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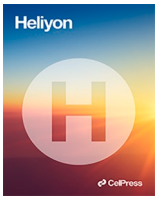

Research article

The ability of SPEEK to promote the proliferation and osteogenic differentiation of BMSCs on PEEK surfaces

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ABSTRACT

To investigate the ability of sulfonated polyetheretherketone (SPEEK) to promote the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and compare the effects of different degrees of sulfonation (DS), SPEEK was made with two different DS. The L-SPEEK group had a lower DS, while the H-SPEEK group had a higher DS. The physicochemical properties of both species were evaluated by scanning electron microscopy (SEM), capitilize Fourier transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), X-ray diffraction (XRD) and differential scanning calorimetry (DSC). Then, proliferation and osteogenic differentiation between the two groups and with pure polyetheretherketone (PEEK) were compared after surface inoculation of bone marrow mesenchymal stem cells (BMSCs). Scanning electron microscopy (SEM) revealed that the surface of the PEEK substrates could be smooth or coarse, and the degree of roughness increased with increasing sulfonation. FTIR spectroscopy showed that both the L-SPEEK and H-SPEEK samples contained sulfonic acid. TGA and XRD revealed that the components in the two groups were the same, but the intensities were different. After BMSC inoculation, a CCK8 assay revealed that the cells proliferated more on the H-SPEEK surface and little on the L-SPEEK surface compared with the PEEK surface. Then, osteogenic differentiation was verified by immunofluorescence staining for OCN and Runx2, which indicated that H-SPEEK had the greatest effect on improving differentiation. The results of alizarin red staining (ARS) and alkaline phosphatase staining (APS) also revealed this trend. Sulfonation can change the microsurface of PEEK, which can improve both BMSC proliferation and osteogenic differentiation.

1. Introduction

Degenerative disc disease (DDD) is a common disease of orthopaedic department, especially the spinal surgery, which could lead to low back pain, neck pain and radiculopathy. Additionally, DDD could disturb urination or defecation when the degenerative disc compresses the nerves and dural sac seriously. These syndromes might decrease the quality of life of patients [1]. To relieve these syndromes, the degenerative disc could be excised, and a cage should be inserted into the intervertebral space as a substitute. The desired effect is the fusion of the adjacent vertebrae through the area of the cage. This kind of spinal fusion surgery is standard for DDD and has been widely applied for several years. The fusion rate is an important factor associated with the outcome of fusion surgery and

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is often affected by several factors [2], such as the condition of the patient [3,4], the technology used by the surgeon [2,5] and the instruments used in the operation [6–8]. Among these factors, the cage used in the operation is an important factor that can be adjusted by technology to improve the fusion rate.

Since the cage is a critical factor in the fusion rate, many studies have been conducted to enhance the ability of the cage to improve the fusion rate. Currently, cages are often made of polyetheretherketone (PEEK) materials. These materials can offer enough strength to withstand the stress applied to the cage and possess a proper elastic modulus. Nevertheless, as a kind of polymeric organic material, PEEK is hydrophobic and does not easily adhere to cells, which might obstruct the course of fusion. Therefore, several studies have attempted to modify the surfaces of PEEK and gotten good results [9,10], for example the introduce of gelatin hydrogel [11] and using special processing technology of combining sulfonation with sub-millimeter laser machining [12]. The using of fast ambient-temperature sulfonation has been verified to modify the surface of PEEK [13]. Among these researches, the fabrication of surface-sulfonated polyetheretherketone have been proved of having positive effect [12,14–18]. For example, polymer coatings based on sulfonated-poly-ether-ether-ketone films were researched for the implant dentistry applications [18]. Similarly, the sulfonate-bearing polyetheretherketone has been approved to have more ideal effect of antibacterial, anti-inflammatory and osteogenic activity [15]. The character of osteogenesis of this modified material inspired us modifying the surface of the cage by the method of sulfonation.

In the present study, PEEK was processed via sulfonation to obtain the material sulfonated polyetheretherketone (SPEEK), after which the PEEK surface was covered with SPEEK. Also, we fabricated two kinds of SPEEK surfaces with different degree of sulfonations (DS). Then, the physicochemical properties of the obtained materials were analysed, and bone marrow mesenchymal stem cells (BMSCs) were cultivated on the SPEEK surfaces to test their adhesion and proliferation ability on the surfaces with different DS. Additionally, the osteogenic differentiation of BMSCs was evaluated and compared between the two kinds of SPEEK, also with that of the BMSCs on PEEK. The current research was approved by the committee of the General Hospital of Northern Theater Command (number Y(2023)085).

2. Materials and methods

The polymer SPEEK was prepared by sulfonation of a PEEK polymer (Jida High Performance Materials Company, Ltd., China) with concentrated sulfuric acid. Briefly, 5 g of PEEK was first dissolved in 50 mL of concentrated sulfuric acid under magnetic stirring at room temperature and then heated to 60 °C for 2 h. Subsequently, the homogenous solution was gently added dropwise to an ice–deionized (DI) water mixture under mechanical stirring and allowed to stand overnight. Afterwards, the ribbon-like polymer was washed with a large quantity of DI water several times before adjusting the filtrate to pH 7. Finally, the SPEEK polymer was obtained after heating in an oven at 60 °C for 12 h. The DS of SPEEK was measured with the titration of released protons from the polymer using a standard solution of sodium hydroxide to be 46 % as the group of L-SPEEK [19]. Similarly, a SPEEK polymer with a DS of 82 % was prepared under the same conditions, but heating to a temperature of 80 °C for a duration of 6 h, as the group of H-SPEEK.

