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Hierarchical Assembly of Nanostructured Coating for siRNA-Based Dual Therapy of Bone Regeneration and Revascularization

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Abstract

Advancing bone implant engineering offers the opportunity to overcome crucial medical challenges and improve clinical outcomes. Although the establishment of a functional vascular network is crucial for bone development, its regeneration inside bone tissue has only received limited attention to date. Herein, we utilize siRNA-decorated particles to engineer a hierarchical nanostructured coating on clinically used titanium implants for the synergistic regeneration of skeletal and vascular tissues. Specifically, an siRNA was designed to target the regulation of cathepsin K and conjugated on nanoparticles. The functionalized nanoparticles were assembled onto the bone implant to form a hierarchical nanostructured coating. By regulating mRNA transcription, the coating significantly promotes cell viability and growth factor release related to vascularization. Moreover, microchip-based experiments demonstrate that the nanostructured coating facilitates macrophage-induced synergy in up-regulation of at least seven bone and vascular growth factors. Ovariectomized rat and comprehensive beagle dog models highlight that this siRNA-integrated nanostructured coating possesses all the key traits of a clinically promising candidate to address the myriad of challenges associated with bone regeneration.

Keywords

Colloidal assembly, Surface functionalization, siRNA, Revascularization, Bone regeneration

1. Introduction

Increases in high-intensity physical activities and overall aging of the population have dramatically increased the incidence of musculoskeletal trauma in recent decades. The related medical and socioeconomic costs of bone-related injuries are therefore also on the rise across different countries [1]. For example, in the United States, over 1 million total hip and knee replacements are performed annually at a cost of over \$25 billion[2]. As such, there is also an increasing need for new and advanced treatments for patients with musculoskeletal diseases such as osteoporosis and bone grafting. Bone implant engineering has been developed as a potential alternative to overcome the critical shortcomings associated with traditionally used autografts and allografts. Although the success rate of the current standard of care in surgical treatments for fractures and joint arthroplasties exceeds 90% at 15 years [2, 3], there is a significant need to further engineer implant function to target the 10% failing as these often involve particularly challenging cases with poor osseointegration such as patients with diabetes and menopausal female patients [3].

Accumulating evidence suggests that the poor osseointegration is attributed to deficient osteogenesis and aggravated osteoclastogenesis. Cathepsin K (CTSK), which is a member of the peptidase C1 protein family, is predominantly expressed in osteoclasts. The encoded protein is a lysosomal cysteine proteinase involved in bone remodelling and resorption [4]. Inhibition of CTSK could effectively inhibit osteoclasts differentiation and increased levels of platelet-derived growth factor (PDGF), stimulating angiogenesis for osteogenesis [5, 6]. To this end, therapeutic manipulation of CTSK expression surrounding the implant should be an ideal therapeutic strategy of improving the implant osseointegration.

The surface functionalization of bone implants has been focused as a potential means for enhancing osseointegration [1, 7], including the approaches of increasing the hydrophilicity, encapsulating osteoprogenitor cells, or releasing biological molecules (growth factors, cytokines, etc.) [8-15]. The assembly of particles into hierarchical structures has recently emerged as a versatile platform for the engineering of functional supraparticles and nanostructured films as superstructures can significantly enhance the interaction on biological-material interface [16-18]. However, there remains few studies focusing to release the nanoparticles from the functionalized implant surface to achieve therapeutic functions. In this study, we have developed a novel hierarchical nanostructure for surface coating of the clinically used titanium implants. The siRNA-CTSK-decorated gold nanoparticles (AuNPs) were assembled on titanium implants through a layer-by-layer (LbL) process with the use of biocompatible natural building blocks, yielding a superstructured multilayer of functionalized nanoparticles. Controlled release of the siRNA-CTSK-decorated AuNPs on the implant-bone interface enables a highly accurate gene silencing process not only for inhibited preosteoclast differentiation but also for stimulated angiogenesis and osteogenesis.

2. Materials and methods

2.1. Surface modification of titanium implant

Titanium disks (15 mm in diameter and 1 mm thick) and titanium implants (1.5 mm in diameter and 5 mm height) of grade 2 commercially pure titanium were prepared by machining (Fullerton Technology Co. Ltd., Beijing, China). After

ultrasonic cleaning, disks were immersed in 10M NaOH and placed in an oil bath maintained at 30 °C for 24 h. The solution in each flask was replaced with ion exchange water (200 ml); this procedure was repeated until the solution reached a conductivity of 5 μ S/cm. The specimens were then dried at room temperature.

