A carboxymethyl chitosan and peptide-decorated polyetheretherketone ternary biocomposite with enhanced antibacterial activity and osseointegration as orthopedic/dental implants†

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Carbon fiber-reinforced polyetheretherketone (CFRPEEK) possesses biomechanical properties such as elastic modulus similar to human bones and is becoming a dominant alternative to replace the traditional metallic implants. The defective osseointegration and bacterial infection risk of CFRPEEK, however, impede its clinical adoption. In the current study, a newly-developed carbon fiber-reinforced polyetheretherketone/nanohydroxyapatite (CFRPEEK/n-HA) ternary biocomposite was functionalized by covalently grafting carboxymethyl chitosan (CMC) followed by the decoration of a bone-forming peptide (BFP) assisted via the polydopamine tag strategy. Antibacterial test with *Staphylococcus aureus* (*S. aureus*) indicated that the CMC and peptide-conjugated substrates (pep-CMC-CFRPEEK/n-HA) significantly suppressed bacterial adhesion. In vitro cell attachment/growth, spreading assay, alkaline phosphatase activity, real-time PCR analysis, osteogenesis-related protein expression and calcium mineral deposition all disclosed greatly accelerated adhesion, proliferation and osteo-differentiation of human mesenchymal stem cells (hMSCs) on the pep-CMC-CFRPEEK/n-HA biocomposite due to the additive effect of the CMC polysaccharide and the small osteoinductive peptide. More importantly, in vivo evaluation of the beagle tibia model by means of micro-CT, histological analysis, SEM observation and fluorescent labeling confirmed the remarkably boosted bioactivity and osteointegration. The CFRPEEK/n-HA ternary composite with the dual functions of bacterial adhesion reduction and osteointegration promotion holds great potential as a bioactive implant material in orthopedic/dental applications based on this scheme.

1. Introduction

Orthopedic and dental implants are much-needed and widely used when irreparable bone loss occurs due to disease, trauma, congenital abnormalities or aging.1,2 Carbon fiber-reinforced polyetheretherketone (CFRPEEK) is becoming a powerful candidate to replace metallic implants made of titanium (Ti) and its alloys.3,4 Different from conventional metallic materials which display a large elastic modulus of over 100 GPa, CFRPEEK has an adjustable elastic modulus close to that of human cortical bone (about 18 GPa), which can mitigate concerns over the potential metal ion release and the risks of osteonecrosis and bone resorption caused by stress shielding as a result of the elasticity mismatch between the adjacent bones and implants.5,6 In addition to its excellent biomechanical characteristics, CFRPEEK inherits the well-known chemical resistance, non-toxicity, natural radiolucrency, and even MRI (magnetic resonance imaging) compatibility from PEEK.7–9 Unfortunately, its clinical adoption is still hindered due to the bioinertness nature of CFRPEEK, eliciting no positive response in the body, although the composite has attracted much attention in spinal, dental and orthopedic applications, since receiving U.S. FDA approval in the late 1980s.4,10

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c5tb02782a
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Received 31st December 2015, Accepted 9th February 2016
DOI: 10.1039/c5tb02782a
www.rsc.org/MaterialsB
The integration of implants with the surrounding bone, a process termed osseointegration, is pivotal for successful bone regeneration and healing in dental/orthopedic applications. Recently, much effort including physical blending (i.e. impregnating bioactive materials, such as hydroxyapatite (HA),\textsuperscript{11,12} titanium dioxide (TiO\textsubscript{2}),\textsuperscript{13} titanium (Ti),\textsuperscript{14} and calcium silicate,\textsuperscript{15} in the PEEK matrix) and surface modification (such as physical treatment, chemical modification, and surface coating) has been devoted to enhance the bioactivity and osseointegration of the PEEK implant.\textsuperscript{16} Nevertheless, there is little work focusing on promoting the biological performance of the CFRPEEK implant. To achieve the goal, a novel carbon fiber-reinforced polyetheretherketone-nanohydroxyapatite (CFRPEEK/n-HA) ternary composite\textsuperscript{7,17} was developed previously using nano-HA as bio-reinforcement into CFRPEEK for load-bearing bone repairing, because n-HA is a constituent of living bone and has been widely used as bioactive coatings on the implant to promote the bone ingrowth.\textsuperscript{18,19} Our lab results have showcased that the cytocompatibility and osteogenic potential of the biocomposite are promoted dramatically compared with bare CFRPEEK.\textsuperscript{7}

Despite the existing superb biocompatibility of the CFRPEEK-based material, there is still a need to further boost bone covering and successful integration of the implant in the human body. Furthermore, the current bioactivity of CFRPEEK/n-HA is not sufficient to achieve complete affinity with the adjacent tissue, which might lead to implant loosening and ultimately implant failure, since there is only a shade of n-HA on the biocomposite surface.\textsuperscript{17} Another important complication that leads to orthopedic and dental implant failure is associated with bacterial infections. Unmodified implant is susceptible to bacterial infections from the patient’s own skin or mucosa during surgical insertion of implants.\textsuperscript{20} Bacterial adhesion and subsequent biofilm formation on material surfaces confer resistance against host defense mechanisms and antibiotic therapies, eventually resulting in implant failure, removal of infected implants and increased patient morbidity, as well as higher treatment expenditure.\textsuperscript{21,22} Thus, it is crucial to accelerate bone formation accompanied by a reduction in the bacterial infection of the newly-fabricated CFRPEEK/n-HA biocomposite for its successful adoption as a synthetic implantable material in clinic.

Surface modification is an effective way to endow enhanced biological properties with materials while the advantageous bulk properties can be preserved. Among the diverse surface modification approaches, biochemical modification of implant surfaces through the introduction of chemical cues by non-covalent or covalent binding to steer the cellular fate has drawn considerable attention in the field of bone regeneration.\textsuperscript{23,24} Reversible immobilization of biomolecules can be achieved via physical adsorption under specific conditions, however, this method suffers from drawbacks of having the necessity for relatively large dosages, and potential undesirable/burst release of the biomolecules from the implantation site.\textsuperscript{25} In contrast, surface modification by covalent binding provides an opportunity for immobilization and orientation of the biomolecules including the extracellular matrix (ECM)\textsuperscript{26,27} and signaling proteins (such as bone morphogenetic protein\textsuperscript{28,29}) to be more effective for prolonged periods of time, allowing successive regulation of cell behaviors at the tissue interface. In order to overcome the two major obstacles for the CFRPEEK/n-HA biocomposite, a promising approach is to modify the implant with an osteo-differentiation promoting factor and an antibacterial agent simultaneously through covalent chemistry.

