A biomimetic gradient porous cage with a micro-structure for enhancing mechanical properties and accelerating osseointegration in spinal fusion

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\textbf{ABSTRACT}

\textbf{Objectives:} Spinal fusion is a widely employed treatment of patients with degenerative disc disease, in which a cage is used to replace the disc for spinal fusion. But it often fails for insufficient mechanical strength and poor osseointegration. Here, we designed a polyether-ether-ketone (PEEK)/tantalum (Ta) composite cage with a biomimetic gradient porous micro-structure, simultaneously enhancing mechanical properties and accelerating osseointegration in spinal fusion.

\textbf{Materials and methods:} In the study, based on the mechanical performances of PEEK and osteogenic potential of Ta, and the three-dimensional (3D) structures of cuttlebone and vertebra, the cages were respectively 3D printed by pure PEEK, PEEK with 5 wt% Ta (PEEK/Ta-5), PEEK with 10 wt% Ta (PEEK/Ta-10) and PEEK with 15 wt% Ta (PEEK/Ta-15), then verified in vitro and in sheep cervical fusion model systematically.

\textbf{Results:} Vertebral Gyroid structure PEEK/Ta-15 cage exhibited superior mechanical properties than Cuttlebone-like structure PEEK/Ta-15 cage, closer to the cervical vertebra. Furthermore, PEEK/Ta-15 cage with higher Ta microparticles in PEEK provided a biomimetic gradient porous micro-structure with higher surface energy, guiding cell biological behavior, promoting new bone penetration, and accelerating osseointegration.

\textbf{Conclusion:} In conclusion, the study designed a biomimetic gradient porous cage with a micro-structure for enhancing mechanical properties, accelerating osseointegration and forming an anatomical lock in the fusion segment through composites, mechanical efficiency, surface extension, and pores.

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1. Introduction

In the field of biomaterials, poly-ether-ether-ketone (PEEK) as the leading polymeric material, has been respectively applied in spinal fusion, dental substitutes, neurosurgical and cranio-maxillofacial repair, mainly for its bio-stability, radioluency and admirable mechanical properties [1,2]. In addition, additive manufacturing (AM) has been a practical manufacturing method of PEEK for the on-demand custom-design and structural complexity [3,4]. However, PEEK is bio-inert and easily covered by the fibrous tissue in vivo, which could cut the contact between PEEK and host bone resulting in the loosening of implants [1]. Therefore, its poor osteogenic activity has raised a high concern in spinal fusion. More recent investigations focused on the methods to improve the bioactive performances but no mechanical strength loss. However, most surface modifications, including physical and chemical ways, were not stable with sterilization procedures and mechanical friction [5,6]. Therefore, the incorporation of bioactive fillers with PEEK became a practiced way to enhance its bioactivity such as tricalcium phosphate and hydroxyapatite into the PEEK matrix [7]; however, most studies mainly performed the procedure by lamination/compression molding, then applied on bone defect models, which are to create a mechanical arrangement for osseointegration under static conditions, lacking further exploration of PEEK based composites in the three-dimensional (3D) printing, biomimetic gradient pores and high-function intervertebral osseointegration [8–10].

With the excellent performances of bioactivity and low bacterial adhesion, tantalum (Ta) has gradually become one of the preferred implantable materials in orthopedics as compared with those of titanium (Ti) and cobalt-chromium-molybdenum [11]. Early studies demonstrated that Ta was bioinert in vivo for its chemical stable surface of passivating oxide [12]. However, recent research has found that Ta could improve cell responses, osteoblasts differentiation and mineralization for its surface roughness, hydrophilicity, and surface energy [13]. Moreover, Ta microparticles allow bone on/in-growth and enhance the early and long-term stability after implantation, for its bioactive tantalum pentoxide layer, which is superior to general bioactive fillers [13]. However, the applications of Ta in orthopedics are still restricted for its difficult manufacturing, high elastic modulus and high density.

Spinal fusion is a widely employed treatment of patients with degenerative disc disease, spinal instability and trauma. In spinal surgery, once the disc is completely removed, a cage is placed in the interspace to replace the disc for the spinal fusion. Though autogenous iliac bone graft transplantation was explored to achieve spinal fusion, it often failed for insufficient mechanical strength as a result of post-operative collapse or absorption. Therefore, spinal fusion cages with good mechanical properties were introduced by Bagby in 1988 [14]. However, the high elastic modulus of Ti and bioinert feature of PEEK both raised the problem of micromotion between the vertebrae and cage, which could not form an anatomical lock in the fusion segment [2,15]. Only 3D printed biomimetic gradient pores could provide a micro-structure for the penetration of new bone (NB).

