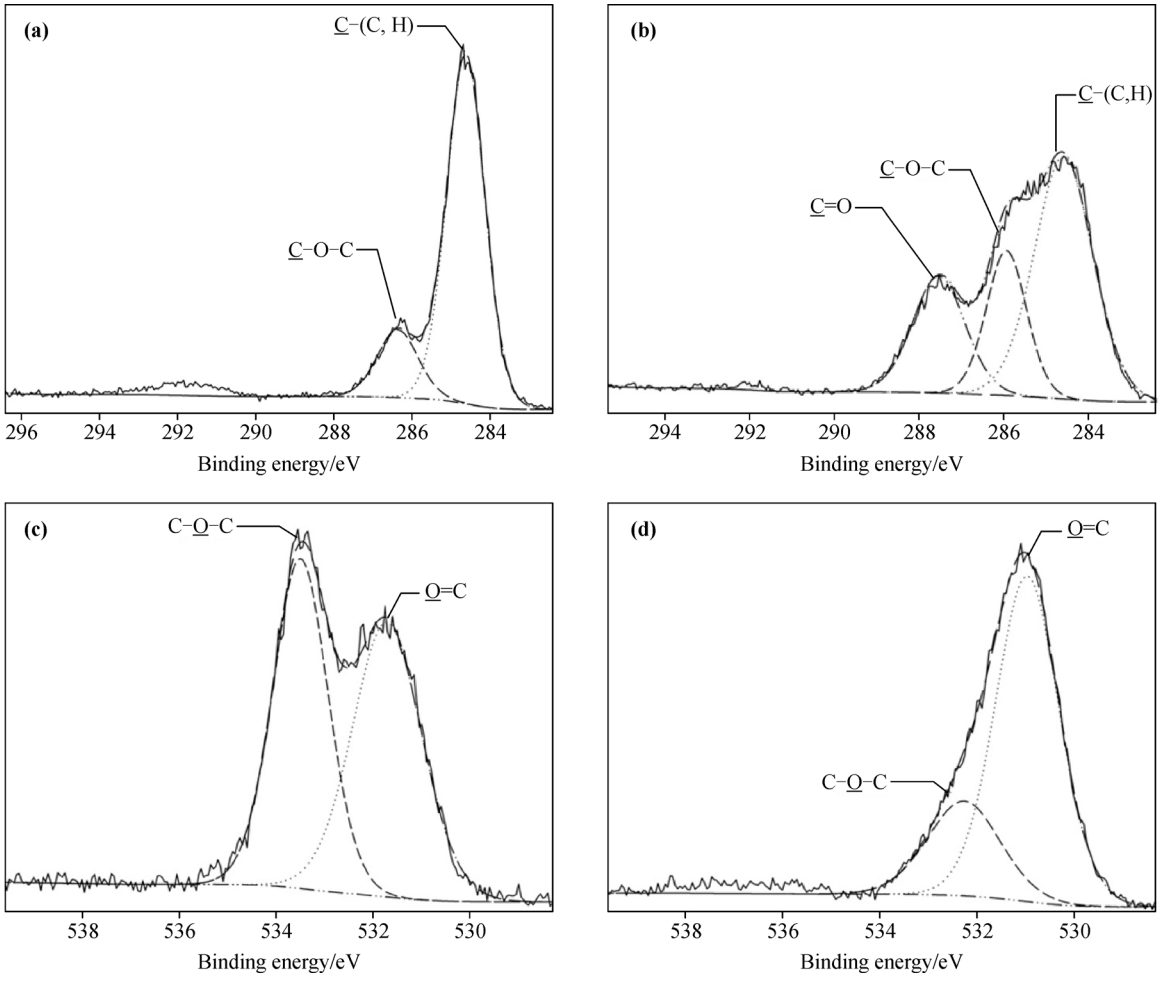

Fig. 7 C 1s spectra of **(a)** PEEK and **(b)** BIPEEK; O 1s spectra of **(c)** PEEK and **(d)** BIPEEK.

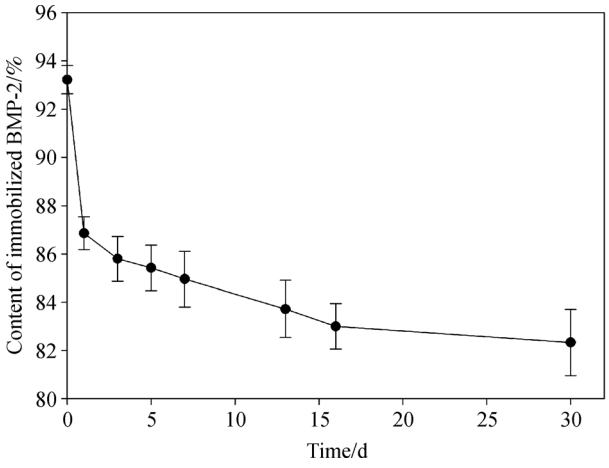

Fig. 8 The residual rate of rhBMP-2 on the surface of BIPEEK.