Bone morphogenetic protein-7 (BMP-7) is known to play a critical role in modulating osteogenic phenotype and proliferation, and in promoting osseointegration during new bone generation.\textsuperscript{30–32} Whereas, the complex multilevel structure of BMP-7 renders it prone to degradation, and tends to lose its bioactivity quickly under physiological conditions.\textsuperscript{13} Recently, a novel bone-forming peptide (BFP, GQGFSYPKYAVFSTQ sequence) from the immature region of BMP-7 identified by Kim \textit{et al.} exhibited more excellent osteogenic efficacy over BMP-7 both \textit{in vitro} and \textit{in vivo}.\textsuperscript{33,34} In addition, the use of synthetic peptides could eradicate concerns regarding immunogenicity derived from proteins and shows greater potential for clinical use.\textsuperscript{36} On the other hand, with excellent biocompatibility, biodegradability, nonimmunogenicity, and intrinsic antimicrobial properties, carboxymethyl chitosan (CMC), a water-soluble chitosan derivative, finds a wide range of applications in medicine.\textsuperscript{37,38} Moreover, it is proved that CMC, a structure similar to glycosaminoglycans, can facilitate osteoconduction and osseointegration. Hence, in the present study, a CMC and peptide-decorated CFRPEEK/n-HA biocomposite was established assisted \textit{via} polydopamine (pDA) technology for co-enhanced antibacterial behavior and osseointegration. We systematically evaluated the biofunctionalities of the prepared decorated CFRPEEK/n-HA both \textit{in vitro} and \textit{in vivo}, in terms of their antimicrobial performance, cellular viability, differentiation capacity, and tissue responses.

2. Materials and methods

2.1. Materials

Commercially-available carbon fibers (TC-35) were supplied from Formosa Plastics Corp. (Taiwan). Medical graded PEEK powders (450G) with a density of about 1.30 g cm\textsuperscript{-3} was purchased from Victrex Plc. (Thornton Cleveleys, UK) Dopamine hydrochloride (DA) was purchased from Sigma-Aldrich (St. Louis, USA), and Tris[hydroxymethyl]-ammonomethane (Tris-HCl) was provided by Sinopharm Chemical Reagent Co. Ltd (Beijing, China). N-(3-(Dimethylamino)propyl)-N'-(ethylenimino)ethylcarboxybiomimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpho)-ethanesulfonic acid (MES), and carboxymethyl chitosan (CMC, $M_w \approx 9.5–20.9$ kDa, degree of carboxymethyl substitution $\geq 86\%$) were obtained from Aladdin (Shanghai, China). To facilitate chemical conjugation on the material surface, the peptide was modified at its N-terminal with a lysine-containing spacer. BFP [Ac-KGGQGFSPYPKAVFSTQ sequence], provided by Chinapeptides...
Co. Ltd (Shanghai, China), were synthesized using a batchwise fmoc-polyamide method and had more than 98% purity for the high-pressure liquid chromatography profile. All other chemicals were of analytical reagent grade, and all aqueous solutions were prepared using deionized water (D.I. water).

2.2. Preparation of the CFRPEEK/n-HA biocomposite

Based on our previously established protocol, the CFRPEEK/n-HA ternary composite was fabricated through a process of compounding and injection-molding. In brief, 15 wt% n-HA powder (synthesized by our group), 20 wt% carbon fiber and 60% PEEK powder were dispersed in alcohol using a ball grinder to obtain a homogeneous mixture. After well dispersed, the mixture was dried in a forced convection oven at 90 °C for 2 h to remove the excess alcohol. The biocomposites were produced at an injection temperature of 380 °C using an injection-molding machine (WMZ-4, Battenfeld). The resulting composite was injected into two specially designed molds, i.e. disks (Φ = 15 × 2 mm²) for physical and chemical characterization and in vitro testing, and cylindrical implants (4.0 mm diameter and 7.0 mm length) for in vivo measurement. Samples of composite were polished with a series of increasingly abrasive SiC papers (400, 1000, 1500, 2000 grit). Then, they were cleaned ultrasonically for 30 min in baths of acetone, anhydrous ethanol and deionized (D.I.) water, respectively, and dried at 50 °C overnight.

2.3. Immobilization of carboxymethyl chitosan and peptide on the surface of CFRPEEK/n-HA

The CFRPEEK/n-HA substrates were first immersed in a dopamine solution (2 mg mL⁻¹ in 10 mM Tris-HCl, pH = 8.5) and shaken for 24 h at room temperature (RT), the resulting substrates were denoted pDA-CFRPEEK/n-HA. After three washes using D.I. water, the substrates were immersed in 3 w/v% CMC solution for another 24 h at 37 °C, and then the treated samples were thoroughly rinsed to remove the physically-adsorbed CMC molecules, which were named CMC-CFRPEEK/n-HA.

The pDA-CFRPEEK/n-HA and CMC-CFRPEEK/n-HA substrates were separately used to prepare peptide-decorated CFRPEEK/n-HA by immobilizing the peptide: (1) pDA-CFRPEEK/n-HA substrates were reacted with BFP in 1 mM concentration dissolved in PBS overnight at RT. BFP was grafted onto pDA-coated CFRPEEK/n-HA via the reaction between the amino groups in peptide and the catechol/quinine groups in pDA through Michael addition and Schiff-base reactions. (2) To conjugate BFP onto the surface, the CMC-CFRPEEK/n-HA samples were firstly pretreated with 2 mM EDC and 5 mM NHS in 0.1 M MES buffer (pH = 5.6) for 40 min. Then, BFP solution (1 mM) was incubated onto the carboxyl-rich CMC-grafted CFRPEEK/n-HA surfaces at 4 °C in a refrigerator for 24 h. The two final peptide-decorated CFRPEEK/n-HA substrates (denoted pep-pDA-CFRPEEK/n-HA and pep-CMC-CFRPEEK/n-HA, respectively) were thoroughly washed by D.I. water and dried under nitrogen influx before characterization and cell experiment.

2.4. Surface characterization

Surface morphologies of various treated substrates were characterized by field emission scanning electron microscopy (SEM, JSM-6701F, JEOL, Japan) at an accelerating voltage of 20 kV. All samples were coated with gold for 1 min before SEM observation. AFM (PI3800/SAPA400, Seiko Instruments, Japan) was used in contact modes (15 μm scanner) under dry conditions to assess morphological characteristics of the bare and modified CFRPEEK/n-HA substrates. Surface roughness (Rₛ) was also calculated from the roughness profile, and each sample was tested in triplicate to the arithmetic average. For the contact measurement, we used a Si₃N₄ cantilever with a spring constant of 0.12 N m⁻¹ (Seiko Instruments) for resolution imaging. The surface hydrophilicity was measured by contact angle goniometry (SL200B, Kono, USA) based on the sessile drop method using 2 μL of D.I. water droplets. Six parallel specimens were used to provide the average and standard deviation. The alteration of chemical constituents and elemental states of different CFRPEEK/n-HA samples were analyzed by X-ray photoelectron spectroscopy (XPS, Kratos Analytical Ltd, UK) recording both survey and high-resolution spectra. The binding energies were calibrated by the C 1s hydrocarbon peak at ~285 eV. Besides, the quantitative analysis and the curve fitting were performed using the CasaXPS software package.