From a comprehensive perspective of research, the purpose of this study was to take advantage of the admirable mechanical properties of PEEK, excellent osteogenesis of Ta, and fully practicing facility of 3D printing and overcome the shortcomings to create 3D printed PEEK/Ta composite cages with biomimetic gradient pores to achieve high-function intervertebral osseointegration in sheep cervical fusion model. We named the 3D printed biomimetic gradient porous structure could not only exhibit excellent custom-design and mechanical properties closer to the cervical vertebra, but also provide a micro-structure that further facilitates the surface performances, cellular responses, and high-function intervertebral osseointegration through mechanical efficiency, surface extension, and biomimetic gradient pores; moreover, PEEK/Ta composites with rougher surfaces for higher Ta microparticles in PEEK could improve the surface hydrophilicity, surface energy, protein adsorption, cell responses, and high-function intervertebral osseointegration in the sheep cervical fusion model.

2. Materials and methods

2.1. Preparation of the 3D printed composite filaments

The 3D printed PEEK/Ta filament with Ta microparticles content of 5 wt% (PEEK/Ta-5), 10 wt% (PEEK/Ta-10) and 15 wt% (PEEK/Ta-15) were prepared by filament extrusion. PEEK powders (JunHua High Performance Specialty Engineering Plastics Products, biomedical grade, China) were first fully comixed with Ta powders for 15min (SAILONG METAL, biomedical grade, China) using a ball-milling machine (Grinder RS-FS1406, China). The rotating speed was set at 3000 rpm and the directions were inverted every 3min. The weight ratios of tantalum microparticles in PEEK matrix were increased from 5% to 15%, until the extrusion of filament became difficult for the aggregation of too many Ta microparticles. Therefore, 15 wt% Ta content was considered as the highest ratio in the PEEK. The mixture was molded into a size of 31 × 26 × 2.5 cm³ under a molding pressure of 5 MPa, then crushed into particles with size of 1–2 mm (Crusher SPC160160, Soyu Machinery, China). Afterwards, the particles were comixed secondly at a melting temperature of 380–400 °C for 12–18 h; then PEEK/Ta composite filaments in 1.75 ± 0.05 mm diameter were produced by extruder (FLD-35, Friend Machinery, China), at a temperature of 340–400 °C and a time of 10–15min. Pure PEEK powders as the control group, also underwent the same process.

In recent years, the porous structures have been subjected to increasing interest as great performances in facilitating the exchange of proteins and cells due to surface roughness and morphology but still far behind many solid materials in the mechanical strength. The vertebral Gyroid structure is based on the 3D reconstruction of micro-computed tomography (Micro-CT) images of cervical vertebra, with biomimetic porous structure of triply periodic minimal surface (TPMS) as well as mechanical properties of cervical vertebra [16] (Fig. 4a and b). 3D printed PEEK in Gyroid structure (G-PEEK), PEEK/Ta-5 in Gyroid structure (G-PEEK/Ta-5), PEEK/Ta-10 in Gyroid structure (G-PEEK/Ta-10), and PEEK/Ta-15 in Gyroid structure (G-PEEK/Ta-15). The Cuttlebone-like structure is inspired by Cuttlebone with biomimetic bone porous structure, which lives in the deep-sea and suffering large compressive force of water [17,18] (Fig. 4c and d). 3D printed PEEK/Ta-15 in Cuttlebone-like structure (C-PEEK/Ta-15). Both of the two structures are characterized by biomimetic gradient porous structure and good mechanical properties.

PEEK 3D printer (Oscan-HP 220, China) was set under the conditions of 420 °C printing temperature, 120 °C platform temperature, 90 °C chamber temperature, 100% filling density, 40 mm/s printing speed, 90 mm/s idling speed, and single skirt platform attachment method, for fused deposition modelling (FDM). The process of printing included depositing layer by layer, print head moving in the Z-direction with a distance of layer thickness (0.2 mm), and the layers fusing to form a 3D biomimetic gradient porous structure (length (mm) = 12, width (mm) = 10, height (mm) = 5, porosity (%) = 68%, outer pore sizes (μm) = 620 μm) [15,19,20].

In the preparation of the 3D printed composite filaments, PEEK powders and Ta powders were uniformly mixed for at least 4 times (including initially the mixing of the two powders before molding, secondly the mixing after being crushed into particles, thirdly the mixing before 3D printed filament extrusion, finally the uniform layout of 3D printing). These repeated mixing methods made the distribution of the two powders more uniform, which not only avoided affecting the strength of PEEK itself, but also improved the distribution of Ta.

2.2. Characterisation of the 3D printed composite filaments

The cold-crystallization temperature, melting temperature and thermal stability parameters of the 3D printed composite filaments
plates with MC3T3-E1 cells at a density of $10 \times 10^5$ cells per well for the cell adhesion ratio, proliferation and osteogenic related genes expressions. The cages completely sank into the medium, which should be renewed at least every 2 days.