2.1. Adherence of a thin SPEEK layer to the PEEK plate

A SPEEK DI water solution (2 wt%) was prepared by dissolving 0.5 g of SPEEK in 24.5 mL of DI water at 50 °C under magnetic stirring. A PEEK (as the substrate) was spin-coated with SPEEK solution at a speed of 100 revolutions per minute (rpm) for 10 s. The final PEEK/SPEEK product was prepared after heating in an oven at 60 °C for 4 h.

2.2. Characterization

Fine microstructure The microstructure of PEEK/SPEEK was observed with a Zeiss scanning electron microscope. All samples were sputter-coated with gold nanoparticles before observation.

Fourier transform infrared spectroscopy The Fourier transform infrared spectra of PEEK/SPEEK and the components were recorded with KBr by a Vertex 70 Spectrometer (Bruker Optics) in the range of 4000–400 cm-1.

Thermal stability To test the thermal stability of PEEK/SPEEK and its components, thermogravimetric analysis curves were recorded on a TGA 290C system (Netzsch Company) under air flow at a rate of 30 mL/min. Approximately 5 mg of sample was heated from ambient temperature to 700 °C at a rate of 10 °C/min. Notably, all the samples were preheated to 100 °C for 4 h.

Differential scanning calorimetry Differential scanning calorimetry (DSC) curves of the samples were obtained using a PerkinElmer DSC 4000 in the range of 25–500 °C.

X-ray diffraction X-ray diffraction patterns were recorded from 5° to 70° with a scan speed of 5°/min by a Part Pro-MPD X-ray diffractometer (Panalytical B.V.) using a Cu Kα radiation source (λ = 1.5418 Å).

$$2\,d\sin\theta = n\,\lambda \tag{1}$$

In the Bragg's equation (Eq. (1)), n is an integer, λ is the wavelength of the incident wave, d is the space between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes. Notably, an increase in the θ value is indicative of a decrease in d owing to the fixed value of nλ.

Water contact angle The water contact angle (WCA) values of the PEEK plate, H-SPEEK and L-SPEEK membranes were measured to evaluate the surface property of hydrophilicity/hydrophobicity. A contact angle goniometer (DSA 25, KRUSS, Germany) was used and

the result was the average value on both sides.

2.3. Comparison of proliferation and osteogenic differentiation

Three groups of samples were used as the blank group of PEEK, PEEK/SPEEK with a DS of 46 %, defining as the L-SPEEK group and the PEEK/SPEEK with a DS of 82 %, defining as the H-SPEEK group. The samples were first immersed in wells with osteogenic medium (Biyuntian Biotechnology Co., Ltd., China).

BMSCs (cat no: CP-M131, Procell, China) in the logarithmic growth phase were inoculated into 96-well plates at a density of 1×10^4/well and cultured. Then, the adhesion and proliferation of the BMSCs were tested by the CCK-8 method (Biyuntian Biotechnology Co., Ltd., China) after 12 h and 24 h.

Then, BMSCs (cat no: CP-M131, Procell, China) in the logarithmic growth phase were inoculated into 24-well plates at a density of 1×10^5/well and cultured. On the 14th day, the growth and morphology of the BMSCs were observed after staining with phalloidin (Biyuntian Biotechnology Co., Ltd., China). Additionally, immunofluorescence staining for OCN and Runx2 was carried out, together with alizarin red staining (ARS) and alkaline phosphatase staining (APS).

3. Results

In the present study, two kinds of SPEEK were obtained with different DS. The SEM images showed that the surface of PEEK changed after the addition of SPEEK. The original surface of PEEK was smooth (Fig. 1A and B), but the surface became coarse after the addition of SPEEK (Fig. 1C and D). Additionally, the degree of roughness increased with increasing DS (Fig. 1C and D). Additionally, the thickness of the SPEEK layer coating was controlled to be 20–23 μm.

The FTIR spectroscopy results showed that the samples from the two groups had similar spectra but different strengths. The characteristic peaks at 1647, 1115 and 1061 cm-1 corresponded to the tensile vibrations of C=O and the asymmetric and symmetrical stretching vibrations of sulfonic acid (O=S=O), respectively. These data showed that SPEEK was successfully synthesized, and that the H-SPEEK group contained more sulfonic acid than the L-SPEEK group (Fig. 2A).

The decomposition and thermal stability characteristics were analysed by TGA and DSC. Both H-SPEEK and L-SPEEK lost a slight amount of mass below 100 °C due to the volatilization of water and solvent. When the temperature increased to 220 °C, the impurities in SPEEK were completely removed, but SPEEK was stable and did not decompose, this resulted in further mass loss. When the temperature increased to 310 °C, the TGA curve decreased, which was caused by the decomposition of SO_3H in SPEEK. When the temperature increased further to 400 °C, the curve decreased again, indicating that the main chain of SPEEK had begun to decompose.