2.5. Peptide content quantification

To compare the peptide density on the two peptide-decorated CFRPEEK/n-HA surfaces, the amount of immobilized peptide was quantified by fluorescamine assay (Sigma-Aldrich). The concentrations of unattached peptides in the retrieved solutions were determined according to the manufacturer’s instructions using a multilabel reader (Perkin Eimer Singapore Pvt. Ltd). Known concentrations of BFP (100–1000 μM) solution were also reacted with fluorescamine to obtain a standard curve. The surface density of bound BFP was calculated from the difference between the BFP in the initial loading solution and that remaining in the supernatant.

2.6. Bacterial adhesion assay

The antimicrobial activity of the functionalized surfaces against *Staphylococcus aureus* (S. aureus) was assessed via confocal laser scanning microscopy (CLSM), SEM and microbial viability assay, respectively. *S. aureus* (UA159, American Type Culture Collection, USA), the most common microbial pathogen encountered in orthopedic infections, was cultured in tryptic soy broth (TSB, Gibco, Carlsbad, USA) overnight at 37 °C. The bacterial suspension was centrifuged at 2700 rpm for 10 min, and after removal of the supernatant, the bacterial pellets were re-suspended at a concentration of 1 × 10⁷ cells per mL in PBS. The substrates after sterilization were placed in a 24-well plate, and each piece was treated with 1 mL of the bacterial suspension and incubated for 6 h at 37 °C. After incubation, the substrates were washed three times with PBS to rinse off loosely adhered bacterial cells. The viability of the bacteria on the surface was assessed using a combination dye (LIVE/DEAD Baclight bacteria viability kit, Molecular Probes, Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol, and observed in the dark under a Zeiss LSM510 CLSM (Germany). The viable bacterial cells would be stained green, while membrane-compromised (dead) cells would be stained red. The adhered bacterial cells
were also observed using SEM (JSM-6701F, JEOL) after fixing with 2.5% glutaraldehyde for 1 h and then dehydrating with serial graded ethanol for 10 min each at RT. Quantification of the viable adhered bacterial cells was further carried out using a microbial viability assay kit-WST. At the scheduled time (6 h), the substrates were taken out, gently rinsed with PBS three times, and placed in a new 24-well plate incubated with 20 μL of WST, which produced a water-soluble formazan dye upon reduction by dehydrogenase in cells, and 380 μL of medium for another 2 h. Then 100 μL of supernatant from each well was transferred to new 96-well plates. Then, the OD value (λ = 450 nm) of the suspension in each well was measured on a microplate reader (Model 680, Bio-Rad, USA).

2.7. **In vitro biocompatibility assays**

2.7.1. Cell culture and osteogenic induction. Human mesenchymal stem cells (hMSCs) obtained from Sciencell Research Laboratories (USA) were isolated from the bone marrow. hMSCs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin, and then incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells before passage five were used for the in vitro experiments. Osteogenic inducing medium comprised fresh DMEM containing 10% FBS, 100 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin, 50 μg mL⁻¹ ascorbic acid, 10 mM sodium β-glycerophosphate, and 10 nM dexamethasone (Sigma-Aldrich, USA). The culture medium was changed every 2–3 days. All samples were sanitized with 75% ethanol for at least 40 min and rinsed twice with sterile PBS before cell culture.

2.7.2. Cell attachment and proliferation. The in vitro adhesion and proliferation of hMSCs were assessed using the cell counting kit-8 assay (CCK-8, Dojindo, Japan). After cell counting, hMSCs were seeded in 24-well plates (Costar, USA) with different functionalized CFRPEEK/n-HA substrates at a density of 2 × 10⁴ cells per well. After incubating for 8 h, and 1, 3, 5, and 7 days, respectively, CCK-8 was added into each well for incubation for 2 h at 37°C, then 100 μL of supernatant was transferred to new 96-well plates. The absorbance value of supernatant optical density was measured using a microplate reader (Model 680, Bio-Rad) at 450 nm wavelength.

2.7.3. Cytoskeletal observation. After 8 h of incubation with the experimental biocomposites, hMSCs were washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at RT. The cells were then permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 5 min, before being incubated with 1% bovine serum albumin/PBS at 37°C for 30 min to block nonspecific binding. This was followed by adding 5 μg mL⁻¹ fluorescein isothiocyanate (FITC)-phalloidin (Sigma-Aldrich, USA) to stain hMSCs for 30 min. The stained signals in the cells were observed by employing osteoblast specific markers including Runx2, ALP, and Col1a1. After grown on the pristine and modified CFRPEEK/n-HA substrates for 7 days, hMSCs were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 10 min and then blocked with 5% BSA in PBS for 30 min. Substrates were then incubated with primary antibodies [Rabbit Anti-Runx2 IgG (CST, USA), Rabbit Anti-Col1a1 IgG (CST) at a dilution of 1:200 and Mouse Anti-ALP IgG (Santa, USA) at a dilution of 1:50] at 4°C overnight, and washed with PBS twice, then incubated with secondary antibodies [Goat Anti-Rabbit Invitrogen 488 IgG, Goat Anti-Mouse Invitrogen 647 IgG (USA)] at 1:500 for 1 h at RT in the dark. Finally, cell nuclei were stained with 10 μg mL⁻¹ DAPI for 15 min at RT. Cells were visualized immediately using a CLSM (LSM510, Carl Zeiss, Germany).

2.8. **In vitro osteogenic potential tests**

2.8.1. Alkaline phosphate activity (ALP) quantification and staining. Cells were seeded onto the pristine and decorated CFRPEEK/n-HA substrates at a density of 2 × 10⁴ cells per cm² in the osteogenic inducing medium. The cellular ALP activity was determined on the 7th and 14th day of osteoinduction using the alkaline phosphatase kit (NJJC-BIO, Nanjing, China). Briefly, the supernatant was removed and 100 μL of lysis solution (1% TritonX-100) was added into each well and incubated for 1 h. Afterwards, 30 μL of cell lysates at each well was transferred to new 96-well cell culture dishes, and cultivated with 50 μL of carbonate buffer solution (pH = 10) and 50 μL of substrate solution (4-aminooantipyrine) at 37°C for 15 min. Then 150 μL of potassium ferricyanide (chromogenic agent) was added into the above solution and the production of p-nitrophenol was determined by the absorbance at 520 nm on a microplate reader. For normalization, the total protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo, USA). Thus, ALP activity was normalized and expressed as the total protein content (μg prot⁻¹). Six specimens were tested for each incubation period, and each test was performed in triplicate. Furthermore, enzyme-histochemistry (EHC) staining was also performed to visualize ALP expression at the same time points using a BCP/NBT ALP color development kit (Beijing ComWin Biotech, China).