### 2.6.1. Cell morphology and adhesion

After the MC3T3-E1 cells were incubated 1 and 3 days, the cages/cells were cleaned in PBS three times, then fixed with 2.5% glutaraldehyde solution for 1 h. After being cleaned again with PBS 3 times, the F-Actin of cells on the specimens were stained with fluorescein isothiocyanate-phalloidin (FITC-Phalloidin, Macklin, China) for 20 min, then the solution was aspirated. Afterwards, the nuclei of cells were stained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Servicebio, China) for 5 min. The MC3T3-E1 cell morphology on cages was evaluated using confocal laser scanning microscopy (CLSM, SUNNY, China). The adherent cell ratio on the cages at 12, 24, and 48 h after inoculation were assessed with optical density (OD) values by the microplate reader (Thermo, USA) at 450 nm. The adherent cell ratio was calculated using the formula: $((OD_{test} - OD_{blank})/ (OD_{control} - OD_{blank})) \times 100\%$ [8].

### 2.6.2. The proliferation and alkaline phosphatase (ALP) activity

The proliferation of MC3T3-E1 cells was assessed by Cell Counting Kit-8 assay (CCK8, Bimake, China). At 1, 3, and 7 days of incubation, the cages/cells were cleaned with PBS 3 times to wash off the unabsorbed cells, then placed in a 12-well plate. Then the solution of 50 μL CCK-8 and 500 μL α-MEM were aspirated into each well. After 4 h of incubation, 100 μL supernatant in each well was transferred into a new 96-well plate to measure the OD values with a microplate reader (Thermo, USA) at 450 nm.

After being incubated 7, 10, and 14 days, the cages/cells were cleaned with PBS three times, the supernatants were aspirated, then the cells were incubated with 50 μL ALP assay buffer solution, which was obtained by dissolving 2 tablets pNPP substrate in 5.4 mL assay buffer to make a 5 mM solution (# ab83369, Abcam). After being incubated 60 min at 25 °C, 20 μL stop solution was added. Afterwards, ALP activity was evaluated using an enzyme-linked immunosorbent assay reader (Thermo, USA) at 405 nm.

### 2.6.3. The expressions of osteogenesis related genes

After being incubated 3, 7 and 14 days on the cages (density of $10^5$ cells per well), the RNA was isolated from cells with a RNA-Quick Purification kit (#RN001, ES Science, China), then was reverse-transcribed into cDNA using StarScript II First-strand cDNA Synthesis Mix with gDNA Remover (GenStar, China) at 42 °C for 15 min. Quantitative real-time PCR assays (RT-PCR) were operated under an ABI7500 Fast Real-time PCR System (Thermo Fisher Scientific, USA). Gene expression levels were calculated by a standard method. The primer sequences of osteogenesis related genes included osteopontin (OPN), osteocalcin (OCN), runt-related transcription factor 2 (Runx2), and ALP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene for normalization (Table 1) [8].

### 2.7. Inner osseointegration in vivo

#### 2.7.1. Surgical procedure

The in vivo sheep experiments were performed under the approval of the Laboratory Animal Ethics Committee (Kantai Medical Laboratory Services, China). Commonly, simple surgical exposure in smaller animals is sufficient to stimulate bone fusion; however, that is difficult in the primate (and human) spine despite complete exposure of spinal fusion bed, due to the evolutionary complexity of species [22]. Moreover, in most studies, bone defect models are to create a mechanical arrangement for osseointegration under static conditions, that is also relatively easy. However, the cervical fusion model represents an anatomical recreation for demand of high-function cervical range of

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motion. Furthermore, the sheep cervical motion segment C3/4 was proven to be a proper model for analogy of human cervical motion segment [23]. Twenty-four female Small Tailed Han sheep (weight: 4 ± 5.0 kg, age: 18 months) were performed the anterior cervical decompression and fusion. The sheep were randomly assigned to four groups according to the cages of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10, and G-PEEK/Ta-15. After intramuscular injection of Xylazine Hydrochloride (2 mg/10 kg, Jinlin Huamu Animal Health, China), anterior cervical site of sheep was sterilized with iodophor disinfectant. A regular anterior Smith-Robinson approach was made to expose the disc level (C3/4), then a complete discectomy was performed. Therefore, the 3D printed biomimetic gradient porous composite cages (G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10, and G-PEEK/Ta-15) were placed in the interspace to replace the disc. Anterior cervical plating was then performed.

The sheep were euthanatized at 12 weeks after operation, and the cervical segments containing cages were fixed with 4% phosphate-buffered formalin solution. Twelve sheep were evaluated for Micro-CT at week 4 and 12 after implantation and histological analyses at week 12 after implantation, and the other 12 sheep were removed anterior cervical plates for biomechanical testing.