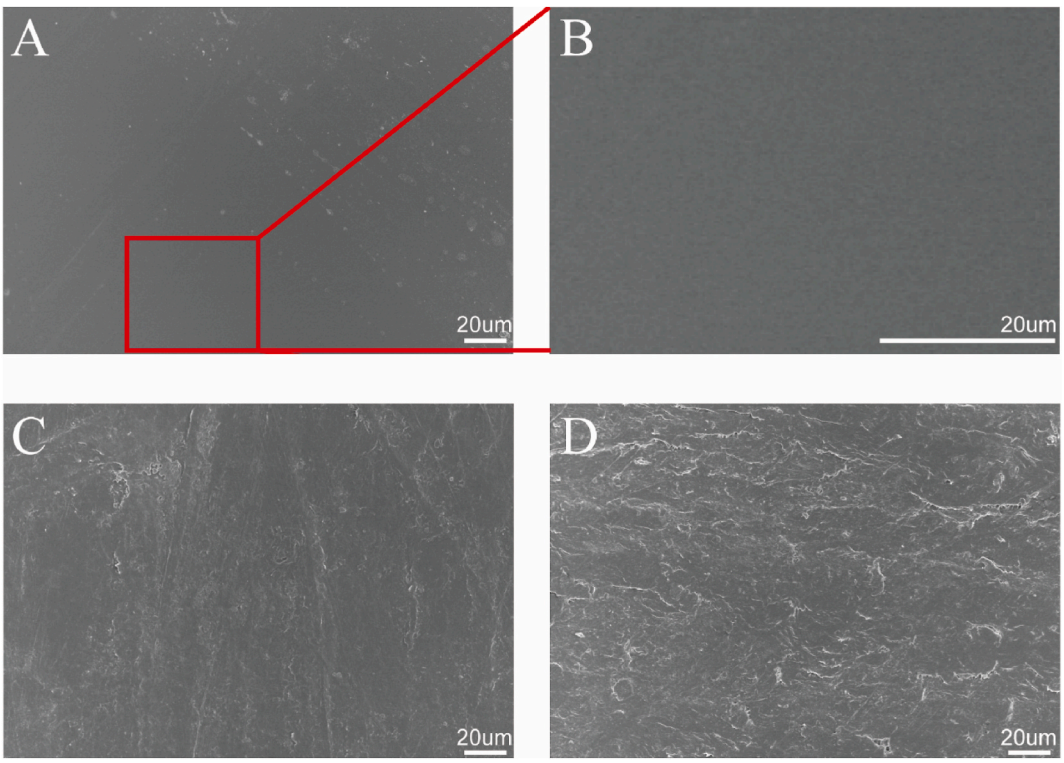

Fig. 1. SEM images of the surfaces of the samples (A–D). The surface of PEEK was smooth (A, B). The surfaces of L-SPEEK (C) and H-SPEEK (D) were coarse. The degree of roughness increased with increasing DS.