2.8.2. RNA extraction and quantitative real-time PCR. After 7 days of osteoinduction, the total mRNA was isolated from cells using TRIzol (Invitrogen, USA) and converted into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). Quantitative real-time polymerase chain reaction (qPCR) analysis was carried out with SYBR Green (Roche, USA) on an ABI 7500 RT-PCR machine (Applied Biosystems, USA). All were performed in triplicate and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for PCR amplification. Primers (5’–3’) provided in this study are listed in Table S1 (ESI†). Primer sets (10 μM final concentration for each primer) were used in a volume of 20 μL per tube. The thermal profile of the PCR was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The comparative CT(2⁻ΔΔCT) method was employed to evaluate fold gene expression differences between groups.

2.8.3. Immunohistochemistry. The osteogenic capability of hMSCs was confirmed using immunofluorescent staining by employing osteoblast specific markers including Runx2, ALP, and Col1a1. After grown on the pristine and modified CFRPEEK/n-HA substrates for 7 days, hMSCs were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 10 min and then blocked with 5% BSA in PBS for 30 min. Substrates were then incubated with primary antibodies [Rabbit Anti-Runx2 IgG (CST, USA), Rabbit Anti-Col1a1 IgG (CST) at a dilution of 1:200 and Mouse Anti-ALP IgG (Santa, USA) at a dilution of 1:50] at 4°C overnight, and washed with PBS twice, then incubated with secondary antibodies [Goat Anti-Rabbit Invitrogen 488 IgG, Goat Anti-Mouse Invitrogen 647 IgG (USA)] at 1:500 for 1 h at RT in the dark. Finally, cell nuclei were stained with 10 μg mL⁻¹ DAPI for 15 min at RT. Cells were visualized immediately using a CLSM (LSM510, Carl Zeiss).
following the manufacturer's instruction by staining with Alizarin Red S (ARS) solution (Sigma-Aldrich). The cell layers were fixed with 4% paraformaldehyde for 30 min at RT, and washed three times with PBS. Then a 2% (wt/v) solution of ARS with pH = 4.2 was added so that it covered the entire surface of the wells containing cells. After incubation for 15 min, the excess ARS was washed with DI water, and the newly formed bone-like nodules on the materials were found to be stained dark red in color. To quantify matrix mineralization, the ARS-stained samples were incubated in hexadecylpyridinium chloride (1 w/v%, Sigma-Aldrich) overnight with shaking to solubilize and release calcium mineral into the solution. 100 μL of supernatant was collected in new 96-well plates, and OD 490 was read using a microplate reader (Model 680, Bio-Rad).

2.9. In vivo animal studies

2.9.1. Surgery. Surgical implantation was performed on four male Beagle dogs aged 10 months and weighing 9–12 kg and observed for a period of 4 weeks after surgery. The in vivo study was conducted according to the ethical principles of Peking University Institutional Animal Care and Use Committee. The sample implants were sterilized prior to surgery as described above for in vivo testing. All dogs were randomly assigned to three groups as pep-pDA-CFRPEEK/n-HA, pep-CMC-CFRPEEK/n-HA and CFRPEEK/n-HA control. General anesthesia was induced using an intravenous injection of 1% pentobarbital 1 mL kg⁻¹, and the amines of CMC could react with the oxidized catechol grafted onto the pDA layer through the catechol chemistry, of which the amine groups on the resulting CMC-CFRPEEK/n-HA composite presenting pDA coating, the CMC molecule was further solubilize and release calcium mineral into the solution. 100 μL of supernatant was collected in new 96-well plates, and OD 490 was read using a microplate reader (Model 680, Bio-Rad).

2.9.2. Micro-CT analysis. High-resolution images of all the specimens were obtained on a micro-computed tomography scanner (Scanco Medical vivaCT40, Switzerland) running at 70 kV and 114 μA. After scanning, the percent bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were calculated using the CT-analyzer program. Radiographs were also synthesized using the CT-analyzer software package (Scanco Medical) to reconstruct three-dimensional (3D) regenerated bone of 0.5 mm width in the marrow bonding to the implants.

2.9.3. Histological evaluation. The bone samples with the implants were harvested and fixed in 10% formalin for 5 days, dehydrated in a series of solutions with different ethanol concentrations (70%, 80%, 90%, 95%, and 100%) for 2 days each, and transferred to a methylmethacrylate solution at 37°C. Afterwards, the embedded samples were cut into sections with a thickness of about 30 μm and examined using CLSM (LSM510, Carl Zeiss) and SEM (JSM-6701F, JEOL). The excitation/emission wavelengths used to observe chelating fluorochromes were 543/620 nm and 405/575 nm for Alizarin Red S (red) and tetracycline hydrochloride (green), respectively. After fluorescence microscopy, the same sections were counter-stained with hematoxylin and eosin (H&E) and optical microscopy (Nikon Eclipse, TE200-E, Japan) was performed to observe the bone ingrowth and integration with the host tissue. Histomorphometric analysis to evaluate the percentage of bone-implant contact (BIC) was also performed on the same sections of each group.

3. Results and discussion

3.1. Preparation and characterization of decorated CFRPEEK/n-HA

In this study, pDA-mediated catechol functionalization was applied to develop a carboxymethyl chitosan and peptide-decorated PEEK-based ternary biocomposite with greater antibacterial activity and better osteoinductivity that can boost the osteogenic potential of hMSCs in vitro, as well as osseointegration in vivo for load-bearing bone repairing (Scheme 1). Dopamine, a repeated motif in the mussel adhesive protein, could initiate self-polymerization and adhere onto almost any solid surface in alkaline solution without surface pretreatments. More importantly, a pDA layer can function as an anchor to graft the secondary functional biopolymers with thiols and amines via Michael addition or Schiff base reactions. To enhance the antimicrobial property and osteogenesis on the surface of the CFRPEEK/n-HA biocomposite presenting pDA coating, the CMC molecule was further grafted onto the pDA layer through the catechol chemistry, of which the amines of CMC could react with the oxidized catechol groups. The –COOH groups on the resulting CMC-CFRPEEK/n-HA

![Scheme 1 Schematic illustration of the preparation of the carboxymethyl chitosan and peptide-decorated CFRPEEK/n-HA biocomposite, and its in vitro biological evaluation and in vivo osseointegration.](image-url)
surfaces were preactivated using the EDC/NHS approach to facilitate the peptide tethering of terminal carboxyl groups to the NHS groups, producing peptide tethering surfaces.

To explore the alteration of morphology and chemical composition after various stages of surface functionalization, the prepared decorated CFRPEEK/n-HA was characterized by SEM, AFM, and contact angle goniometry, as well as by XPS analysis.