2.2. Synthesis of siRNA-integrated nanoparticles

Gold nanoparticle (AuNP, diameter in 10 nm) were purchased from Sigma-Aldrich, USA. For the LbL coating of the nanoparticles, we envisioned a strategy that is illustrated in **Fig. 1**. Briefly, thiolated siRNA was conjugated to gold nanoparticles named AuNP@siRNA-CTSK as described before. Briefly, 50 μ l siRNAs at the concentration of 30 μ mol/L (5' labeled with Cy3 or not, 3' modified with thiol, Ribbio, Guangzhou, China) was dissolved in 450 μ l phosphate saline buffer (PBS, pH = 8.5 adjusted with NaOH) and then processed by DTT to reduce the disulfide bond, followed by purification. Then, aliquot of 250 μ l nucleic acid solution was mixed with gold nanoparticles (10 nm in diameter, Sigma, St Louis, MO, USA) in 1.5 ml citrate buffer and allowed to stand for 1 hour. PBS (0.1M, pH=7.0) was added stepwise in 10 μ l increment with 5 minutes interval until the final concentration of sodium reached 10 mM. NaCl solution was also added stepwise with the same increment and interval to reach a concentration of 100 mM before incubation at 4°C for 16 hours. The oligonucleotide gold nanoparticles conjugate was harvested by centrifuging at 17,000 g for 30min. Polyelectrolyte multilayer coating on AuNP@siRNA-CTSK was performed as follows. Chitosan was dissolved in 1% (v/v) acetic acid to a final concentration of 5 mg/mL. Gelatin (5 mg/mL) was prepared with phosphate buffered saline (PBS, pH = 7.4). AuNP@siRNA-CTSK were suspended in 20 mL chitosan solution and stirred for 15 min at room temperature. Next, the mixtures were centrifuged and washed with distilled water, followed by gelatin coating, forming a (AuNP@siRNA-CTSK)_{cg}. Particle size and Zeta-potential were measured using dynamic light-scattering (DLS) (Malvern ZS90 particle analyzer). The loading capacity was examined by qPCR analysis of the siRNA in the supernatant before and after the loading process. The drug loading efficiency was expressed as the percentage of siRNA loaded and count of siRNA per nanoparticle.

2.3. Engineering of siRNA-integrated nanostructured coating

The titanium disk was initially immersed into PEI solution (5 mg/mL in distilled water) for 20 min. Then, the titanium disks were immersed into gelatin solution 2%HAc/0.15M NaCl and chitosan solution (0.15 M NaCl/PBS), followed by rinsing two times with distilled water for 1 min, until the desired multilayered films of Ti/PEI/(Gel/Chi) 2 /Gel were deposited. Next, (AuNP@siRNA-CTSK)_{cg} (10^8 in 1 ml distilled water) were deposited onto pre-deposited substrates in the same manner for 15 min. Subsequently, and other Gel/(Chi/Gel) 2 layers were coated on (AuNP@siRNA-CTSK)_{cg} layer. The process was then repeated to get multilayer of AuNP on titanium to obtain hierarchical nanostructured coating [(AuNP@siRNA-CTSK)_{cg}]_{CG}.

2.4. Characterizations

The morphologies of the prepared specimens of [(AuNP@siRNA-CTSK)_{cg}]_{CG} functionalized titanium implant were visualized by scanning electron microscopy (SEM; S-4800; Hitachi, Japan). The size of gold nanoparticles was determined by

transmission electron microscopy (TEM; HT770; Hitachi, Japan) and laser particle size analyzer (Mastersizer 3000; Malvern Panalytical; UK). Contact angle measurements were examined using video contact angle measurement system model (K100, Kruss, Germany) at room temperature. Ultra-pure water was used in the contact angle measurements.

2.5. AuNP and siRNA release assay

To evaluate the releasing behaviors of AuNP and the associated siRNA from the titanium, the implants functionalized with [(AuNP@siRNA-CTSK)_{cg}]_{CG} were soaked in 5 mL of DEPC treated phosphate buffer saline (PBS, pH 7.4; supplemented with 1 pmol NC siRNA) solution and placed in a shaker table (100 rpm) at 37 °C. At predetermined time intervals, the solution was taken out and the released AuNP was harvested. The siRNA on the AuNP was isolated by Trizol and reverse transcribed, followed by qPCR analysis of the siRNA. The NC served as the control. The cumulative release of siRNA was calculated.

2.6. Preparation of OVX model and placement of modified implants

Female Sprague Dawley (SD) rats weighing 200~230 g were obtained from the animal center were grown and processed in accordance with the Animal Handling Guideline and approved by the Institutional Animal Care and Use Committee (IACUC) of Capital Medical University, School of Stomatology.

All the animals were housed in an environment with temperature of 22 ± 1 °C, relative humidity of $50 \pm 1\%$ and a light/dark cycle of 12/12 hr, and were placed on standard diet. After 7 days of adaptation to the environment, the experimental group underwent bilateral ovariectomy (OVX), while the SHAM group had the same mass of fat tissue removed. After 12 weeks, the osteoporotic model was established. To confirm the successful establishment of osteoporosis model, 4 rats of either OVX or SHAM group were sacrificed to obtain the femurs for micro-CT scanning (Inveon CT, Siemens) three months post- OVX and SHAM surgeries. The quantitative parameters of volume ratio (BV/TV) were obtained.