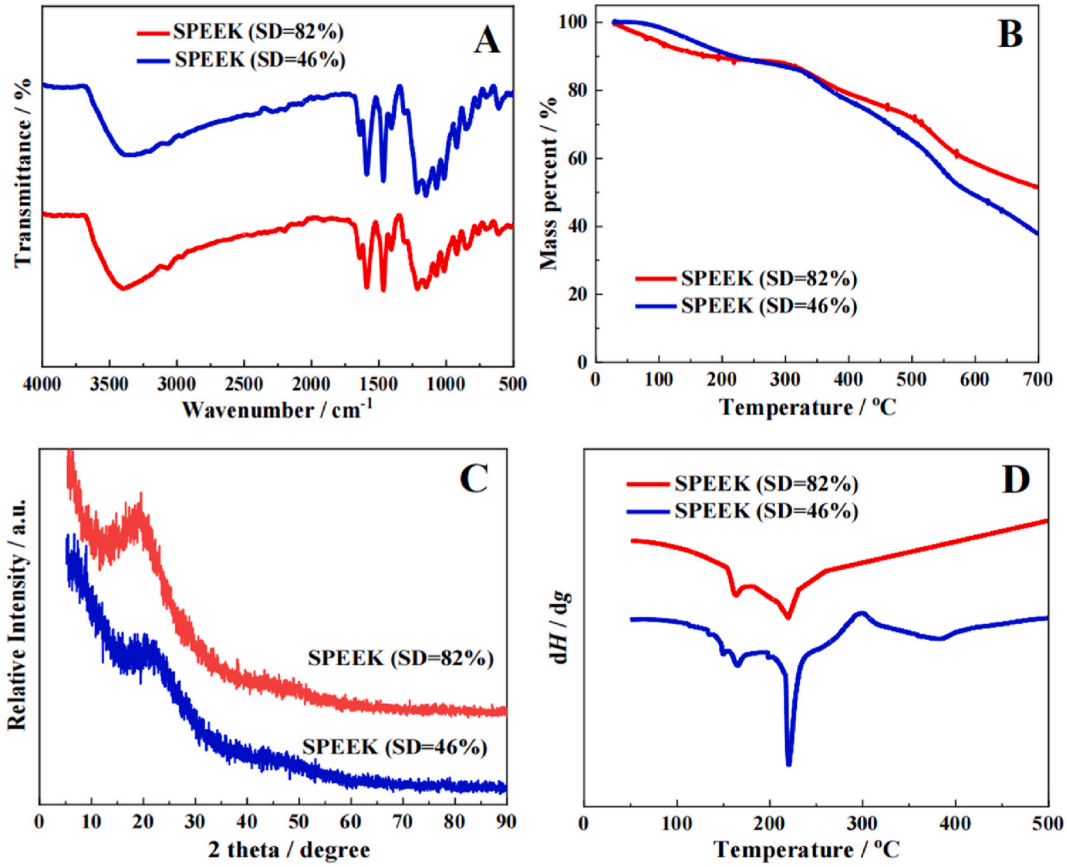

Fig. 2. Characterization of the surfaces of L-SPEEK and H-SPEEK. SPEEKSPEEKSPEEK

Also, the decomposition temperature of L-SPEEK was higher than that of H-SPEEK, which might be caused by the enhancement of main chain stability related to the lower DS. (Fig. 2B). Additionally, these SPEEK membranes exhibited the similar DSC curves with the exothermic peaks (Fig. 2D), which was coincided with the TGA results.

XRD analysis verified that the components in the two groups were the same, but the intensities were different due to the different degrees of sulfonation (Fig. 2C). Additionally, the SPEEK (SD = 46 %) membrane possessed the wide peak centering at 2θ =20.2°, which was higher than 2θ = 18.3° of the SPEEK (SD = 82 %) membrane. According to the Bragg's equation (Eq. (1)), the result meant that the SPEEK (SD = 46 %) membrane has more compact microstructure.

This characteristic was also verified by DSC (Fig. 2D).

The hydrophilicity/hydrophobicity on the surface of L-SPEEK and H-SPEEK was evaluated with the water contact angle (WCA). From Fig. 3, the WCA values of L-SPEEK and H-SPEEK were respectively 63.1° and 45.6°. By contract, the WCA of the surface of the PEEK plate was 131.2°. The results meant the hydrophilicity was increased with the introduction of SPEEK layer on the surface on the PEEK plate. Additionally, the H-SPEEK exhibited the stronger hydrophilicity due to more hydrophilic sulfonated goups in SPEEK. From our perspective, the hydrophilic surface facilitated to the growth of bone cell.

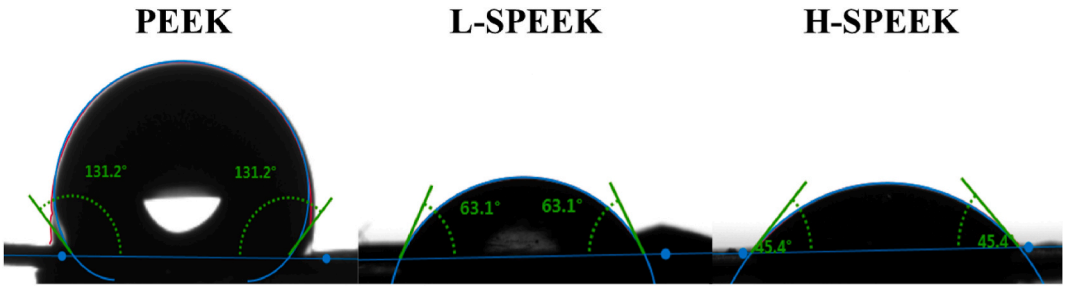

Fig. 3. The water contact angles of PEEK, L-SPEEK and H-SPEEK.

The CCK8 results showed the numbers of BMSCs that proliferated on the samples. The most active proliferation was observed on the surface of H-SPEEK, with the least active proliferation observed on the surface of L-SPEEK (Fig. 4).

Phalloidin staining indicated that the BMSCs could proliferate on the surfaces of L-SPEEK (Fig. 5B–E, H) and H-SPEEK (Fig. 5C–F, I). However, few cells were observed on the surface of PEEK (Fig. 5A–D, G). There were more BMSCs on H-SPEEK than on L-SPEEK. Additionally, though the detail morphology of the cells could not be displayed through the technology using in the current research, it could be observed that the BMSCs on the surfaces of L-SPEEK and H-SPEEK were more orderly, which might indicate osteogenic differentiation.

OCN (osteocalcin) and Runx2 (Runt-related transcription factor 2) were chosen to determine the osteogenic differentiation trend of the cells. These two transcription factors are related to osteogenic differentiation. Immunofluorescence staining for OCN showed similar results in the L-SPEEK (Fig. 6B–E, H) and H-SPEEK (Fig. 6C–F, I) groups. The BMSCs in these two groups exhibited osteogenic differentiation, as indicated by the positive staining results. However, the BMSCs in the PEEK group (Fig. 6A–D, G) did not exhibit this trend. Moreover, the results of immunofluorescence staining for Runx2 were similar. The H-SPEEK group (Fig. 7G–F, I) displayed more positive staining than the L-SPEEK group (Fig. 7B–E, H), and the degree of positivity was similar between the PEEK (Fig. 7A–D, G) and H-SPEEK groups.

Osteogenic differentiation was also verified by alizarin red staining (ARS) (Fig. 8A–C) and alkaline phosphatase staining (APS) (Fig. 8D–F). ARS revealed nearly no positive signals on the surface of PEEK (Fig. 8A) and few signals on the surface of L-SPEEK (Fig. 8B), while more positive signals were detected on the surface of H-SPEEK (Fig. 8C). For the APS test, few positive results were detected on the surfaces of PEEK (Fig. 8D) and L-SPEEK (Fig. 8E), while obvious signals were detected on the surface of H-SPEEK (Fig. 8F).