As depicted in Fig. 1a, the homogeneous dispersion of carbon fibers and nano-HA particles (white spots) was found on all the CFRPEEK/n-HA surfaces. The average roughness $R_a$ value of the decorated CFRPEEK/n-HA surfaces, as measured by AFM, indicated that the coating of pDA and CMC enhanced the surface roughness of CFRPEEK/n-HA substrates ($R_a \approx 44.3$ nm and 64.7 nm, respectively). However, the further addition of a small molecule peptide slightly increased the surface morphology/roughness of substrates. The water contact angle on a substrate can provide information on the wettability and surface energy of the substrate and has been widely used to track and evaluate the effectiveness of surface modification protocols. The surface wettability of pDA-coated substrates has been documented well previously. For example, the water contact angle of clean glass ($11 \pm 1°$) increased to $44 \pm 2°$ after 24 h of pDA coating, and the pDA-coated polyvinylidene fluoride film showed a dramatic decrease in the water contact angle.\(^\text{45}\)

In good agreement with previous reports, the water contact angle on the CFRPEEK/n-HA substrate after dopamine anchoring significantly decreased from $76 \pm 1°$ to $47 \pm 3°$, and further to $32 \pm 1°$ after CMC attachment (Fig. 1c). This could be due to the hydrophilic groups (–OH, –COOH, and –NH$_2$) of the grafted polymers on the CFRPEEK/n-HA surfaces. In addition, immobilization of the peptide on the coated CFRPEEK/n-HA continuously reduced the water contact angle to below $18°$, attributed to the super-hydrophilic property of peptide, which implied that the BFP peptide was successfully grafted onto the CFRPEEK/n-HA surface via pDA and the CMC intermediate layer.

These findings were further verified by an XPS survey scan (Fig. 1d, Table S2 and Fig. S1, ESI\(^\text{†}\)). In the full scan spectrum of pristine CFRPEEK/n-HA, carbon and oxygen elements were the predominant components, while a certain amount of calcium and phosphorus were also recognizable, attributed to the presence of nano-HA on the surface. Successful anchoring of pDA was indicated by an increase in the N 1s and O 1s content, and a corresponding decrease in the C 1s content from 70.27% to 65.47% as shown in Table S2 (ESI\(^\text{†}\)). The nitrogen-to-carbon (N/C) ratio was about 0.121 on pDA-CFRPEEK/n-HA which was similar to the theoretical N/C of 0.125 for dopamine. Furthermore, the complete suppression of photoelectron peaks unique to Ca 2s, 2p and P 2p confirmed the formation of the pDA thin film. These changes, nevertheless, were further aggravated after the grafting of CMC onto the pDA-treated substrate, indicating the immobilization of CMC. Upon attachment of peptide on the surface, notably in the wide-scan spectra, the appearance of a sodium signal (because the peptide was dissolved in PBS buffer), and the enhancement of nitrogen peaks (N 1s, 2.48% and 2.78%, respectively) on the surface of pep-pDA-CFRPEEK/n-HA and pep-CMC-CFRPEEK/n-HA samples indicated successful tethering of the peptide. Furthermore, an evident change in the carbon bond composition observed in the high-resolution narrow carbon spectra (C 1s) clearly supported these conclusions (Fig. S1, ESI\(^\text{†}\)). The high-resolution C 1s spectrum of the pristine CFRPEEK/n-HA was deconvoluted into three different curves. The binding energies centered at 284.8 eV, 286.4 eV and 291.5 eV could be assigned to the $-C-C/-C-H,-C-OH$, and $-C(O)O-$ bonds, respectively.\(^\text{46,47}\)

After pDA coating, the intensity of the carbon skeleton ($-C-C/-C-H-$) decreased dramatically, and the peaks of the hydroxyl ($-C-OH$) and carbonyl ($-C=O$) groups increased as shown in Fig. S1b (ESI\(^\text{†}\)). This should be attributed to the catechol/quinone groups of pDA. Whereas a broad peak of the $-C-N-$ bond at about 285.5 eV was newly recorded on CMC-grafted and pDA-treated surfaces.

3.2. In vitro antibacterial activity evaluation

It is known that the interface between the implant surface and human tissues is vulnerable and can easily become the hotbed
for bacteria growth which is gradually becoming one of the main reasons for post-surgical implant failure.\textsuperscript{21} The initial adhesion of bacteria to implant interfaces is the initial and crucial step for biofilm formation, which plays an imperative role in the pathogenesis of infection.\textsuperscript{48} The prevention of bacterial adhesion to implanted biomaterials such as PEEK-based implants, during the early post-implantation period is vital to the efficacy and long-term success of an implant. Hence the investigation of initial bacterial adhesion, was carried out on the substrates incubated for 6 h, which was deemed sufficient to reflect the bacterial contact in the early ‘decisive period’ of implant infection.\textsuperscript{49} In the present work, \textit{S. aureus} was selected as the target bacterium since it is the main pathogenic microbe responsible for implant-related infections, accounting for 34\% of these infections. Representative CLSM images of different substrates after incubation in \textit{S. aureus} suspension (1 \times 10^7 cells per mL) for 6 h are shown in Fig. 2. It was obvious that there was a clear difference between the number of \textit{S. aureus} cells on the pristine CFRPEEK/n-HA and the functionalized substrates. The presence of many viable cells (stained green) could be seen on the pristine CFRPEEK/n-HA surface (Fig. 2a), while at the same time, there were hardly any dead cells. With regard to the CMC-functionalized CFRPEEK/n-HA substrate, both viable and dead cells were detected on the surface, but the number of viable cells was substantially less than that on the pristine CFRPEEK/n-HA. The considerable reduction in the amount of viable bacteria on CMC-CFRPEEK/n-HA together with the increase in dead cells demonstrated the antibacterial property of the immobilized CMC, consistent with previous studies.\textsuperscript{50,51} Similar findings with pep-CMC-CFRPEEK/n-HA substrates were found. Unlike what was observed with pep-CMC-CFRPEEK/n-HA, there was a large amount of viable \textit{S. aureus} without dead cells on the pep-pDA-CFRPEEK/n-HA sample, since BFP peptide was not bactericidal. Furthermore, the pep-pDA-CFRPEEK/n-HA surface showed an elevated number of bacterial cells than on the CFRPEEK/n-HA counterpart. SEM analysis (Fig. 2b) confirmed the results obtained from CLSM observation, where more \textit{S. aureus} cells were evidenced on the bare CFRPEEK/n-HA and pep-pDA-CFRPEEK/n-HA substrates compared with the groups functionalized with CMC (i.e. CMC-CFRPEEK/n-HA and pep-CMC-CFRPEEK/n-HA).