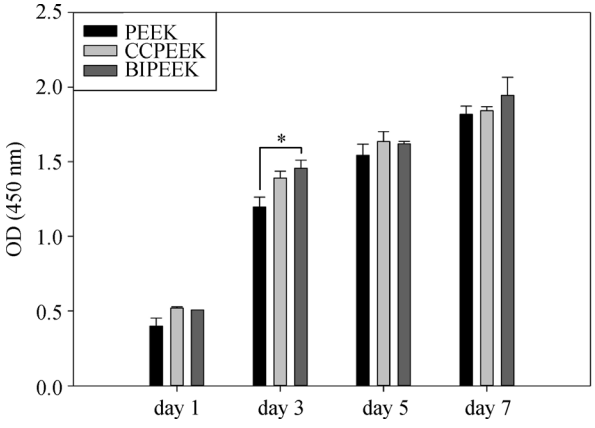

Fig. 9 Cell proliferation of MC3T3-E1 cells cultured on PEEK, CCPEEK and BIPEEK after 1, 3, 5 and 7 d. (* denotes significant difference: $P < 0.05$)

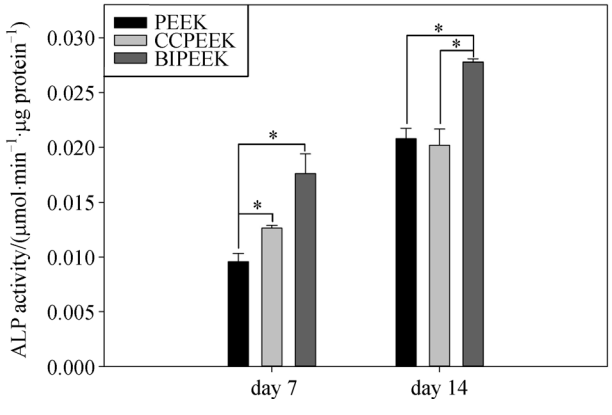

Fig. 10 ALP activity of MC3T3-E1 cells cultured on PEEK, CCPEEK and BIPEEK after 7 and 14 d. (* denotes significant difference: $P < 0.05$)

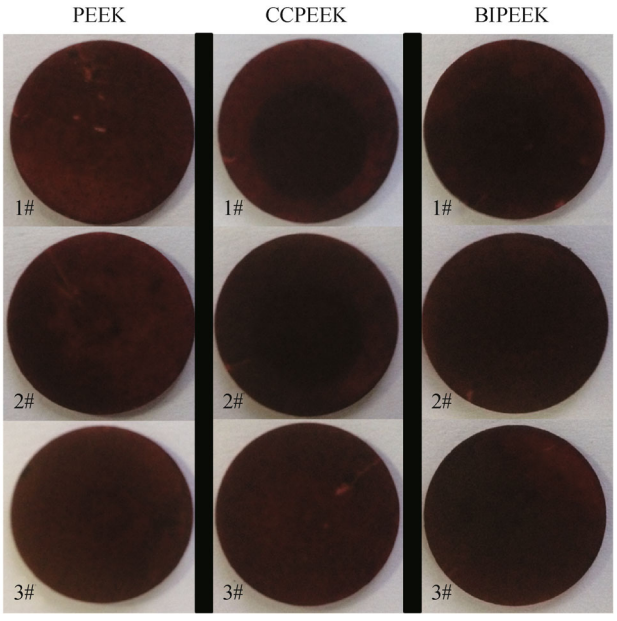

Fig. 11 Alizarin red S staining of PEEK, CCPEEK and BIPEEK cultured with MC3T3-E1 cells after 14 d.

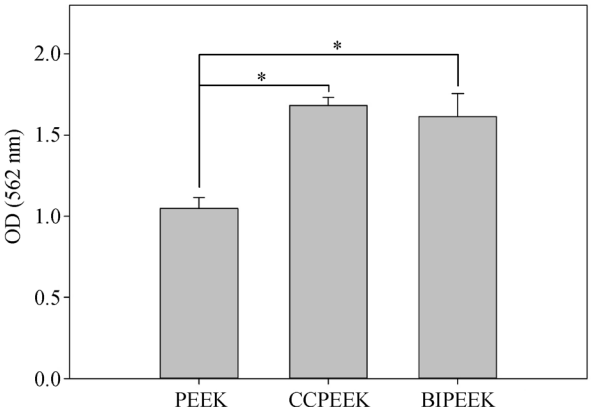

Fig. 12 Semi-quantitative analysis of alizarin red S staining of PEEK, CCPEEK and BIPEEK cultured with MC3T3-E1 cells after 14 d. (* denotes significant difference: $P < 0.05$)

3.3 MicroCT analysis

Lumbar spine specimens from prostheses implanted animals were analyzed by microCT. From the 3D reconstruction images (Fig. 13), a larger callus was formed in the experimental group (Fig. 13(b)) than that in the control group (Fig. 13(a)) after 4 weeks. At 8 weeks, both experimental group and control group showed similar size of callus.