4. Discussion

The aims of DDD surgery include the effective decompression of nerves and reliable fusion of adjacent vertebrae. Solid fusion can ensure better prognosis of the patients. To improve the fusion rate, several factors should be considered. For example, patients should be in an ideal general state, including the nutritional status, blood glucose level, and bone mineral density. For surgeons, appropriate surgical methods should be chosen, and unnecessary trauma during surgery should be avoided. Many studies have described treatments that can improve the fusion rate, such as total removal of the cartilage without any injury to the end plates, sufficient filling of the intervertebral space using autogenous bone and artificial bone, stable fixation with internal fixators such as pedicle screw systems [20].

To fill the intervertebral space, a cage is introduced during spine fusion surgery. The cages used between the vertebrae should have some certain characteristics. For example, the material used to construct the cages should be hard sufficiently to bear the stress applied to the cages. Moreover, the material should have an elastic modulus similar to that of bone to avoid complications such as subsidence, nonfusion or pseudoarticulation. The metallic materials can supply enough stiffness, but their elastic modulus is not a match. Some organic materials have excellent elastic moduli but are not stiff enough. Therefore, many studies have attempted to use polymer materials to construct cages with ideal results [21]. Among these materials, PEEK is an ideal material. The first PEEK cage was developed by AcroMed in the 1990s [22]. These PEEK cages avoided the stress-shielding effect caused by the previous metal intervertebral cages and had an appropriate elastic modulus, which could improve the fusion rate. So, it is one of the most widely used materials for the production of cages has been for several years. Despite the advantages, PEEK also has several shortcomings. For example, cages made with PEEK could not be clearly observed by X-ray, which makes it difficult for surgeons to determine the position during surgery. Since then, small metal lines were added to the cages, which could be observed via X-ray. This modification allows the surgeons to observe the position of the cage in the operation. However, another shortcoming of PEEK still remains unsolved, as the lack of cytotropism.

To improve cytotropism, researchers have attempted many methods and got different results. Yamagishi A [23] tested

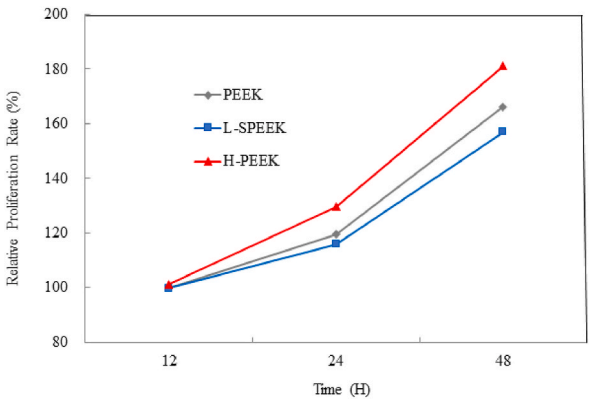

Fig. 4. CCK8 assay results indicating the proliferation of BMSCs in the three groups.