In addition to the qualitative CLSM and SEM analysis of bacterial adhesion on the different substrates, quantitative determination of the number of viable cells on these substrates was further conducted using the microbial viability assay kit-WST, and the results are depicted in Fig. 3. The quantitative bacterial density on different substrates was congruent with the qualitative results shown in Fig. 2. The CMC- and pep-CMC-eluted CFRPEEK/n-HA substrates showed a remarkable reduction in the number of adherent bacterial cells compared to pristine CFRPEEK/n-HA (\(p < 0.05\)). For instance, at 6 h the amount of viable \textit{S. aureus} cells on the CMC-CFRPEEK/n-HA and pep-CMC-CFRPEEK/n-HA decreased to approximately 29\% and 31\% of that on pristine CFRPEEK/n-HA, respectively. Several aspects such as chemical composition, surface charge, hydrophilicity, and surface roughness have been reported to influence bacterial adhesion on a surface. It is widely accepted that chitosan harbors antibacterial behavior as a result of its positively charged amino groups which can interact with the negatively charged bacterial membrane to change the membrane’s permeability, and the CMC has been proved to retain the antimicrobial property of chitosan.\textsuperscript{52,53} In accordance with the CLSM and SEM investigations, more bacteria were detected on pep-pDA-CFRPEEK/n-HA than CFRPEEK/n-HA. This was possibly due to the reason that the presence of specific proteins or peptides on an implant surface such as fibrinogen and fibronectin peptide can promote bacterial adhesion via interactions between the
protein and adhesion receptors on the bacterial cell membrane.\textsuperscript{54,55} However, in the present study, no significant alteration in bacterial adhesion was observed among the CMCS-functionalized surfaces with and without BFP peptide, indicating that the immobilized BFP on the CMC chains did not promote bacterial adhesion and the bacterial inhibition properties of CMC were preserved even after further functionalization with BFP.

### 3.3. Cell adhesion and proliferation on the decorated CFRPEEK/n-HA

As we know, initial cell adhesion plays a major role in cellular functions and eventual tissue integration, while cell proliferation is closely correlated with the amount of new bone formation. Better pre-osteoblast adhesion and proliferation probably produce a larger mass of bone tissues around the implants. Thus, to explore the potential of the decorated CFRPEEK/n-HA biocomposites in dental and orthopedic applications for load-bearing bone repair, the evaluation of cell adhesion and interactions with bone cells is essential. The hMSC cells exist abundantly in human bone marrow and are considered ideal cells for the evaluation of cytocompatibility and osteogenic properties of bone substitutes. Cell attachment and growth on different samples were quantified using a colorimetric CCK-8 test, in which the adherent cell numbers on all the modified CFRPEEK/n-HA substrates were enhanced compared to bare CFRPEEK/n-HA control at 8 h. In particular, pep-CMC-CFRPEEK/n-HA displayed the highest initial cell adhesion (Fig. 4a), indicating superb cell binding affinity. The time-related proliferation of hMSCs cultured on the studied samples was evaluated and the data are presented in Fig. 4b. Cells grown on all the biocomposites displayed a persistent increasing proliferation tendency from day 1 to day 7, though there was no significant difference between them at day 1. However, noticeably augmented cell viability could be seen on the peptide-eluted PEEK-based biocomposites (i.e., pep-pDA-CFRPEEK/n-HA and pep-CMC-CFRPEEK/n-HA) than the bare CFRPEEK/n-HA at days 3 and 5, which revealed the improved cytocompatibility after peptide decoration. The number of cells on different CFRPEEK/n-HA matrices was similar on day 7. This result indicated that the cells on different substrates reached confluence and began to differentiate along an osteogenic lineage.

The adhesion morphology and cytoskeletal organization of hMSCs observed by CLSM on the pristine CFRPEEK/n-HA and functionalized CFRPEEK/n-HA substrates are shown in Fig. 4c–g. Most of the cells adhered to the substrates after an 8 h attachment period and appeared to be spread. The spreading and the number of hMSCs on pDA-CFRPEEK/n-HA substrates were superior to those on pristine CFRPEEK/n-HA. Previous reports have demonstrated that pDA coating could enhance the adhesion and survival of cells via anchoring the extracellular matrix protein from culture medium, which play vital roles in cell adhesion/proliferation.\textsuperscript{56,57} In addition, there were increased cell numbers on two peptide-decorated substrates, which suggested that BFP was more advantageous over hMSC adhesion. In comparison with pep-pDA-CFRPEEK/n-HA, much more hMSCs were detected on the pep-CMC-CFRPEEK/n-HA group, consistent with CCK-8 results. Furthermore, as cell adhesion was enhanced, hMSCs cultured with the pep-CMC-CFRPEEK/nHA group extended more adhered filopodia and spread more with visible presentation of more mature F-actin intracellular stress fibers. There are several reports demonstrating the peptide-modulated adhesion of bone cells by immobilized peptide. Chen et al. proved that a short peptide from BMP-7 enabled the increase of osteoblast proliferation due to the peptide–integrin interaction.\textsuperscript{58} Balasundaram et al. had shown that anodised nanotubular titanium (\textit{TiO}$_2$) bearing CKIPKASSVPTELSAISTLSYL peptides from BMP-2 boosted the adhesion of human fetal osteoblasts.\textsuperscript{59} Our results showcased that peptide-decorated CFRPEEK/nHA exhibited excellent in vitro cytocompatibility, and the pep-CMC-CFRPEEK/nHA substrate offered a more favorable cell environment for both cell attachment and proliferation.

### 3.4. Enhanced osteogenic differentiation of hMSCs on peptide-decorated CFRPEEK/n-HA

The osteogenic differentiation activity of cells on the bio-interfaces, as far as bone-repair biomaterials were concerned, is a key event in bone formation. To determine the extent of osteogenesis in hMSCs on carboxymethyl chitosan and peptide-decorated CFRPEEK/n-HA substrates, the ALP activity, gene expression, and the corresponding marker protein expression as well as the amount of calcium deposition were evaluated at specific time intervals.

Among the major osteogenic hallmarks, the up-regulation of ALP activity is a central event occurring during the early time...
points of osteogenesis. As shown in Fig. 5f, clearly all bio-
composites exhibited good time-dependent ALP expression. The pDA-CFRPEEK/n-HA and CMC-CFRPEEK/n-HA groups pos-
sessed higher ALP activity than the bare CFRPEEK/n-HA group
during culture time, indicating the positive influence of pDA
coating and CMC on the osteogenic phenotype of stem cells,
which coincided with earlier literature. Furthermore, an
increase in the expression of ALP was detected in pep-pDA-
CFRPEEK/n-HA samples at days 7 and 14 due to the immobi-
lization of the BFP peptide on the pDA-grafted CFRPEEK/n-HA
surfaces. This finding suggested that the presence of peptide
on the CFRPEEK/n-HA surfaces was capable of triggering an
up-regulation of ALP, correlated with the first check-point for
osteo-differentiation. It was noteworthy that more than
about 1.5-fold increase in ALP activity was observed in pep-CMC-
CFRPEEK/n-HA samples compared with Pep-pDA-CFRPEEK/n-HA
to day 14, highlighting a significant synergistic effect between the
CMC and the peptide on the osteo-differentiation of hMSCs. These results were further qualitatively confirmed via EHC stain-
ing of ALP at days 7 and 14 (Fig. 5a–e), in which the ALP-positive areas were obviously larger on pep-pDA-CFRPEEK/n-HA and
pep-CMC-CFRPEEK/n-HA than on pristine CFRPEEK/n-HA after
culturing for 7 days. After 14 days, more ALP was generated on
the pep-CMC-CFRPEEK/n-HA surface, suggesting the long-term
stimulating effect of the presence of CMC and peptide on hMSC
osteodifferentiation.