2.7.2. Histological images and bone-implant contact (BIC)

The cervical segments were dehydrated by ascending concentrations of ethanol (70–100 v/v%), then sank in polymethyl methacrylate. After that, the cervical segments were sliced into 150 μm thickness sections along the long axis of cervical segment by a microtome (Leica SP1600, Germany), then were polished to 50 μm thickness. Therefore, the thin slices were stained by toluidine blue basic magenta staining (TB) to reveal the NB (bluish violet). The histological images were taken by a polarized optical microscope (POM, Olympus, Japan). and BIC was evaluated with machine-assisted software.

2.7.3. 3D-reconstructed images from Micro-CT

The NB around the cages were visualized by Quantum GX Micro-CT Imaging System (PerkinElmer, USA). The scanning parameters were 70 kV X-ray source voltage, 120 μA beam current and 14min exposure time at a resolution of 10 μm. The 3D images were reconstructed with machine-assisted software at week 4 and 12 after implantation. New bone volume/total volume (BV/TV) in the cages as well as the bone mineral density (BMD) of cages were evaluated with machine-assisted software at week 4 and 12 after implantation.

2.7.4. Biomechanical testing

The biomechanical performances were evaluated by the sheep C3/4 intervertebral range of motion (ROM). The cervical muscles were removed, the ligaments were kept intact, then the visual capture speckle pattern was sprayed on the entire cervical vertebra. The base of the occipital condyle and the cervical 7 vertebral body were embedded with polymethyl methacrylate. Then the two ends of the specimen were fixed with clamps, and placed on the spine 3D motion testing machine (MTS858 Mini Bionix, USA). Under a non-destructive manner, 2.0 Nm was applied to the cervical segment under six motion modes of flexion and extension, left or right bending, and left/right axial rotation. Three loading/unloading cycles were repeated for each test, and kinematic measurements were taken at the third cycle to reduce the effects of

viscoelastic action of the cervical segments [22,23]. The cervical intervertebral motions (C3/4) were captured by digital image correlation (DIC) systems (High resolution binocular stereo-DIC, China).

2.8. Statistical analysis

SPSS 26.0 (SPSS Inc) was performed by two independent orthopedic surgeons. Continuous variables were described as means and standard deviation. Data results were collected from at least three independent experiments and assessed with one-way analysis of variance (ANOVA). Significance was indicated by p values at * represents p < 0.05, ** represents p < 0.01, *** represents p < 0.001 and **** represents p < 0.0001.

3. Results

3.1. DSC and TGA analysis of the 3D printed composite filaments

Fig. 1 shows the thermal properties of PEEK, PEEK/Ta-5, PEEK/Ta-10 and PEEK/Ta-15 in DSC and TGA. In DSC, the peaks of 296 ◦C were attributed to the cold-crystallization temperature (Tc) of PEEK, and the valleys at 340 ◦C were related to the melting temperature (Tm) of PEEK. The transition points at 549 ◦C in TGA were attributed to the thermal decomposition temperature of PEEK. The characteristic peaks of PEEK/Ta-5, PEEK/Ta-10 and PEEK/Ta-15 were the same as PEEK; however, no related characteristic peaks appeared in Ta.

3.2. Micro-FTIR, XRD and Micro-CT of the 3D printed composite filaments

Fig. 2 a is the Micro-CT images of Ta distribution in PEEK, PEEK/Ta-5, PEEK/Ta-10 and PEEK/Ta-15, which shows the characteristic peaks of 69.7 cm−1, 28.7 ◦C and 38.5 ◦C in TGA were attributed to the thermal decomposition temperature of PEEK. The characteristic peaks of PEEK/Ta-5, PEEK/Ta-10 and PEEK/Ta-15 were the same as PEEK; however, no related characteristic peaks appeared in Ta.

The XRD patterns of PEEK, PEEK/Ta-5, PEEK/Ta-10 and Ta are shown in Fig. 2 b. The characteristic peaks at 69.7 ◦C, 55.6 ◦C, and 38.5 ◦C corresponded to Ta (high crystallinity). The characteristic peaks at 22.5 ◦C, 20.7 ◦C and 1160 cm−1 corresponded to the group of diphenyl ketone. However, no characteristic peaks were revealed in Ta.

The SRD patterns of PEEK, PEEK/Ta-5, PEEK/Ta-10, PEEK/Ta-15 and Ta are shown in Fig. 2 b. The characteristic peaks of 69.7 ◦C, 55.6 ◦C, and 38.5 ◦C corresponded to Ta (high crystallinity). The characteristic peaks of 22.5 ◦C, 20.7 ◦C and 1160 cm−1 were attributed to PEEK (low crystallinity). All of these peaks appeared simultaneously in PEEK/Ta composites.

Fig. 2 c-f shows the Micro-CT images of Ta distribution in PEEK, PEEK/Ta-5, PEEK/Ta-10, and PEEK/Ta-15 filaments. The images reveal that Ta microparticles are approximately 1 μm in size exhibiting irregular shape, with diameters ranging from 0.4 to 5 μm. Ta microparticles were uniformly distributed in the PEEK/Ta composites while not found in PEEK.