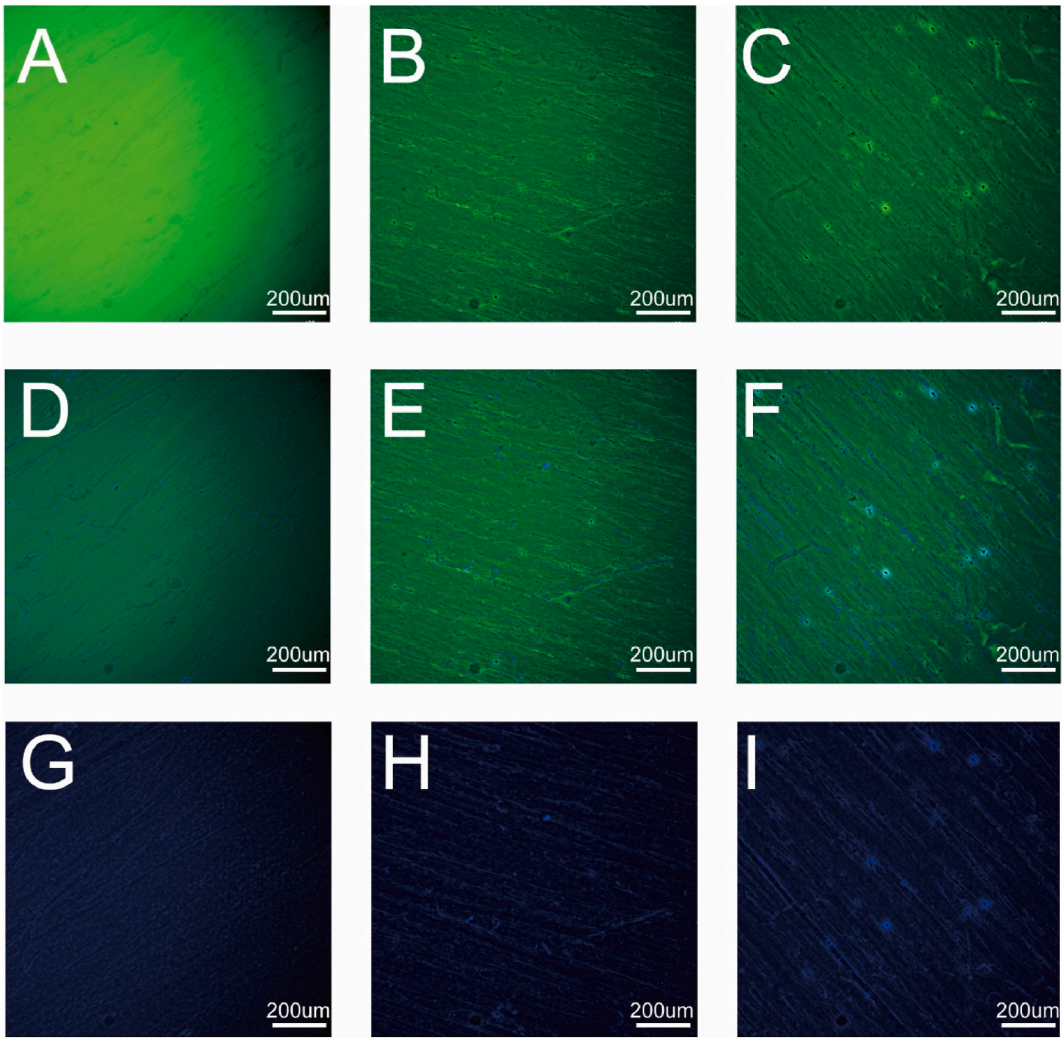

Fig. 5. Images of phalloidin staining. Few BMSCs were observed on the surface of PEEK (A, D, G). SPEEKSPEEKSPEEKSPEEK

titanium-coated PEEK cages and found that this kind of cage could improve the 1-year fusion rate compared with carbon fibre-reinforced PEEK cages. Johnson J W [24] found that manufactured titanium cages could increase the fusion rate and concluded that the structures of the surface could impact the fusion rate. While, a comparison by Vanek P [25] between titanium-coated PEEK cages and uncoated PEEK cages showed nearly same fusion rate. Similarly, Sultana T [26] also reported that three-dimensional-printed titanium cages had a similar fusion rate to PEEK cages. Then, Patel N A [27] conducted a systematic review and cautiously concluded that 3D-pTi might improve the fusion rate, but he suggested that additional human investigations should be performed. Due to these results, the methods of adding metals to PEEK are unreliable. Unpredictable effects might occur on the contact surface between PEEK and the metal material. Then, researchers tried other methods to modify the surface of PEEK. Among these methods, sulfonation of the PEEK has been verified to have desired result.

In 2023, Chen Z [28] found that sulfonation could change the topological morphology of PEEK and effectively determine the osseointegration of implants. These result demonstrated that using appropriate high polymer materials and changing the micro-structure of the contacting surface of the cage might have the potential of improving the fusion rate of spinal surgery [29]. Traditional methods of modifying the surface of PEEK often associated with relative high temperatures which might cause damage to PEEK substrates. While the method of sulfonation could avoid the high temperature by using concentrated sulfuric acid to attach sulfonate groups to the aromatic rings in the PEEK structure [30]. The added acidified surface could create pores which might make the cells more easily to adhere on the surface and bring some affect to the differentiation. Brum R [18] assessed the bioactivity of PEEK coating with SPEEK films. According from the results, they concluded that these methods could be used for implant dentistry applications. Wang. W [13] tried fast ambient-temperature sulfonation and verified it to be an effective method without higher temperature. So, the authors recommended the method to modify the traditional bone implant. Similarly, Yun [31] tested different time of sulfonation, they found that the group of 2 min showing superior biocompatibility and significantly stronger osteogenic activity when co-cultured with