Cells are capable of sensing and responding to the changes
in biochemical stimuli from the surrounding environment,
triggering a cascade of intracellular events regulating the gene
expression associated with cell differentiation. An in-depth
study on the gene expression at the transcript level is instru-
mental to better understand the cellular interactions with
biomaterials and their subsequent effect on various cell func-
tions related to osteogenic differentiation. Therefore, our study
revealed the typical osteogenesis-related gene expressions of
hMSCs in different groups including Runx2, ALP, Col1a1, and
OCN, assessed by quantitative real-time PCR for 7 days. Runx2
is the early and master transcription factor, and it regulates the
transcription of various osteogenesis-related genes (such as
Col1a1 and OCN) via binding to the core site of their enhancers or
promoters. Of all the osteo-related genes, OCN is regarded as the
most specific marker for the mature osteoblast and mineralization
during the course of osteogenesis, and it reaches the maximum
expression during mineralization and accumulates in the mineral-
ized bone due to its high affinity to HA crystals. The other two
osteo-specific genes whose expressions were probed include ALP,
which is a membrane-bound enzyme and plays an essential role in
the mineralization of the bone matrix via hydrolysis of organic
phosphates, and Col1a1, which is involved in the biosynthesis of
the extracellular matrix (ECM). As found in Fig. 6, CFRPEEK/n-HA
control was the least at every time point. The hMSCs on CMC-pDA-
CFRPEEK/n-HA showed stronger mRNA expression of Runx2, ALP
and OCN than the neat CFRPEEK/n-HA, although the Col1a1
expression showed no difference statistically. At the same time, the
higher level of four genes indicated that osteoblast differentiation
happened ubiquitously and more actively on pep-pDA-CFRPEEK/

Fig. 5 ALP activity assays of the hMSCs cultured on different CFRPEEK/n-HA
surfaces for 7 and 14 days: ALP staining of the bare CFRPEEK/n-HA (a), pDA-
CFRPEEK/n-HA (b), CMC-CFRPEEK/n-HA (c), pep-pDA-CFRPEEK/n-HA (d),
and pep-CMC-CFRPEEK/n-HA (e); and the corresponding quantitative ALP
activity determined from the ALP kit (f). * represents $p < 0.05$ between
groups, and # represents $p < 0.05$ compared with other groups.

Fig. 6 Real-time PCR detection of osteogenesis-related gene expression
of the hMSCs cultured on the samples for 7 days. * represents $p < 0.05$
between groups, and # represents $p < 0.05$ compared with other groups.
The osteogenic differentiation of hMSCs on different CFRPEEK/n-HA substrates was further confirmed by the expression of osteogenesis proteins (Runx2, ALP and Col1a1) through immunofluorescence staining. As shown in Fig. 7, in accordance with real-time PCR results, cells subjected to the BFP peptide were characterized by an enhanced production of the osteo-related proteins. Moreover, Runx-2 (pink), ALP (green) and Col1a1 (green) expression levels in hMSCs were strongly improved on pep-CMC-CFRPEEK/n-HA than those on pep-pDA-CFRPEEK/n-HA at 7 days. Our current data also supported the hypothesis that the combined employment of CMC and BFP peptide up-regulated these osteo-related protein markers, subsequently resulting in the acceleration of the osteogenesis on the composite surface.

The production of calcium deposit at the late stage of differentiation is another critical indicator for osteogenic efficiency of hMSCs. ARS stain which specifically binds to highly enriched calcium deposits is generally employed to confirm the presence of mineralized cells. As the osteogenic culture of hMSCs on different CFRPEEK/nHA substrates progressed to 21 days, cells started to aggregate together and form bone-like structures which were stained for ARS. The hMSCs cultured on the bare CFRPEEK/n-HA surface formed a small number of bone nodules in osteogenic inducing medium as seen in Fig. 8a. Similar to the trend observed for ALP activity, the CMC surface induced denser/larger red staining than bare CFRPEEK/n-HA. The structure of CMC is similar to glycosaminoglycans, and it has been reported that CMC can promote the expression of ECM in human osteoblasts and chondrocytes and stimulate the differentiation of osteoprogenitor cells.39,40 On the other hand, a significant increment in the amount of mineralized matrix was observed due to the covalent conjugation of peptide on the two peptide-decorated CFRPEEK/n-HA substrates. BFP-1 peptide derived from the immature region of BMP-7 protein holds the similar osteogenic inducing property and may bind to the same functional sites (BMP receptors), triggering the up-regulation of target osteogenic genes through Smads or mitogen-activated protein kinase (MAPK).64,65 In addition, it is noteworthy that pep-pDA-CFRPEEK/n-HA facilitated more production of calcium deposition than the CMC-pDA-CFRPEEK/n-HA group, suggesting that growth factors arising from BFP peptide may play a greater role in the osteogenic differentiation of hMSCs than glycosaminoglycans imparted by CMC. In particular, hMSCs on the peptide-decorated CMC-grafted CFRPEEK/n-HA showed the highest production of calcium nodules (red area) in all groups (Fig. 8e and f). These findings reinforced the statement that BFP and CMC exerted an additive effect in promoting osteogenesis on hMSCs, but more studies were needed to elucidate the accurate mechanism of the collaborative effect of CMC and osteoinductive peptide. According to our above results, after the CMC and peptide were co-immobilized on the CFRPEEK/n-HA surface, adhesion, proliferation, and osteoblastic differentiation of hMSCs were all improved. Therefore, in the present study, our developed peptide-conjugated CFRPEEK/n-HA assisted by pDA, particularly pep-CMC-CFRPEEK/n-HA, has endowed poorly bioactive CFRPEEK/n-HA with both outstanding osteo-compatibility and osteogenesis potential in vitro.
3.5. *In vivo* osseointegration and tissue responses

Besides *in vitro* evaluation, *in vivo* tissue response to the carboxymethyl chitosan and peptide-decorated CFRPEEK/n-HA ternary biomaterials, the gold standard for osseointegration, is crucial to the success of our composites as artificial implants. A beagle tibia implantation model was adopted here. The implants were inserted into the cancellous bones of the tibial narrow cavity where hMSCs exist abundantly. New bone formation and implant change after operation were evaluated at prescribed time points using micro-CT. The high resolution micro-CT scans of the defect regions in Fig. 9a clearly exhibited discontinuous parts of the adjacent bone around the implanted bare CFRPEEK/n-HA, and more formation of new bone was found around the peptide-eluted ternary biocomposite compared with the pure CFRPEEK/n-HA counterpart. Moreover, the implanted pep-CMC-CFRPEEK/n-HA biocomposite was thickly surrounded by the natural bone, which showed the trabeculae about 0.5 mm thickness vertical to the longitudinal axis of cylindrical implants.