3.3. SEM and EDS micrographs of the 3D printed composite filaments

Fig. 3 a–d shows the SEM micrographs of the cross section of the 3D printed composite filaments (PEEK, PEEK/Ta-5, PEEK/Ta-10 and PEEK/Ta-15). PEEK showed a smooth cross section while PEEK/Ta composites

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|------|----------------|----------------|
| OPN  | GAGGTGATGGACGACGAGATGAC | GTGTCGGACGATGAAGGACTC |
| OCN  | AGACTGAGACGACGACGAC | TGGATAGCGGCGGATCTATTTC |
| Runx2| AACGAGGCGGCACGCCGAC | GCGAGGACGACGAGATTG |
| ALP  | CAGATTAGACGGGAGAAGA | CAATTACGGGAGAGGAGTC |
| GAPDH | ACAGCAAGGTTGGTGGACAG | TTTTGAGGGTGCAGCGAACTT |
revealed rougher cross section. Additionally, more Ta microparticles were dispersed in PEEK/Ta-15 composite filament. Fig. 3 e–h reveals the EDS mapping of Ta (cyan dots) element dispersed in the specimens. The intensity of Ta element increased from PEEK to PEEK/Ta-15.

3.4. Architecture and mechanical properties of the 3D printed cages

Fig. 4a-d shows the architecture of Gyroid structure and Cuttlebone-like structure. Fig. 4e shows the mechanical properties of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10, G-PEEK/Ta-15, and C-PEEK/Ta-15 \[22,24\]. The compressive strengths of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10, G-PEEK/Ta-15, C-PEEK/Ta-15, and cervical vertebra were 50.92 ± 1.38 MPa, 51.65 ± 2.12 MPa, 52.17 ± 2.45 MPa, 52.38 ± 2.29 MPa, 44.04 ± 1.14 MPa, and 7.14 ± 0.17 MPa respectively (Fig. 4f). Elastic modulus of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10, G-PEEK/Ta-15, C-PEEK/Ta-15, and cervical vertebra were 104.04 ± 13.36 MPa, 104.98 ± 14.92 MPa, 109.11 ± 13.79 MPa, 113.48 ± 14.27 MPa, 64.77 ± 3.93 MPa and 116.41 ± 11.18 MPa, respectively (Fig. 4g). Under the same printed
conditions and porous parameters, we observed Cuttlebone-like structure usually experienced uniform interlayer deformation under compressive stress, which was not accepted in spinal fusion for sacrificing the strength and height of the structure [17]. Finally, vertebral Gyroid structure with biomimetic gradient pores exhibited higher mechanical properties, and was determined as 3D structure of PEEK/Ta composite cages to further investigate the bioactivity of PEEK matrices incorporated with different ratios of Ta.

3.5. Biomimetic gradient porous structure and surface roughness

Fig. 5 a-d reveals the images of biomimetic gradient porous G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15. Fig. 5e–l reveals the SEM micrographs of biomimetic gradient porous G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15. G-PEEK showed a smooth surface while G-PEEK/Ta composites revealed rougher surfaces. Additionally, more Ta microparticles were dispersed on G-PEEK/Ta-15 surface. Fig. 5m shows the gradient porous properties of Gyroid structure. The range of pore sizes are from 0.006 to 620 μm and the outer pore size is 620 μm. Fig. 5n–q shows the surface roughness of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15. The surface roughness S of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 were 0.50 ± 0.05 μm, 0.90 ± 0.04 μm, 1.27 ± 0.07 μm and 1.76 ± 0.05 μm, respectively (Fig. 5r).

3.6. Surface hydrophilicity, surface energy and protein adsorption

Fig. 6a, b shows the water and diiodomethane contact angles of the specimens, respectively. The water contact angles of the specimens were 76.00 ± 1.63°, 66.81 ± 1.56°, 54.34 ± 0.48° and 40.79 ± 0.12°, respectively, representing the hydrophilicity of the specimens. The diiodomethane contact angles of the specimens were 60.16 ± 0.59°, 56.12 ± 0.77°, 37.20 ± 0.40° and 30.74 ± 0.14°, respectively. The surface energy was evaluated with water and diiodomethane contact angles (Fig. 6c).

Fig. 6d shows the adherent protein on the cages. The adherent BSA on G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 were 36.22 ± 1.58 μg/cm², 44.13 ± 1.63 μg/cm², 69.34 ± 1.01 μg/cm² and 100.24 ± 1.72 μg/cm². The adherent Fn on G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 were 18.24 ± 0.97 μg/cm², 24.51 ± 0.98 μg/cm², 31.30 ± 1.03 μg/cm² and 49.84 ± 1.90 μg/cm², respectively.