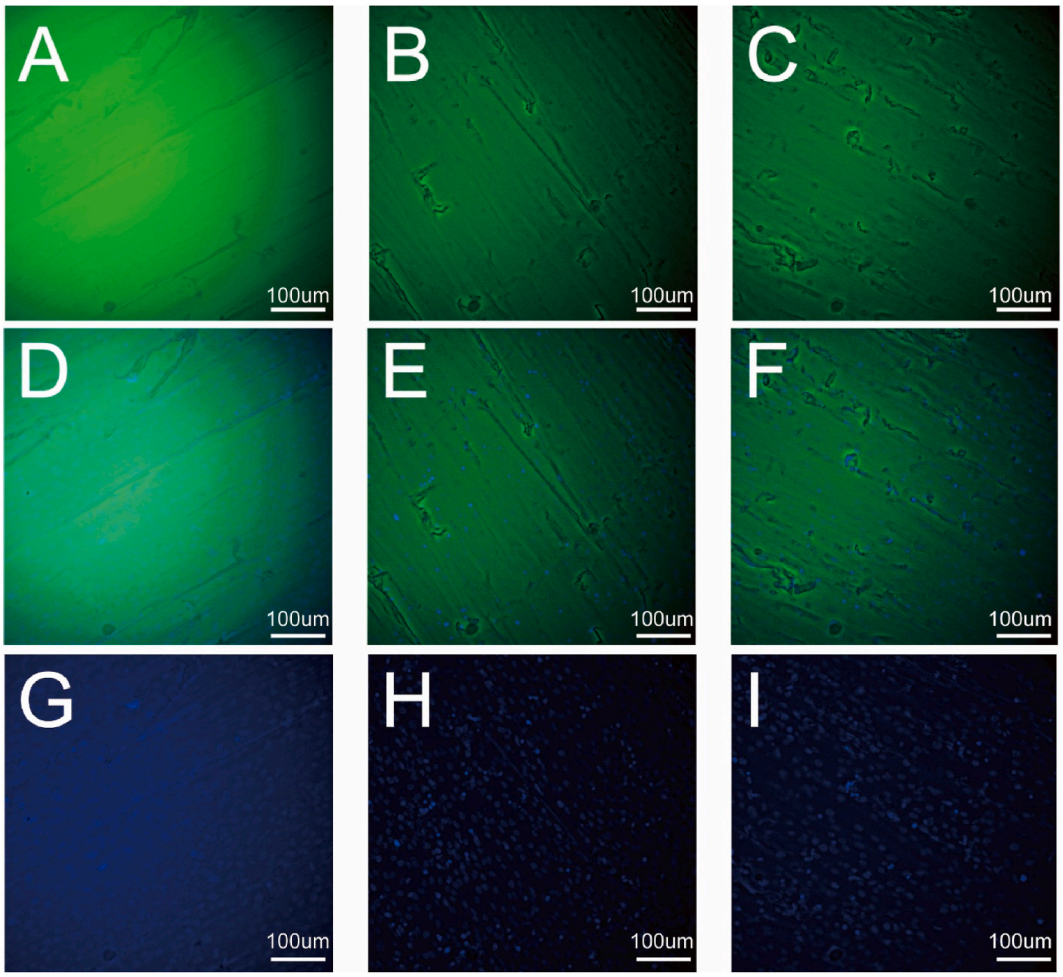

Fig. 6. Immunofluorescence images of OCN (osteocalcin), which revealed similar results in the L-SPEEK (B, E, H) and H-SPEEK (C, F, I) groups. The BMSCs in the PEEK group (A, D, G) exhibited a relatively weak trend towards osteogenic differentiation.

stem cells from human exfoliated deciduous teeth (SHED). Using the cells of MC3T3-E1, which was a kind of pre-osteoblasts, Ying Zhao [32] produced 3D porous and nanostructured network with bio-functional groups by the method of sulfonation and subsequent water immersion. This structure change facilitates the adhesion and proliferation of the cells, also the SPEEK film expressed the character of improving the cells' osteogenic differentiation. Rui Ma [33] used 5-min sulfonation time and removing the residual sulfuric acid by post-treatment method to improve the cytocompatibility of SPEEK. Also, the cell they used was MC3T3-E1 cells. Lei Fan [34] developed a BMSC-derived Exos-functionalized implant to modified SPEEK and achieved the results of promoting osteogenesis. Reviewing the researches above, we thought that the sulfonation of PEEK should have the ability of improving the adhere and proliferation of the cells on the modified surfaces. Also, the SPEEK might have the ability of stimulate the osteogenic differentiation. Inspired by these researches above, we had the idea of construction the modified surfaces of the cage made by PEEK, then using the cells of BMSCs to identified the promoting effect of the topological structure of SPEEK to the proliferation and differentiation.

So, in the present study, we tried the modification method to the surface of PEEK via sulfonation. Then, several methods, such as SEM, FTIR, TGA and XRD, were used to analyse the characteristics of the changed surfaces and verify the differences between L-SPEEK and H-SPEEK. The results showed that the surface was rougher in the group of H-SPEEK, in which more complicated topological morphology was observed. Then, BMSCs were inoculated and proliferated on both modified cage surfaces. Compared with those on PEEK, the cells on SPEEK were arranged in more orderly manner, which might be associated with the fibres of the SPEEK lining on the surface. Additionally, more cells were observed in the H-SPEEK group, which had a greater DS. Moreover, the topological morphology of SPEEK might mediate the osteogenic differentiation of BMSCs, which was verified by OCN and Runx 2 staining, ARS and APS.

5. Conclusions

Sulfonation can change the surface character of PEEK, which could complicate the topological morphology of the surface. This topological morphology could make it easier for the cells to adhere and proliferating. Also, the surfaces with higher DS might mediate