For implant surgery, SHAM and OVX SD rats were intraperitoneally injected with 1% pentobarbital (0.4 ml/100 g) and then subjected to local anaesthesia with primacaine (Pierre Rolland, Bordeaux, France). The surgical field was shaved and sterilized with iodophors, and 1.5 cm long incisions were made at the proximal part of the bilateral tibia. Then, perforating planting holes with 1.0 mm in diameter were drilled for placed implants (1.5 mm×5 mm, titanium implants). During hole drilling, iced saline irrigation was applied. Following implant placement, suture and postoperative antiinflammation treatment with gentamycin, animals were kept for 4, 8 or 12 weeks' recovery before further osseointegration strength test, microCT and histology analysis.

The Canine study protocol was also approved by the Institutional Animal Care and Use Committee of Capital Medical University, School of Stomatology. Six healthy female beagle dogs (at the age of 9) with permanent dentition were selected because of their anatomical size and bone remodelling characteristics. After arrival, the animals were quarantined to allow 5 days of adaptation and were randomly selected for each healing period. On the days of surgery, antibiotic prophylaxis (amoxicillin by mouth, 500 mg. twice a day) were initiated 12 hours preoperative and continued 5 days postoperative. Animals were sedated with acepromazine (0.1 mg/kg).

After surgeries, animals were fed a soft diet for 3 days followed by 1 to 2-day soft weaning back to dry food and were monitored at least once every 24 hours throughout the study.

During the first surgical phase, third mandibular premolars were extracted bilaterally using an atraumatic technique to help preserve the alveolar sockets. The wound sites were sutured for closure and extraction sites were allowed to heal for 3 months. Three days before the second phase surgery, fluoroscopy (OEC 9800; GE Medical Systems, Salt Lake City, UT) was used to evaluate extraction site healing.

During the second surgical phase, crestal and vertical releasing incisions were made, and full-thickness flaps were elevated to expose the alveolar bone in the edentulous areas. The osteotomies were prepared without a surgical template in the interalveolar margins between the mandibular extraction sites bilaterally. Control or experimental implants were placed in each dog using a randomized, reversed, or mirrored sequence between each quadrant and between every 2 animals. A total of 12 implants (4 siRNA-NC, 4 siRNA-CTSK and 4 siRNA-MOCK) (5x10 mm) were used in this study.

2.7. Cell culture

Bone marrow cell suspensions were isolated by flushing femurs and tibias of 8- to 12-week-old SD rat with complete RPMI1640 (+10% FCS, +1% Pen/Strep). For mesenchymal cell isolation, the aspirate of bone marrow was cultured for 3 h and nonadherent cells are removed carefully after 3 h and fresh medium is replaced. When primary cultures become almost confluent, the culture is treated with 0.5 ml of 0.25% trypsin containing 0.02% ethylenediaminetetraacetic acid for 2 min at room temperature. A purified population of MSCs at passage 3 were used. For isolation and macrophage differentiation of bone marrow derived monocytes, the aspirate was adjusted to give a suspension of 10^6 cells/mL, and seeded on 6-well ultra-low attachment surface plates in DMEM medium supplemented with 20 ng/mL M-CSF for 1 week.

Adherent cells (differentiated macrophages) were harvested by gentle pipetting and repeated washing of the wells with phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM EDTA to detach adherent cells. For isolation of endothelial progenitor cells, the aspirate was filtered and stained with CD31 and CD34 fluorescence labelled antibodies. CD34⁺ and CD31⁺ cells, which are considered as endothelial progenitor cells, were sorted by FACS.

RAW264.7 macrophages were cultured in DMEM medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% antibiotics (Hyclone, Logan, Utah, USA) in humidified atmosphere of 5% CO₂ at 37°C. The cells were passaged when reaching 90% confluency.

2.8. Real-time quantitative PCR analysis

Total RNAs from cells or tissues were isolated using the Trizol (Invitrogen, Carlsbad, California, USA) and the isolated RNAs were then subjected to reverse transcription using PrimeScript RT Reagent kit (Takara, Dalian, China). Real-time PCR analysis (*qPCR*) was conducted with SYBR Premix Ex Taq II (Takara, Dalian, China) using a CFX96 Real Time System (Bio-Rad, USA). Relative expression was calculated with $2^{-\Delta\Delta Ct}$ method. The referred primer sequences are listed in **Table S1**.

2.9. Removal torque values test

The removal torque values test was conducted immediately after implants together with the tibias were harvested. The tests were performed with a torque meter (Aigu, Hongkong, China) and torque applied on the implants was increased gradually after the samples fixed well. As the strength went up, the adhesion between implants and its host bodies was destroyed. The torque value at the critical moment was recorded as the removal torque values of implant.

2.10. Micro-CT scanning and reconstruction

At the end of experiments, rats or dogs were euthanized and the implant together with the surround bone was excised and fixed in 4% paraformaldehyde. For further imaging examination, a high-resolution micro-CT scanner (Yxlon, Hamburg, Germany) was employed. The specimens were placed vertically into a sample holder and scanned. Region of interest (ROI) was defined as a ring with a 500 μm (for rat) and 1500 μm (for dog) radius starting from the implant surface in the coronal plane. The two- (2-D) and three-dimensional (3-D) views of the implant with the surrounding new bone were reconstructed by Cobra_Exxim Cone Beam Reconstruction Apparatus. Quantitative data of the ROI from images, including BV/TV, Tb.Th, Tb.Sp and Tb.N, were analysed automatically in the Inveon Research Workplace (SIEMENS, Munich, Germany).