3.7. Cell morphology, adhesion and proliferation, ALP activity

Fig. 7a–h shows the cytoskeletal morphology of cells on the cages. The cytoskeleton (red) and cell nuclei (blue) were respectively stained under FITC-Phalloidin and DAPI. After culturing 1 and 3 days, more filopodia and lamellipodia of the MC3T3-E1 cells were found on G-PEEK/Ta composite cages than G-PEEK, which increased as higher Ta microparticles in PEEK.

The amount of the adherent cells and proliferation on the cages were evaluated with CCK-8 at different points of time after culturing. It was found that the adherent cells on the G-PEEK/Ta composite cages enhanced with time; however, no obviously enhanced for G-PEEK (Fig. 7j). In addition, at different points of time after culturing, the cell adhesion for G-PEEK/Ta composite cages enhanced as higher Ta microparticles in PEEK. The OD values of cells on the cages enhanced with time; however, no obviously enhanced for G-PEEK (Fig. 7j). Moreover, after culturing 1, 3, and 7 days, the OD values for G-PEEK/Ta composite cages enhanced as higher Ta microparticles in PEEK.

Fig. 7k reveals the activity of ALP in the cells on the cages. The activity of ALP for G-PEEK/Ta composite cages enhanced with time; however, no obviously enhanced for G-PEEK. Moreover, after culturing 7, 10, and 14 days, the activity of ALP for G-PEEK/Ta composite cages increased as higher Ta microparticles in PEEK.

3.8. The expressions of osteogenesis related genes

As shown in Fig. 8a–d, the expressions of osteogenesis genes on G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 were evaluated by RT-PCR. The gene expressions for G-PEEK/Ta composite cages enhanced with time; however, no obviously enhanced for G-PEEK. At different points of time, the gene expressions for G-PEEK/Ta composite cages increased as higher Ta microparticles in PEEK.

3.9. Inner osseointegration in vivo

3.9.1. Histological images and BIC

Fig. 9a–h is the histological images of NB around cages at week 12 after implanted in vivo. The NB tissues (bluish violet) accumulation for G-PEEK/Ta-15 increased as higher Ta microparticles in PEEK. Additionally, the growth trend of NB from top to bottom was found more obvious in cages.

Fig. 9i shows the quantitative evaluation of BIC at week 12 after cages being implanted in vivo. The NB were closely in touch with G-PEEK/Ta-15, indicating good osseointegration around the cage. At week 12, the BIC for G-PEEK/Ta composite cages increased as higher Ta microparticles in PEEK.

3.9.2. 3D-reconstructed images from Micro-CT

Fig. 10a–h reveals the 3D-reconstructed images of NB (orange)
around the cages at different points of time. The NB accumulation around cages improved as the implanted time. Furthermore, at different points of time after implantation, the accumulation of NB around G-PEEK/Ta composite cages increased as higher Ta microparticles in PEEK.

The BMD and BV/TV at different points of time after implantation are shown in Fig. 10i, j. The BMD and BV/TV for G-PEEK/Ta enhanced as time; however, no obviously enhanced for G-PEEK. The NB formation for G-PEEK/Ta composite cages enhanced as higher Ta microparticles in PEEK.

3.9.3. Biomechanical testing

Fig. 11 reveals the sheep cervical biomechanical performances in ROM. The C3/4 ROM of sheep cervical segment at week 12 after implantation with G-PEEK/Ta-15 were smaller than other cages. Moreover, osseointegration for G-PEEK/Ta composite cages enhanced as higher Ta microparticles in PEEK.

4. Discussion

In spine surgery, cage is an important substitute for intervertebral
discs, which is markedly related to the long-term performance of the spinal fusion [14]. It should play key roles in providing sufficient mechanical support, possessing excellent surface performances, improving cell behaviors, and enhancing osteogenic differentiation until the NB are completely combined with the cages to achieve high-function intervertebral osseointegration [25, 26]. Although PEEK has been widely applied as cages in spine for its superior mechanical properties and 3D printed performances, its performance in bioactivity is still unsatisfactory [27]. In this study, to design a 3D printed PEEK/Ta composite cage with biomimetic gradient pores, which could take advantage of the admirable mechanical properties of PEEK, excellent osteogenesis of Ta, and fully practicing facility of 3D printing, and provide a biological micro-environment for surface performances, inner cell responses, and high-function intervertebral osseointegration through mechanical efficiency, surface extension, and biomimetic gradient pores. Moreover, PEEK/Ta-15 composite with a rougher surface could enhance the hydrophilicity, surface energy, protein adsorption, cell behaviors, and inner osseointegration in sheep cervical fusion model for the higher Ta microparticles in PEEK.