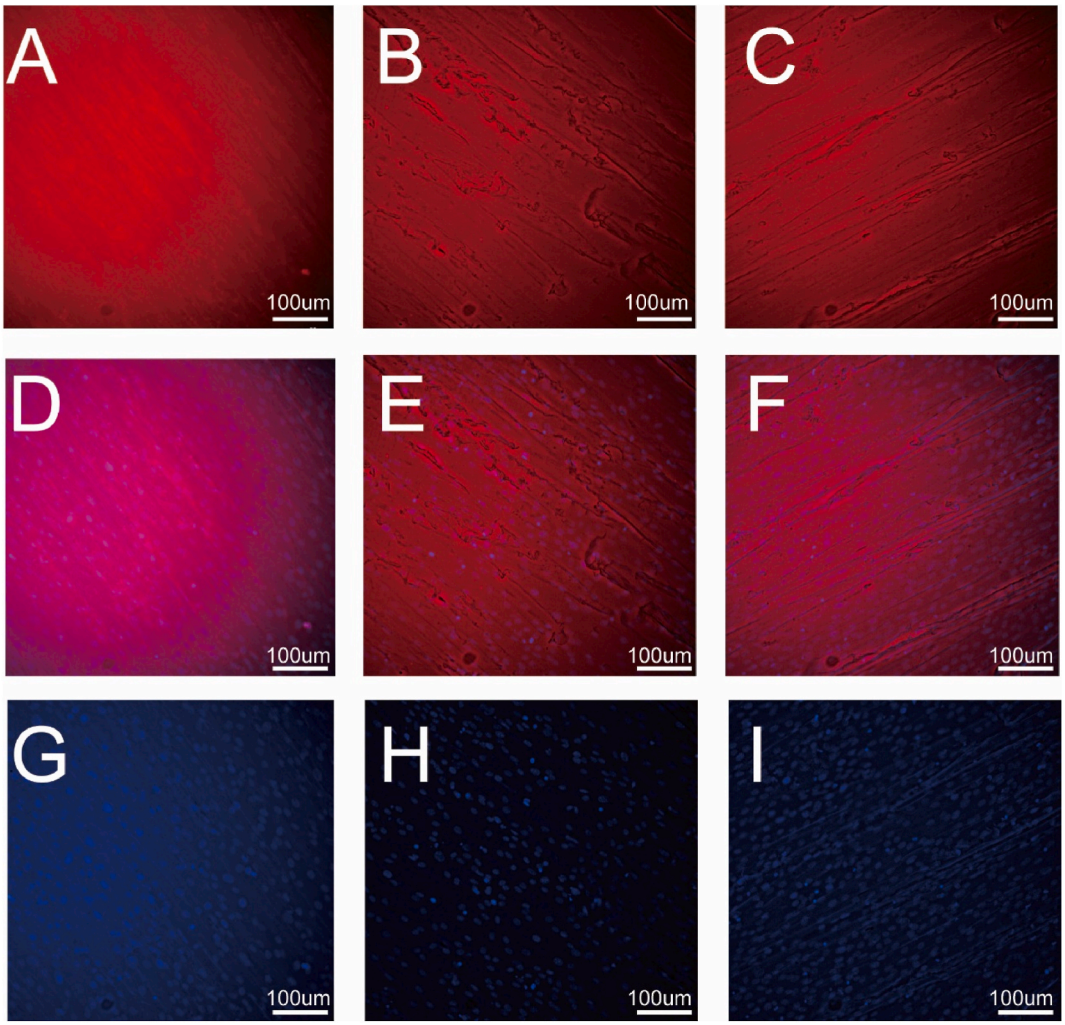

Fig. 7. Immunofluorescence staining images of Runx2 (Runt-related transcription factor 2). The BMSCs in the H-SPEEK group (C, F, I) exhibited the most positive staining, indicating the most obvious osteogenic differentiation. Moreover, the BMSCs in the L-SPEEK group (B, E, E, H) exhibited the least osteogenic differentiation. The BMSCs in the PEEK group (A, D, G) tended to undergo osteogenic differentiation to an extent between those in the other two groups.

the osteogenic differentiation of BMSCs, which should be verified more deliberately.

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Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the committee of the General Hospital of Northern Theater Command (Y2023065).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

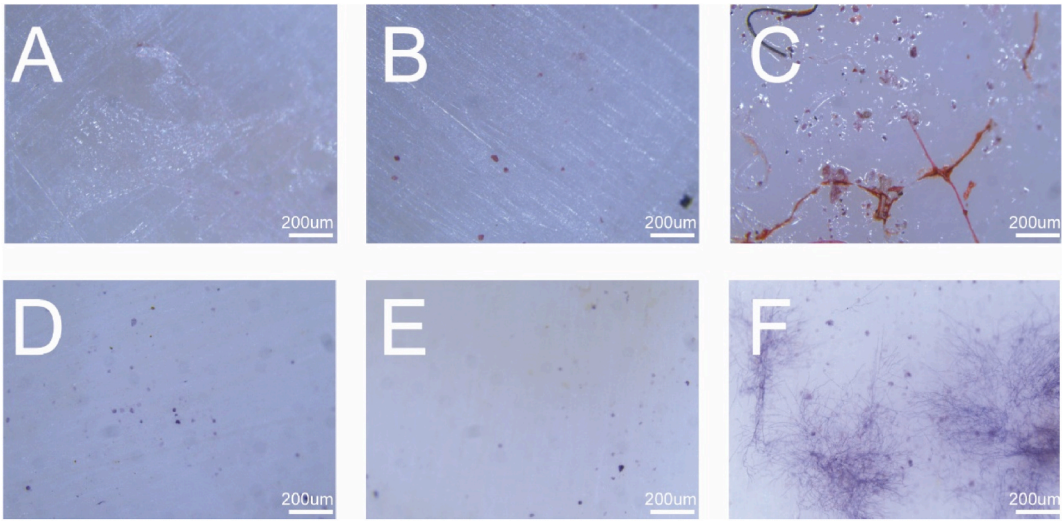

Fig. 8. Alizarin red S staining (ARS) (A–C) and alkaline phosphatase staining (APS) (D–F) results. ARS revealed nearly no positive signals in the PEEK group (A) and only few in the L-SPEEK group (B), while more positive signals were detected in the H-SPEEK group (C). For the APS test, few positive signals were detected on the surfaces of PEEK (D) and L-SPEEK (E), while obvious signals were detected on the surface of H-SPEEK (F).

CRediT authorship contribution statement

Shuang Wang: Writing – original draft, Investigation, Conceptualization. **Jun-xiong Ma:** Writing – original draft, Investigation. **Liang Zheng:** Writing – original draft, Investigation. **Hong Wang:** Formal analysis, Data curation. **Hai-long Yu:** Writing – review & editing. **Yu Chen:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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