To assess bone remodeling, the indices of bone histomorphometry from the 3D micro-CT data were further analyzed. Higher trabecular bone volume/tissue volume (BT/TV) was maintained in the pep-CMC-CFRPEEK/n-HA cylindrical implant group than other two implant groups (*p* < 0.05) at 4 weeks after surgery. The trabecular number (Tb.N) and the trabecular thickness (Tb.Th) of the pep-CMC-CFRPEEK/n-HA implant group were also dramatically higher than in the bare CFRPEEK/n-HA implant (*p* < 0.05). In addition, a decrease in trabecular spacing (Tb.Sp) which corresponded to the distance between adjacent trabecula was detected for the pep-CMC-CFRPEEK/n-HA implant (*p* < 0.05). These results all showed that the quantity of newly formed bone in contact with the implants of pep-CMC-CFRPEEK/n-HA was drastically higher than that for the bare CFRPEEK/n-HA group, which was closely correlated with hMSCs’ proliferation and differentiation *in vitro*. Thus, the CFRPEEK/n-HA polymer after immobilization of CMC and BFP not only positively affected the osteointegration between the implant and the bone, but also boosted the bone formation surrounding the implant. Our results provide an unequivocal proof that the pep-CMC-CFRPEEK/n-HA composite benefits the enhancement of *in vivo* bioactivity and ossification.

Fig. 10 shows the tissue responses to the implants after 4 weeks with immunohistochemical/immunofluorescent staining and SEM. In the dyeing image of histotomy, the red area represented the newly formed bone and the dark black area represented the CFRPEEK-based implants. All implants showed direct contact with the newly formed bones, and there was no sign of a fibrous layer which implied the loosening of the implants (Fig. 10a). Moreover, no inflammation or necrosis was observed on the CFRPEEK-based samples, suggesting that the implants did not produce observable toxic effects in the surrounding tissues although a longer time point was necessary prior to clinical acceptance and fathom the healing process. On the other hand, there were more bones found around peptide-decorated CFRPEEK/n-HA implants than CFRPEEK/n-HA control (Fig. 10a), which indicated that the BFP peptide was osteoinductive that could generate more osteoblasts from hMSCs surrounding the implants *in vivo*. At the same time, in the areas where the implants were in contact with native cortical bones, the new bone formed well and could grow along the implants, thus confirming

![Reconstructed 3D models (a) and Micro-CT results (b) of the bare CFRPEEK/n-HA, pep-pDA-CFRPEEK/n-HA, and pep-CMC-CFRPEEK/n-HA implants, after 4 weeks of implantation. * represents *p* < 0.05 between groups.](image1)

![Histological assessment (a), SEM observation (b), and sequential fluorescent labeling (c) of the pristine CFRPEEK/n-HA, pep-pDA-CFRPEEK/n-HA, and pep-CMC-CFRPEEK/n-HA implants. White arrows in SEM images marked the direct contact between implants and new bones. Green and red represented labeling by tetracycline hydrochloride (Te) and Alizarin Red S (Ar), respectively. Imp: implant.](image2)
excellent osteoconductivity on the peptide modified biocomposite surface. Notably, much more newly-formed bone layers were seen around the pep-CMC-CFRPEEK/n-HA implant. This result was verified by SEM images (Fig. 10b), where there was an obvious gap between the bare CFRPEEK/n-HA implant and the neighboring bone. However, the gap disappeared and the new bone bonded to the pep-CMC-CFRPEEK/n-HA implant surface tightly and directly. It is believed that good osseointegration subsequently improves the bonding strength. In the end, sequential fluorochrome labeling has been used to locate the site of new bone formation since it is designed to bind with calcium ions and get incorporated into the site of mineralization. The fluorochrome bone marker labels (tetracycline and Alizarin Red S) were clearly observed for bone tissue bonded to the cylindrical implants (Fig. 10c). At 4 weeks, the CFRPEEK/n-HA implant surface showed the least tetracycline (green) and Alizarin Red S (red). However, most bone deposition and remodeling were found around the pep-CMC-CFRPEEK/n-HA implant, suggesting a greater degree of bone regeneration than other two implants, which showcased the phenominal osteoinductive integration. All in all, sequential fluorescent labeling indicated that peptide decoration stimulated more new bone formation, especially pep-CMC-CFRPEEK/n-HA, consistent with those obtained by the Micro-CT and histological assessments. The bone-implant contact (BIC) was measured from six different specimens of each group and the results are presented in Fig. S2 (ESI†). The BIC index of pep-CMC-CFRPEEK/n-HA was 56.98 ± 4.15% that was significantly larger than that of both pep-pDA-CFRPEEK/n-HA (32.54 ± 2.21%) and bare CFRPEEK/n-HA groups (16.90 ± 4.61%), suggesting that the pep-CMC-CFRPEEK/n-HA induced the best bone-implant contact among the three groups. As a consequence, in vivo tests clearly indicated that our CFRPEEK/n-HA biocomposite after combined decoration of CMC and BFP peptide possessed the superior ability to bond with host bones and significantly boosted osseointegration thereby boding well for application to dental/bone tissue engineering.

4. Conclusion

In summary, the CFRPEEK/n-HA ternary biocomposite was decorated with peptide and CMC via polydopamine chemistry to improve its antibacterial properties and osteogenic activity. The use of CMC resulted in high disinfection efficacy, and of particular importance is the fact that the interaction of BFP peptides with CMC exerted an additive effect on hMSC differentiation towards osteoblast phenotype. Our results demonstrated that cell adhesion, proliferation, and osteoblastic commitment of hMSCs on the carboxymethyl chitosan and peptide-decorated polyether-etherketone were promoted in vitro. In the dog tibia implantation model, faster and better osteointegration could be observed around the pep-CMC-CFRPEEK/n-HA biocomposite implants in vivo. Our findings have paved the way for the CFRPEEK-based biocomposite to be used as a potential bioactive implant material in many challenging orthopedic/dental tissue engineering applications with combined improvement in antibacterial activity and osseointegration.

Acknowledgements

This work was supported by the Strategic Emerging Industry Development Special Funds of Shenzhen [No. JCYJ20130402090759059], Natural Science Foundation of China (Grant 30973317), and Peking University’s 985 Grant.

References