As for remarkable bioactive performances of PEEK, more recent investigations have practiced in the incorporation of bioactive fillers with PEEK by lamination/compression molding, but lack further exploration of PEEK based composites in the 3D printing in fear of mechanical strength loss [8]. However, in terms of custom-design, open and interconnected porous framework, and mechanical properties, new-generation 3D printed porous structure has evolved to the intent of superior mechanical efficiency [17]. In the study, the two architectures were 3D printed with PEEK/Ta-15 composites under the same printed and porous parameters. We observed Cuttlebone-like structure usually experienced uniform interlayer deformation under compressive stress (Fig. 4). Although the layer-by-layer deformation could avoid catastrophic failure, it sacrificed the strength and height of the structure, which was not accepted in spinal fusion [17]. Finally, vertebral Gyroid structure with biomimetic gradient pores exhibited higher mechanical properties, and was determined as 3D structure of PEEK/Ta composite cages to further investigate the bioactivity of PEEK matrices incorporated with different ratios of Ta [16, 18].

Generally, surface roughness of implantable materials could provide more binding sites for adhesive protein [27–29]. The study showed that G-PEEK/Ta composites with higher Ta microparticles content revealed rougher surface but G-PEEK with a smooth surface (Fig. 5); therefore, G-PEEK/Ta-15 presented higher roughness for more Ta microparticles distributed on composite surface. In addition, the 4 times mixing in the preparation of specimens improved the distribution of Ta, and 3D biomimetic gradient porous structure remarkably extended the inner rough surface area, both significantly increasing binding sites of adhesive

![Fig. 5. The images of biomimetic gradient porous G-PEEK (a), G-PEEK/Ta-5 (b), G-PEEK/Ta-10 (c) and G-PEEK/Ta-15 (d). The biomimetic gradient porous micro-structure of G-PEEK (e, i), G-PEEK/Ta-5 (f, j), G-PEEK/Ta-10 (g, k) and G-PEEK/Ta-15 (h, l); enlarged porous micro-structure images (i, j, k, l) of small yellow frame in images (e, f, g, h). The gradient porous properties of Gyroid structure (m). The surface roughness of G-PEEK (n), G-PEEK/Ta-5 (o), G-PEEK/Ta-10 (p) and G-PEEK/Ta-15 (q). The surface roughness $S_a$ (r) of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 (**** represents $p < 0.0001$).

![Fig. 6. Water contact angles (a), diiodomethane contact angles (b), and surface energy (c) of the specimens. Protein adsorption (d) on the cages of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 (* represents $p < 0.05$, *** represents $p < 0.001$, **** represents $p < 0.0001$).]
Hydrophilic surface of implantable materials could influence the behaviors and functions of cells by promoting the initial blood contact and cell attachment [31]. PEEK is hydrophobic as a polymer; however, Ta is hydrophilic as a metal material [32]. In this study, the Ta microparticles distributed in the PEEK matrix increased the hydrophilicity of PEEK/Ta composites; therefore, G-PEEK/Ta-15 is more favorable for cell attachment and spreading due to more Ta microparticles dispersed on its surface (Fig. 6a). Furthermore, implantable materials with higher Ta microparticles content exhibit higher surface energy, which facilitates adherent proteins and cell spreading in the biological environment, and further accelerates NB formation [33]. In this study, G-PEEK/Ta-15 with higher surface energy provided a biological environment for protein adsorption and cell attachment [30] (Fig. 6c). Moreover, the adsorbed proteins on implantable materials as ligands could bind to protein receptors on the cell membrane, further promoting cell adhesion [34]. In spine surgery, albumin and Fn from initial blood contact, could bind to osteogenic cells, improving the preliminary cell adhesion and spreading on cages [34]. In this study, G-PEEK/Ta-15 exhibited higher performance of albumin and Fn adsorption for more Ta microparticles

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dispersed on cage surface (Fig. 6d). Additionally, the 3D biomimetic gradient porous structure provides huge space to facilitate the surface ability of hydrophilicity, surface energy and protein adsorption on the cages [35]. The surface topographical features and chemical composition of implantable materials could induce the interaction of osteogenic cells with the materials, promoting cell adhesion, spreading and proliferation [36]. In this study, G-PEEK/Ta-15 composite cages containing higher Ta microparticles revealed more obvious MC3T3-E1 cell adhesion and spreading than other cages (Fig. 7). Moreover, the efficacious cell adhesion and spreading is crucial for the subsequent cell proliferation [34]. In the study, G-PEEK/Ta-15 with higher Ta microparticles content revealed higher accumulation of cells than other cages (Fig. 7j). Therefore, G-PEEK/Ta-15 composite cages exhibited highest cell

Fig. 9. Histological images of G-PEEK (a, e), G-PEEK/Ta-5 (b, f), G-PEEK/Ta-10 (c, g) and G-PEEK/Ta-15 (d, h) at week 12 after implanted in vivo under different magnifications; enlarged inner NB (bluish violet) images (e, f, g, h) of small yellow frame in images (a, b, c, d). Bone implant contact (i) on the cages of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 at 12 weeks after implantation in vivo (** represents \( p < 0.001 \)).