2.11. Histology analysis and immunofluorescence microscope analysis

For histological analysis of osteo-implant interface, the specimens without decalcification were embedded in resin and then cut into 50 μm sections. The sections were stained with methylene blue/acid fuchsin. The digital section images were captured with the aid of light microscope (Olympus, Tokyo, Japan). For immunostaining of macrophage in the bone tissue, OCT-embedded bone was decalcified and sectioned, followed by staining with F4/80 primary antibody, and then stained with the secondary antibody (Life Technologies, Carlsbad, CA, USA). Briefly, 10- μm -thick OCT-embedded cryosections were fixed with 4% PFA, permeabilized with 0.2% Triton X100, and blocked with 4% bovine serum albumin. Sections were then incubated with primary antibody overnight (4°C in a humidified chamber) and then washed and incubated with secondary antibody for 1 hour at 37°C. The nuclei were counterstained with DAPI, while the siRNA itself was pre-labeled with Cy3. After these processes, the sections were visualized using a fluorescence microscope.

2.12. Statistical analysis

Data were expressed as mean $\pm$ standard deviation (*SD*). Data were analyzed with one-way ANOVA using SPSS 16.0. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Assembled siRNA-conjugated nanoparticles as nanostructured implant coatings

In addition to the fundamental role of CTSK in osteoclast differentiation [5, 6], CTSK was also found abundantly expressed in preosteoclasts and differentiated

macrophages (**Fig. S1**), while rarely expressed in other cell types in the bone marrow, such as mesenchymal stem cells and endothelial progenitor cells (**Fig. S1**). The highly specific expression profile further suggests that CTSK should be a rational target *in vivo*. To this end, we tried to load the thiolated siRNA against CTSK (siRNA-CTSK) onto the AuNP (**Fig. 1a**). AuNP@siRNA-CTSK showed a $\sim 15\text{-}20$ nm increase in particle diameter compared with bare AuNPs (**Fig. 1b** and **Fig. S2**). The conjugation of siRNA-CTSK on the AuNPs was also supported by the shift of zeta potential towards more negative values (from -3.4 ± 1.7 to -15.3 ± 3.6 mV) (**Table S2**). After layering of the biocompatible and antibacterial chitosan and gelatin (cg), the resultant (AuNP@siRNA-CTSK)_{cg} nanoparticles showed a further increase in hydrodynamic diameter and a more negative charged surface (**Fig. 1b**). In addition, the loading amount of the siRNA on the functionalized AuNP was measured as 35 ± 6 molecules per particle.

The siRNA-decorated gold nanoparticles (AuNPs) were assembled on etched titanium implants to a superstructured multilayer of particles (**Fig. 1c**). Scanning electron microscopy (SEM) images revealed that the etched titanium implant showed features ranging from 0.5 to 10 μm (**Fig. S3**), which facilitated the homogenous and hierarchical deposition of multilayers of (AuNP@siRNA-CTSK)_{cg} (**Fig. 1d**) using the (cg) biopolymers as intermediate layers. Contact angle measurements indicated an increase in hydrophilicity of the [(AuNP@siRNA-CTSK)_{cg}]_{CG}-coated surface compared with the native titanium implant surface (**Fig. S4, S5**), which should promote biological interactions between the implant and bone tissue. The designed hierarchical layered structure of the [(AuNP@siRNA-CTSK)_{cg}]_{CG} coating enabled a long-term and sustainable release of therapeutic siRNA molecules under physiological conditions (**Fig. S6**).

Fig. 1. Particle-based hierarchical nanostructured implant coatings. (a) Facile synthesis of siRNA-CTSK-loaded AuNPs via layer-by-layer assembly of biocompatible and antibacterial chitosan and gelatin (cg) multilayers. (b) TEM images of monodispersed native AuNPs, siRNA-loaded AuNPs, and (AuNP@siRNA-CTSK)_{cg}. Scale bars are 100 nm. (c) Formation of hierarchical nanostructured [(AuNP@siRNA-CTSK)_{cg}]_{CG} coatings via the assembly of (AuNP@siRNA-CTSK)_{cg} on the bone implant surface. (d) SEM images of the titanium implant surface functionalized with [(AuNP@siRNA-CTSK)_{cg}]_{CG} at different magnifications. Scale bars are 2 μm and 200 nm.

The effect of the $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ -coated implant on the proliferation of various cell lines was determined using a CCK-8 assay. The cell viability of macrophage cells (RAW264.7), bone mesenchymal stem cells (MSCs), and vascular endothelial progenitor cell (EPCs) showed negligible differences between bare implants, $(\text{AuNP}@siRNA\text{-}CTSK)_{cg}$, and $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ -coated implants (**Fig. 2a-c**), highlighting the high biocompatibility of $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$. To confirm that the siRNA-CTSK could be released and internalized by preosteoclast cells, macrophage RAW264.7 cells were seeded on the $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ -coated titanium substrate where siRNA-CTSK was pre-labeled with Cy3. **Fig. 2d** shows the cellular internalization of $\text{AuNP}@siRNA\text{-}CTSK$ and strong fluorescence around the nuclei of the attached macrophages (**Fig. 2e**). Real-time PCR analysis of the gene expression of the macrophages indicated that siRNA-CTSK could be efficiently delivered into the cells from the nanostructured implant coating (**Fig. 2f**).

