



An in vivo study on the effect of coating stability on osteointegration performance of collagen/hyaluronic acid multilayer modified titanium implants



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ABSTRACT

Aseptic loosening of implant is one of the main causes of Ti-based implant failure. In our previous work, a novel stable collagen/hyaluronic acid (Col/HA) multilayer modified titanium coatings (TCs) was developed by layer-by-layer (LBL) covalent immobilization technique, which showed enhanced biological properties compared with TCs that were physically absorbed with Col/HA multilayer *in vitro*. In this study, a rabbit model with femur condyle defect was employed to compare the osteointegration performance of them. Results indicated that Col/HA multilayer with favourable stability could better facilitate osteogenesis around implants and bone-implant contact. The Col/HA multilayer covalent-immobilized TC may reduce aseptic loosening of implant.

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1. Introduction

In the joint replacement surgery, aseptic loosening of implant is one of the main causes of implant failure [1]. Although plasma-sprayed titanium coatings with rough surfaces and macroporous structures possess good mechanical properties and biocompatibility, it does not have bone-inductive potential and cannot induce bone-bonding with living bone tissue because of bioinert, which may cause aseptic loosening [2–4].

Lots of studies have indicated that the desirable method to reduce aseptic loosening is ameliorating bioinert of Ti-based implant through surface modification [5–7]. Among various surface modification technologies, biochemical methods, by immobilizing the main components of the extracellular matrix (ECM), enzymes or peptides on biomaterials surfaces, have shown great potential to induce specific cell responses and reinforce the tissue-implant interface [8]. Approaches for immobilizing ECM components onto the surface of Ti-based implants include adsorptive immobilization, covalent immobilization and layer-by-

layer (LBL) self-assembly technique [9–11]. The mechanism of the LBL technique involves two kinds of oppositely charged polyelectrolytes that are alternately absorbed on the material surface by electrostatic interaction, and finally form a polyelectrolyte multilayer (PEM) film [12]. However, the multilayer film formed by electrostatic interaction is unstable in physiological conditions, which may affect function of PEM film [13].

In our previous study [14], a novel stable collagen/hyaluronic acid multilayer modified titanium coating was developed by LBL covalent immobilization technique, and compared with another PEM film modified TCs prepared by LBL technique on physical properties and biological performance *in vitro* study. Results showed that there was no significant difference in the composition and surface morphology. *In vitro*, the Col/HA multilayer covalently immobilized TCs showed excellent biological properties and promoted hMSC attachment, spreading, proliferation and differentiation compared with the Col/HA multilayer absorbed TCs obtained by the traditional method. However, the *in vivo* effect of Col/HA multilayer stability on osteointegration performance of multilayer modified titanium coatings should be further investigated.

In this study, a rabbit model with femur condyle defect was prepared. Micro X-ray computed tomography (Micro-CT), scanning electron microscopy (SEM) and histological examinations were

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employed to compare the osteointegration performance of Col/HA multilayer covalently immobilized TCs to that of TCs that were physically absorbed with Col/HA multilayer.

2. Materials and methods

2.1. Materials

Porous Ti coatings on Ti-6Al-4V substrates (TCs, Φ 2.6 mm \times 10 mm of rods) were fabricated by vacuum plasma spraying (F4-VB, Sulzer Metco, Switzerland) as in previous literature [15]. Type I collagen (Col) from calf skin was obtained from Sigma-Aldrich, China. Hyaluronic acid (HA) was purchased from Bloomage Freda Biopharm CO., LTD., China. Silane-coupling agent aminopropyltriethoxysilane (APS) and N-hydroxysuccinimide (NHS) were obtained from Shanghai Sinopharm Chemical Reagent Corp., China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was produced by Tokyo Chemical Industry Co. LTD., Japan.

2.2. Preparation of Col/HA multilayer modified titanium coatings

The two kinds of Col/HA multilayer modified titanium coatings were prepared as previously described [14]. Briefly, TC samples were immersed in 5 M NaOH at 80 °C for 12 h. After dipped in deionized water at 60 °C for 7 days with the water changed daily, the samples were dried under vacuum. The alkali-treated TCs were denoted as TC-A.

TC-A samples were dipped into the 1 mg/ml Col/5 mM acetic acid solution for 30 min, rinsed with deionized water and then soaked into the 1 mg/ml HA/5 mM acetic acid solution for 30 min, followed by rinsing with deionized water. The cycle was repeated six times. After the final assembly cycle, the samples were ultrasonically cleaned in deionized water and dried under vacuum, these samples were denoted as TC-A(C/H)₆ (see Fig. 1).

TC-A samples were immersed in a boiling APS/toluene solution (APS concentration of 10%) for 12 h. The APS-coated samples were ultrasonically washed once in methanol and twice in deionized water, and then dried prior to further modification. The APS coated samples were denoted as TC-AA. Then, TC-AA samples were dipped alternately into the Col solution and HA solution for 30 min, both included 2.5 mg/ml EDC and 0.63 mg/ml NHS. Each dipping process was followed by rinsing with deionized water. After the cycle repeated six times, the samples were ultrasonically cleaned with deionized water and dried under vacuum; the Col/HA PEM covalently immobilized TCs were denoted as TC-AA(C/H)₆ (see Fig. 2).