Control and experimental groups showed some differences in the bone tissue near the implants at both 4 and 8 weeks after implantation (Table 2). Statistical difference

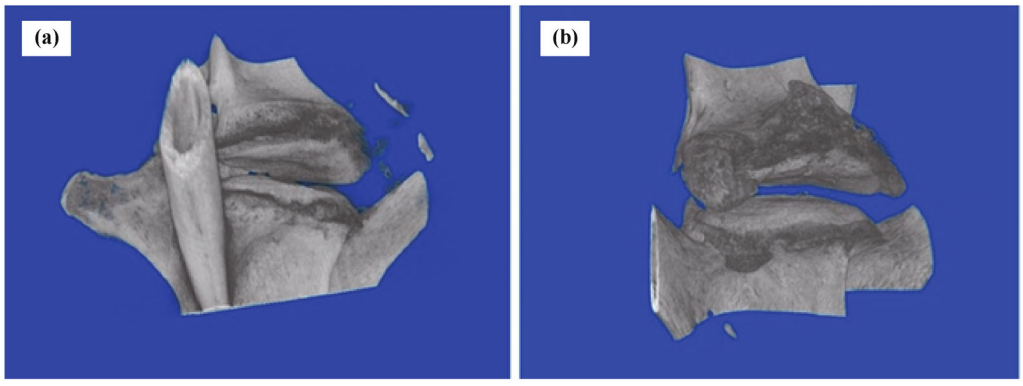

Fig. 13 Calluses in specimens in **(a)** the control group and **(b)** the experimental group after implanting prostheses for 4 weeks.

($P < 0.05$) of the percent of BV/TV between the experimental group and the control group appeared as early as 4 weeks after surgery, while the differences in other parameters were not statistically significant ($P > 0.05$). At 8 weeks after implantation, compared with the control group, BIPEEK implants displayed a more significant difference in the percent of BV/TV ($P < 0.001$). In the meantime, the BMD value of BIPEEK implants was significantly higher than that of the control group ($P < 0.05$), and the BS/BV value of BIPEEK implants was significantly lower than that of PEEK implants ($P < 0.05$). Compared with the control group, the Tb.Sp value of the experimental group was significantly lower than that of unmodified implants ($P < 0.05$), and the Tb.Pf of BIPEEK prostheses were significantly higher than that of untreated ones ($P < 0.05$).

3.4 Tensile strength test

After 8 weeks after implantation, a significant higher strength was observed on the experimental group ((17.06±5.48) N) than that on the control group ((8.37±5.16) N) ($P < 0.05$).

4 Discussion

The bioinertness of PEEK limits its application in spinal implantation. To overcome this, BMP-2 was considered to modify the surface of PEEK in this study. However, it is difficult to immobilize BMP-2 onto PEEK due to lack of available functional groups on the surface of PEEK. Irradiation treatments such as plasma ion immersion implantation (PIII) technology have been developed to introduce different functional groups to the surface of PEEK, but subsequent processing cannot effectively solve the problem. Traditional covalent bond linking strategy is quite inefficient [29–30] and surface-initiated polymerization technology is sophisticated for industrial mass production [31–32]. In this study, a simple and viable method to modify PEEK was investigated.

Hydrophobic surface of PEEK makes it suitable for stable collagen-coating by hydrophobic interaction. Moreover, acid-soluble type-I collagen does not dissolve well in neutral liquid, which would benefit the physical adsorption process. Thus, collagen-coating was designed to facilitate the immobilization of BMP-2 onto the surface of PEEK. As no in-depth study has been conducted to explore the optimal collagen concentration for PEEK adsorption, we investigated the concentrations ranging from 1 μg/mL to 1 mg/mL and found that the concentration of 1 mg/mL was the optimized concentration for the PEEK coating.