Fig. 2. Biocompatibility of the nanostructured hierarchical coatings and the efficient delivery of siRNA-CTSK to the surrounding tissue in vitro and *In vivo*. Cell viability assay as determined by CCK-8 after incubation with $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ of (a) a macrophage cell line (RAW264.7 cells), (b) cells responsible for bone regeneration (MSC cells), (c) cells related to bone vascular system repair (EPC cells). Bare titanium implant and dispersed $(\text{AuNP}@siRNA\text{-}CTSK)_{cg}$ nanoparticles

were used as controls in (a) – (c). (d) Confocal micrograph of preosteoclast cells cultured on Cy3-labelled [(AuNP@siRNA-CTSK)_{cg}]_{CG}. Cell nuclei were stained with 4',6-diamidino-2-phenylindole. (e) Magnified fluorescence image showing the internalization of AuNP@siRNA-CTSK by tissue around the implant. (f) Real-time PCR analysis showing the PCR cycle threshold of gene expression in the macrophages cultured on the [(AuNP@siRNA-CTSK)_{cg}]_{CG}. (g) *In vivo* setting of [(AuNP@siRNA-CTSK)_{cg}]_{CG}-functionalized titanium implant in the tibia bone of a healthy rat. (h) Confocal micrograph of the tissue surrounding the functionalized implant. The inset image shows the delivery and localization of siRNA-CTSK to the nuclei. F4/80 is green, siRNA-CTSK is red, and the nuclei is blue. Scale bar is 50 μ m. (i,j) Real-time PCR analysis of the cells surrounding the implant indicates the inhibition of mRNA transcription of CTSK and TRAP. (k) Real-time PCR shows the increase of gene expression of vascular-related PDGF-BB. The variation is represented by the standard deviation of four independent replicates in all graphs, * vs siRNA-MOCK, # vs siRNA-NC ($P < 0.05$; MOCK: blank control group, NC: negative control group).

3.2. Biological interaction of nanostructured implant coatings with bone tissues

Next, we explored if the delivery of siRNA-CTSK translates to inhibition of mRNA and an increase of vascular growth factor *in vivo*. Specially, an [(AuNP@siRNA-CTSK)_{cg}]_{CG}-functionalized implant was placed into a tibia bone of a healthy rat to characterize the inhibition of mRNA and the increase of vascular growth factors. The *in vivo* tissue samples were analyzed by an RNA sequence assay (Fig. 2g). Consistent with the *in vitro* experiments, the Cy3-labeled AuNP@siRNA-CTSK on the titanium implant was endocytosed by the surrounding preosteoclast cells, which is supported by the correlation of the dual staining of Cy3-labeled siRNA-CTSK and macrophages labeled by anti-F4/80 antibody (Fig. 2h). Accordingly, the knockdown of CTSK could further inhibit the function of osteoclasts and promote angiogenesis, as seen from the reduced expression of osteoclast marker genes TRAP and CTSK (Fig. 2i, j) (CTSK itself is also a marker for osteoclasts) and through the increased expression of regeneration factor PDGF-BB (Fig. 2k).

RAW264.7 cells are commonly used in the literature to study biological effects on macrophages and osteoclast [19]. To further confirm the effects of [(AuNP@siRNA-CTSK)_{cg}]_{CG} on bone osteogenesis, macrophage RAW264.7-conditioned medium was used to culture MSCs seeded on the functionalized titanium microchips (Fig. 3a). Fig. 3b-f shows that the conditioned medium had a significant promotion of the expression of multiple bone regeneration growth factors, including ALP, COL-1, RUNX2, and OCN in MSCs [8]. In addition, the conditioned medium from cells grown on [(AuNP@siRNA-CTSK)_{cg}]_{CG}-functionalized titanium also dramatically increased VEGF expression and promoted the proliferation of vascular-related EPCs (Fig. 3g-i). We noticed that [(AuNP@siRNA-CTSK)_{cg}]_{CG}-functionalized titanium itself had minimal effects on MSC osteogenesis (Fig. S7) and VEGF expression as well as the proliferation of EPCs (Fig. S8), which is consistent with the low expression of CTSK in these cell types. Together, the above data suggest the crucial role of the surrounding macrophages on the siRNA-based regulation process.