Fig. 10. 3D reconstructed images of NB (orange) around the cages of G-PEEK, G-PEEK/Ta-5, G-PEEK/Ta-10 and G-PEEK/Ta-15 from Micro-CT at week 4 (a, b, c, d) and 12 (e, f, g, h) after implantation. The bone mineral density (BMD) (i) and bone volume/total volume (BV/TV) (j) from Micro-CT at week 4 and 12 after implantation (* represents \(< 0.05\), ** represents \( p < 0.001 \), **** represents \(< 0.0001\)).
adhesion, spreading and proliferation for the surface characteristics. Moreover, the 3D biomimetic gradient porous structure was markedly conducive for the initial retention of cells, improving the cell adhesion and spreading, effectively extending the time of cell relevant responses [37].

In the initial time of osteogenesis, the ALP activity is a trigger to motivate the main osteogenic marks [38]. In the study, G-PEEK/Ta-15 exhibited higher ALP activity than other cages for higher Ta microparticles in PEEK (Fig. 7k). Additionally, at day 7 and 14, the expression of the most important osteogenesis related genes OPN, OCN, Runx2 and ALP on G-PEEK/Ta-15 were also higher than other cages due to higher Ta content in PEEK (Fig. 8). Obviously, G-PEEK/Ta-15 with higher Ta microparticles content remarkably improved the ALP activity and expressions of osteogenesis genes [39]. Additionally, the 3D biomimetic gradient porous structure provides a biology micro-environment for the transportation of nutrients and waste, facilitating the trigger of ALP activity and osteogenesis expression [30].

As an intervertebral substitute in spinal fusion, the direct contact between the cage and vertebra is vital to the rapid initial NB formation on the cage [15]. The histological images exhibited that accumulation of NB for G-PEEK/Ta-15 were higher than other cages due to higher Ta microparticles in PEEK. Moreover, the NB was more closely in contact with G-PEEK/Ta-15 surface, indicating higher osseointegration. Additionally, good bone to implant contact (BIC) is a favor for achieving the stable anchorage between the cage and vertebra [40]. In this study, the BIC of G-PEEK/Ta-15 was significantly higher than other cages at week 12 as higher Ta microparticles in PEEK (Fig. 9). Moreover, the 3D biomimetic gradient porous structure provides an open and interconnected porous framework, facilitating the penetration of NB.

Commonly, simple surgical exposure in smaller animals is sufficient to stimulate bone fusion; however, that is difficult in the primate (and human) spine despite completed exposure of spinal fusion bed, due to the evolutionary complexity of species [22]. Furthermore, in most studies, the bone defect models are to create a mechanical arrangement for osseointegration under static conditions, that is also relatively easy [22]. However, the in vivo experiment of NB formation and biomechanics conducted in sheep cervical fusion model is an anatomical recreation for demand of high-function cervical range of motion, as a proper model for analogy of human cervical motion segment. In the study, the NB accumulation around G-PEEK/Ta-15 were significantly higher than other cages at week 4 and 12 after implantation as higher Ta microparticles in PEEK (Fig. 10). Additionally, in comparison with other cages, the ROM in G-PEEK/Ta-15 was significantly smaller than other cages, indicating the higher osseointegration (Fig. 11).

5. Conclusions

In the study, taking advantage of the admirable mechanical and 3D printed performances of PEEK and excellent osteogenesis of Ta, overcoming the bio-inertness of PEEK and difficult processing/high elastic modulus of Ta, and fully practicing facility of 3D printing, the designed biomimetic gradient porous PEEK/Ta composite cages were 3D printed by filaments incorporated PEEK with Ta microparticles, then implanted in sheep cervical fusion model for high-function intervertebral osseointegration. The 3D printed Gyroid structure exhibited more superior mechanical properties than Cuttlebone-like structure, closer to the cervical vertebra, and provided a micro-structure that further facilitated the surface performances, cell biological behaviors, and high-function intervertebral osseointegration in sheep cervical fusion model through mechanical efficiency, surface extension, and biomimetic gradient pores. Moreover, PEEK/Ta-15 composite with a rougher surface markedly enhanced the hydrophilicity, surface energy, protein adsorption, cell behaviors, and inner osseointegration in sheep cervical fusion model for the higher Ta microparticles in PEEK. In short, incorporating Ta microparticles into PEEK created 3D printed PEEK/Ta-15 composite cage with biomimetic gradient pores, facilitating custom-design, enhancing mechanical properties and providing a micro-structure for surface performances, inner cell responses, and osseointegration.

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Ethics approval and consent to participate

All procedures performed in this study involving animals were approved by the Laboratory Animal Ethics Committee (Kangtai Medical
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