Further studies are required to confirm the cross-linking mechanism of collagen cross-linking by EDC/NHS. The increasing concentration of EDC/NHS would result in

Table 2 Differences between control and experimental groups at the time of 4 and 8 weeks after implantation

Time /week	Group (n = 6)	BMD /(mg·cc^{-1})	(BV/TV) /%	(BS/BV) /mm^{-1}	Tb.Th /μm	Tb.N /mm^{-1}	Tb.Sp /μm
4	control group	174.35±6.43	68.40±3.78	11.12±1.22	0.29±0.02	2.37±0.11	0.19±0.02
	experimental group	179.60±9.78	76.40±3.82	9.33±1.37	0.32±0.02	2.41±0.11	0.17±0.02
	P-value	0.364	0.016	0.076	0.094	0.560	0.149
8	control group	170.95±5.00	66.33±3.17	11.44±1.36	0.29±0.02	2.27±0.10	0.19±0.01
	experimental group	178.28±4.35	76.40±12.84	9.78±1.22	0.31±0.04	2.45±0.21	0.16±0.01
	P-value	0.022	< 0.001	0.05	0.026	0.075	0.003

decreased amount of coated collagen [33]. And the collagen fibril consists of triple helix (tropocollagen) of approximately 300 nm in length and 1.5 nm in diameter [34]. Five tropocollagen composed microfibers first, which formed subfibril in the next moment with the separation distance of 1.3–1.7 nm [35]. The diameter of microfiber is 3.5–4.0 nm, but EDC/NHS could only cross-link two groups located within 1.0 nm, so only inter-helical cross-linking would happen theoretically [35].

Our results of cell proliferation test proved that the unmodified PEEK was nontoxic and there was no obvious effect of collagen-coating on cells proliferation, which was consisted with the results previously reported [12–13]. This is probably because microscopic amounts of rhBMP-2 immobilized, BIPEEK also showed limited capacity promoting cell proliferation.

ALP activity assay and alizarin red S staining was used for cell differentiation test. ALP is a protein marker expressed early in the development of osteoblast, while calcifying nodules were formed in a later stage, so the ALP activity assay was carried out on days 7 and 14 and the alizarin red S test was done on day 14. In alizarin red assay, the absorbance of CCPEEK mathematically higher than that of BIPEEK, but the difference was not significant. It was not very accurate to access the quantity of calcifying nodules by the semi-quantitative analysis using (1-hexadecyl)pyridinium chloride monohydrate. From the results, equal amount of calcifying nodules on CCPEEK and BIPEEK were observed and both of them were significantly higher than those on PEEK. The effect of collagen coating on cell differentiation was for sure based on our data. It has been proved both in previous reports and our investigation that collagen coating has a positive effect on cell proliferation [10–11]. As a result, collagen coating enhanced both cell proliferation and differentiation into osteogenic leakage [10–13]. It sounds contradictory and the reason and mechanism are still unclear. Meanwhile, although rhBMP-2 did not show significant effect on cell proliferation, its immobilization resulted in significant increase of ALP activity on BIPEEK than that on CCPEEK on day 14. By the simple immobilization method, the bioactivity of a small amount of rhBMP-2 could preserve for a relative long period.

MicroCT is a sensitive and effective analytic tool, which is more and more applied in the *in vivo* study of orthopedic biomaterials [36–37]. In our microCT test, the most important parameter we concerned is the percent of BV/TV which could perceivably reflect the change in bone mass. The notable change of the percent of BV/TV as well as other parameters proved a positive bone change in the surrounding bone tissue of modified implants when compared with the results of unmodified prosthesis.

Although the pullout force of the prostheses was very small, the measurement reliability was guaranteed by the high-precision tensile strength machine with the precision of 0.1 N. As the most persuasive evidence, the results of tensile strength test directly demonstrated the stability of implant fixation, which was the original intention of our modification.

5 Conclusions

In this study, we successfully prepared a CCPEEK with an immobilization of rhBMP-2 by hydrophobic interaction and covalent binding method. The effects of immobilization of rhBMP-2 on collagen-coating were investigated *in vitro* and *in vivo*. The results proved that rhBMP-2 immobilized PEEK facilitated the differentiation of cultured MC3T3-E1 cells and promoted osteogenesis surrounding the implanted sites of lumbar spines. This method could be used to improve the biocompatibility of PEEK materials for orthopedic applications.

Abbreviations

ALP	alkaline phosphate
BIPEEK	BMP-2 immobilized collagen-coated PEEK
BMD	bone mineral density
BMP-2	bone morphogenetic protein-2
BS/BV	bone surface/bone volume ratio
BV/TV	bone volume/total volume
CCPEEK	collagen-coated PEEK
ECM	extracellular matrix
EDC	N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
α-MEM	α-minimum essential medium
NHS	N-hydroxysuccinimide
PBS	phosphate buffer saline
PEEK	polyetheretherketone
PET	poly(ethylene terephthalate)
PIII	plasma ion immersion implantation
PS	polystyrene
rhBMP-2	recombinant human bone morphogenetic protein-2

ROI	region of interest
SD	standard deviation
SEM	scanning electron microscopy
Tb.N	trabecular number
Tb.Pf	trabecular bone pattern factor
Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
XPS	X-ray photoelectron spectroscopy

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