Fig. 3. Microchip-based experiments on macrophage-induced enhanced regulation of bone and vascular growth factors. (a) Schematic of the release of multiple growth factors by the conditioned medium from RAW264.7 treated with different [(AuNP@siRNA-CTSK)_{cg}]CG. (b) Schematic of the osteogenic effect by three major growth factors. (c-f) Specific gene expression of MSCs cultured on titanium surfaces receiving conditioned media, including ALP (c), Col1a (d), OCN (e), and Runx2 (f). Cells were additionally induced by osteogenic medium and harvested for qPCR analysis after 1-, 4-, and 7-day incubation. Relative expression was normalized to GAPDH. (g) Schematic of the angiogenic effect induced by the knockdown of CTSK involving the increase of key vascular growth factors. (h) VEGF expression in vascular EPCs cultured on titanium surfaces receiving the indicated conditioned medium. Relative expression was normalized to GAPDH and expressed as mean $\pm$ standard deviation. (i) EPC growth on titanium surfaces receiving the indicated conditioned medium. The variation is represented by the standard deviation of three independent replicates in all graphs. *, vs siRNA-MOCK, #, vs siRNA-NC ($P < 0.05$).

3.3. Promoted bone regeneration and revascularization by nanostructured implant coatings *in vivo*

We then examined the comprehensive therapeutic effects of [(AuNP@siRNA-CTSK)_{cg}]CG-functionalized implant for bone regeneration *in vivo*. Ovariectomized (OVX) rat model was commonly used to mimic the corresponding bone conditions to menopausal women [20]. As expected, osteoporosis was observed in the OVX rats (Fig. 4a,b, and Fig. S7). Then, native and functionalized titanium implants were placed in both control and OVX rat tibia. At different healing phases, the implant samples combined with the intact tibia were harvested, and the osseointegration condition was examined by critical removal torque assay, micro-CT scanning

followed by 3D reconstruction, and tissue histology. There were much less trabeculae covered the control implant in the OVX mice compared with the SHAM mice (**Fig. S9**). However, the implants functionalized with $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ were covered by more trabeculae of different sizes in the OVX group (**Fig. 4c, S9**). Notably, there was no significant increase in the trabecular bone volume and thickness in the whole tibia of the OVX mice. After hard tissue sectioning and processing, methylene blue/acid fuchsin staining was applied to provide more accurate and detailed features regarding the osseo-implant interface. The newly formed bone surrounding the titanium implant was clearly visible (**Fig. 4c, S10, S11**). Consistent with the micro-CT results, more bone structure was observed on implants functionalized with $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ in comparison with the controls in the OVX group. The improved implant trabeculae coverage was further confirmed by the qualitative analysis of the bone tissue fraction, trabecular number, and trabecular spacing of the new bone around the implant (**Fig. 4d,e, and Fig. S12**). Confocal microscopy images showed the vascular growth conditions of surrounding tissues near the implants. **Fig. 4f** demonstrated that $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ coating significantly promoted the growth of vascular systems in the new bone which was generally recognized as challenges in previous studies. The normalized fluorescence intensities of the signals of vascular systems further show the therapeutic outcome of our $[(\text{AuNP}@siRNA\text{-}CTSK)_{cg}]_{CG}$ coating on the implant. Moreover, functionalized titanium implant showed significantly enhanced mechanical properties than the control groups (**Fig. S13**). These enhanced effects followed the same pattern in the both control and OVX group (**Fig. S13**).

Fig. 4. *In vivo* comprehensive therapeutic effects of titanium implants with nanostructured coatings on an osteoporosis rat model. (a) Schematic of implantation and release of siRNA-CTSK for bone regeneration. (b) Micro-CT image of the control and OVX rat. Osteoporosis was observed in an OVX rat. (c) Formation of new bone around the titanium implants based on 3-D reconstructed views of the micro-CT analysis 4- and 8-weeks post-implantation. The light blue color indicates osteoids. Histology of the interface between the bone and the modified implants and

removal torque value for the implant samples. Representative images of methylene blue/acid fuchsin staining showing that the new bone (blue) formed around the titanium implant (black) after 4- and 8-week implantation. NB: new bone, MB: mineralized bone, OS: osteoid. The scale bars are 50 μm . **(d)** The quantitative data of bone volume/total volume (BV/TV) percentage obtained from the micro-CT analysis. **(e)** The quantitative data of trabecular thickness (Tb.Th.) obtained from the micro-CT analysis. The individual data was plotted as against bar graphs. **(f)** Confocal microscopy images of surrounding tissue near the coated implants. 4',6-diamidino-2-phenylindole (DAPI) stain (blue) represents the cell nuclei and anti-CD31 antibody conjugated with Alexa Fluor 647 were used to stain the generation of vascular structures. **(g)** Normalized anti-CD31 antibody fluorescence intensity between the implants used with different siRNA sequences. The variation is represented by the standard deviation of four independent replicates (d, e) and three independent replicates (g), * vs siRNA-MOCK, # vs siRNA-NC, & vs SHAM, & vs OVX-based siRNA-MOCK, \$ vs OVX-based siRNA-NC, @ vs OVX-based siRNA-CTSK ($P < 0.05$).

3.4. Application of nanostructured implant coatings in an aged beagle dog model

Canine study is also commonly used to study the bone formation surrounding the implant [21]. To further confirm the findings revealed above, an old beagle dog model was established which can be relevant to aged dental implant patients (**Fig. 5**). Three months after extraction surgery (**Fig. S14**), [(AuNP@siRNA-MOCK)_{cg}]_{CG}, [(AuNP@siRNA-NC)_{cg}]_{CG}, and [(AuNP@siRNA-CTSK)_{cg}]_{CG} implants were placed in tooth extraction sockets (**Fig. 5a,b**). Consistent with the findings in the OVX rat model, the newly formed bone in the dogs showed compromised therapeutic effect with the use of siRNA-MOCK and siRNA-NC. A minimal level of new bone formation around the implants could be observed in the [(AuNP@siRNA-MOCK)_{cg}]_{CG}- and [(AuNP@siRNA-NC)_{cg}]_{CG}-functionalized implants (**Fig. 5c**). While the [(AuNP@siRNA-CTSK)_{cg}]_{CG}-functionalized implant showed obvious trabeculae. The rat and beagle dog models both confirm that the implants functionalized with [(AuNP@siRNA-CTSK)_{cg}]_{CG} produce significant beneficial effects on the bone regeneration in a wide range of clinical parameters. Together, these data suggest that the siRNA-based hierarchical nanostructured implant coating could be a promising treatment for challenging bone regeneration, such as for implant therapy in aged people and menopausal women.

Fig. 5. Comprehensive beagle dog model revealed the application of the siRNA-CTSK loaded hierarchical nanostructured coating on dental implant and bone regeneration. (a) Extraction of fourth premolar from both sides of a beagle dog. The [(AuNP@siRNA-MOCK)_{cg}]_{cg}, [(AuNP@siRNA-NC)_{cg}]_{cg}, and [(AuNP@siRNA-CTSK)_{cg}]_{cg}-functionalized titanium implants were placed into the tooth extraction sockets. (b) Micro-CT images of dog maxillary bone three months after implant insertion. (c) Formation of new bone around the titanium implants based on 2-D images and 3-D reconstructed views of the micro-CT analysis in 12-week post-implantation. The bright green area represents the growth of new bone around the implants. (d) The quantitative data of the newly formed bone around the implants in the dog model as obtained from the micro-CT analysis, including BV/TV, Tb.Th., Tb.N. (trabecular number), and Tb.Sp. (trabecular separation). The variation is represented by the standard deviation of four independent replicates in all graphs (n = 4), **P* < 0.05 vs corresponding siRNA-MOCK; #*P* < 0.05 vs corresponding siRNA-NC.

4. Discussion

Bone defect or teeth loss patients who need implantation therapy, usually have been complicated with other diseases or functional deficiency, such as osteoporosis and diabetes mellitus, which is especially prevalent in menopausal women and the aged [22-25]. The discovery of the common targeting cells/genes/pathways responsible for the deficiency or capable to reverse the deficiency would certainly be therapeutic desired. In this study, we have developed a novel hierarchical nanostructured coating on the metallic surface of implants. In this system, siRNA-CTSK-decorated gold

nanoparticles (siRNA-CTSK@AuNPs) were assembled on titanium implants, yielding a superstructured multilayer of particles. The inhibition of CTSK through the controlled release of the siRNA-CTSK enables a highly accurate gene silencing process and an efficient bone regeneration. *In vivo* studies based on rats and dogs demonstrated that the nanostructured implant coating showed an osseointegration effect and enhanced angiogenesis of the vascular system on the osteoporosis and dental implant models. This particle-assembled coating strategy holds great promise in future dental implant and bone regeneration applications.

Fig. 6. The delivery of siRNA for synergistic therapeutic effects in vascularization and bone regeneration. (a,b) Schematic illustration of the musculoskeletal system and siRNA-loaded particles interacting with the repairing bone tissue. (c) Localized release of siRNA-CTSK enables the establishment of a functional vascular system during bone regeneration. (d) [(AuNP@siRNA-CTSK)_{cg}]_{CG} can interact with the surrounding biological environment and effectively facilitate synergistic therapeutic revascularization and bone regeneration through multiple growth factors (green boxes).

Therapeutic siRNAs are of priority not only due to its small size, but also for its great specificity. In terms of siRNA delivery, although various RNA carriers have been developed and applied under experimental conditions, including liposomes, exosomes, and gold nanoparticles [26-29]. The assembly of particles into hierarchical structures has recently emerged as a versatile platform for the engineering of functional supraparticles and nanostructured films as superstructures can significantly enhance the interaction on biological-material interface [16-18]. We recently developed a natural polyphenol-based particle assembly method to superstructure various nanoscale building blocks into complex hierarchical structures and inorganic-biological hybrids for various applications [30-33]. However, there were few studies applying these particle-assembled nanostructured coatings as therapeutic delivery platforms in bone implants. Previous study has tried to load the chitosan/hyaluronic

acid nanoparticles to the microarc oxidation (MAO)-treated titanium implant surface by a gel-based coating method for the purpose of releasing functional nanoparticles [34]. However, the study had no demonstration *in vivo*, which might be explained by the theoretical burst release of the nanoparticles. LbL technique has been widely used for nanoparticle fabrication or surface coating [35]. In this study, we combined the advantages of LbL and siRNA-AuNPs for the engineering of therapeutic implant coating for bone regeneration (**Fig. 6a**). We assembled the siRNA-CTSK@AuNPs on clinically used titanium implants through a highly biocompatible LbL technique. The hierarchical structure of the [(AuNP@siRNA-CTSK)_{cg}]_{CG} implant coating provides a unique multi-scaled topography on the bio-nano interface between bone tissue and implant (**Fig. 6b**). The controlled release of siRNA-CTSK mainly relies on the gradual degradation of biocompatible building blocks constructed in the multiple coating layers in the context of bone remodeling. These results suggest that the designed strategy could be a promising method for releasing functional siRNAs from the implant (**Fig. 6c**).

The augmentation of the osteogenic capacity *in vivo* is greatly desired in clinic, while previous studies focused on the function of mesenchymal stem cells (MSCs) and osteoclasts, which have been viewed as particularly promising therapeutic targets [36-43]. For example, topical delivery of siRNA can be used to promote MSC differentiation [44, 45]. Osteoclast, which is one of the key cell types which function importantly in the osteogenesis and bone remodeling [46], have been found overactivated and the excessive osteoclasts have been considered as the main cause for osteoporosis, and poor bone regeneration, in many conditions. Therapeutically blocking the function of osteoclast is now commonly used for osteoporosis management. In addition, the limitation in revascularization has significantly restrained the therapeutic effects of treatments for bone-related injuries [47-49]. The incorporation of cues to augment the vascularization response by means of a highly accurate gene silencing process could greatly enhance the efficacy of bone implants while diminishing their limitations [50, 51]. Compromised bone repair and regeneration in many patients can be attributed to the impaired blood supply and unhealthy vascular system recovery; thus, modulation of VEGF levels in bones represents a potential strategy for treating compromised bone repair and improving bone regeneration [50]. These numerous observations highlight the critical need to develop a novel therapeutic implant coating that can achieve efficient delivery of siRNA with the synergistic goals of revascularization and bone regeneration [52]. In this study, we additionally identified that preosteoclast, the precursor of osteoclast, should be a cell target of great advantages (**Fig. 6d**). As a precursor of osteoclast, the preosteoclast cells display great ability to taken up nanoparticles [53-55]. Thus, nanoparticle mediated drug delivery would be preferentially endocytosed by those cells and thus the toxic effects and side-effects would be minimized. On the other hand, our study here confirmed that targeting the preosteoclast could coordinate the processes of osteoclastogenesis, osteogenesis, and angiogenesis through a synergistic strategy.

In the study, we have identified that CTSK is one of the ideal targets for the improvements of osteogenesis and osteointegration. Inhibition of CTSK effectively increases preosteoclasts and the levels of PDGF-BB, stimulating angiogenesis for osteogenesis [56]. Namely, CTSK orchestrate the osteogenesis, osteoclastogenesis, and angiogenesis by regulating the CTSK-PDGF-BB-bone remodeling pathways [5, 6, 56]. By local delivery of siRNA-CTSK, our study here has identified that CTSK

should be such a candidate. Moreover, our study here revealed that CTSK is predominantly expressed in pre-osteoclasts and osteoclasts in the bone marrow, further suggesting the advantages of the target in terms of off-target effects. Together, our study suggests that therapeutically targeting CTSK would be an attractive, now proven method for the bone regeneration and corresponding revascularization.

5. Conclusions

In summary, we have developed and characterized a hierarchical nanostructured coating through the assembly of siRNA-loaded nanoparticles on a metallic implant. This [(AuNP@siRNA-CTSK)_{cg}]_{CG} coating system provides substantial advantages for the efficient localized release of siRNA and enhanced bone-implant interfacial interaction. The current study has revealed that CTSK is a suitable target to increase osteogenesis in the context of post-menopause and aged subjects. siRNA-CTSK targeting cathepsin K could be released from the nanostructured coating and internalized by the adjacent macrophages, demonstrating a therapeutic improvement on the osteointegration with synergetic effects on bone regeneration and blood vessel system repair. [(AuNP@siRNA-CTSK)_{cg}]_{CG} effectively promoted multiple osteogenesis-related gene expressions in a microchip-based experimental setting. Our results showed that the nanostructured coating efficiently enhances the key vascular regeneration factors. Similarly, *in vivo* results based on an OVX rat model revealed that the nanostructured coating dramatically increased osseointegration effects with enhanced angiogenesis of blood vessels integrated with the regenerated bone. A more comprehensive beagle dog model demonstrated dental bone recovery promoted by the functionalized premolar implant. Compared with conventional method to directly affect mesenchymal stem cells and endothelial cells, our results showed that macrophages are also an appropriate target for enhanced osteogenesis and angiogenesis, through indirect but more effective ways. Furthermore, the use of multi-scaled bio-functional coatings as presented herein may find applications in regenerative medicine for other tissue due to their ability to favor complex stem cell differentiation paths.

Data availability

All of the data reported in this work as available upon request.

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Supplementary data

Supplementary data including detailed experimental information as well as supplementary figures and tables to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2019>.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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