2.3. In vivo study

2.3.1. Surgical procedure

A rabbit model with femur condyle defect was used to compare the in vivo function of the two kinds Col/HA multilayer modified titanium coatings. Eighteen adult white New Zealand rabbits were obtained from Laboratory Animal Center of Shanghai Ninth People's Hospital (male, 2–2.5 kg body weight). The use of animals and the experimental protocol were approved by the Animal Experimental Ethics Committee of Ninth People Hospital, School of Medicine, Shanghai Jiao Tong University. And the surgery was performed according to the Guide of the Care and Use of Laboratory Animals published by the National Academy of Sciences. As described as Fig. 3, rabbits were anesthetized by injecting 3% Nembutal (30 mg/kg) via the ear vein and a longitudinal incision was made by scalpel in the rabbit femur under rigorous aseptic conditions. Circular holes with 2.9 mm diameter and 10 mm deep were drilled using a surgical electronic drill and thoroughly rinsed with physiological saline to remove shards of bone. Implants of TC, TC-A(C/H)₆ and TC-AA(C/H)₆ after sterilized were used in this study. The wound was sutured with nylon thread. Rabbits were euthanized 3 months after implantation.

2.3.2. Micro X-ray computed tomography analysis

After sacrifice, distal femurs with implants were collected under aseptic condition, fixed in 4% paraformaldehyde for 2 days, and then rinsed with running water for 24 h. For assessment of the bone architectural around different implants after 3 months of implantation, femur condyles with implants were examined using a desktop micro-CT (GE Locus SP), equipped with an 80 kV X-ray source with camera pixel size of 15 μ m. During scanning, the femur condyles were placed in polyethylene tubes filled with alcohol 75 vol%. Micro-CT images along the midsagittal plane in the region around implant were obtained. The scans resulted in reconstructed data sets with a voxel size of 28.79 μ m. To determine the trabecular volume of interest (VOI) in the axial direction, the region of interest was chosen with its closest edge at 3.5 mm distally from the growth plate. The bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were calculated as measurements of trabecular bone mass and its distribution.

2.3.3. Scanning electron microscopy of modified titanium coatings-bone interface

After Micro-CT analysis, specimens were subsequently dehydrated through a series of graded ethanol solution (50%, 60%, 70%, 80%, 90%, and 100%), and embedded in polymethylmethacrylate resin. Undecalcified sections with a thickness of 200 μ m were cut using a saw microtome (Leica SP 1600, Germany). The specimens

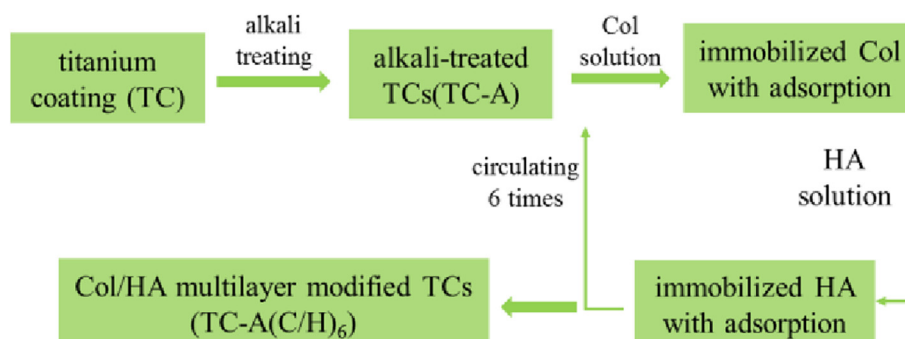


Fig. 1. The fabrication process of Col/HA multilayer modified TCs.

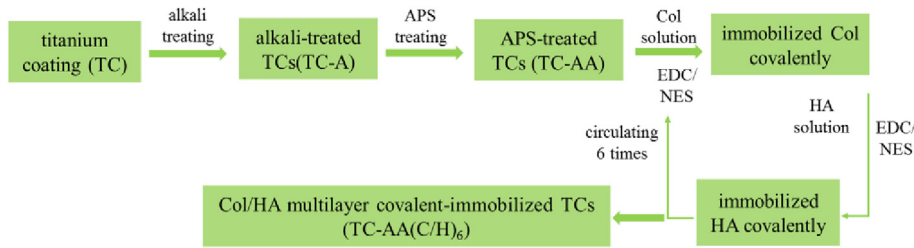


Fig. 2. The fabrication process of Col/HA multilayer covalent-immobilized TCs.



Fig. 3. Main surgical procedure of rabbit model with femur condyle defect. a: a longitudinal incision was made by scalpel in the rabbit femur; b: Circular holes with 2.9 mm diameter and 10 mm deep were drilled using a surgical electronic drill; c: materials were implanted into the holes.

were then sputter-coated with gold and the interface between bone cement and bone tissue was observed using a scanning electron microscope (SEM, JEOL JSM-6700F, Japan).

2.3.4. Histological examinations

Sections were prepared as described above. The thickness of sections was polished to 50 μm using P300, P800 and P1200 abrasive paper, and then burnished with abrasum. After dipped in 1% methane acid solution for 3 min and 20% methanol solution for 2 h, burnished specimens were stained with Van Gieson's picro fuchsin, and then observed by laser scanning confocal fluorescence microscopy (Leica TCS.SP5).

2.4. Statistical analysis

The data were expressed as the mean \pm standard deviation (SD) for all the experiments ($n = 5$) and statistical differences were determined by an analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Micro-CT image analysis

Fig. 4 shows the micro-CT images along the midsagittal planes in the region around implants after 3 months of implantation.

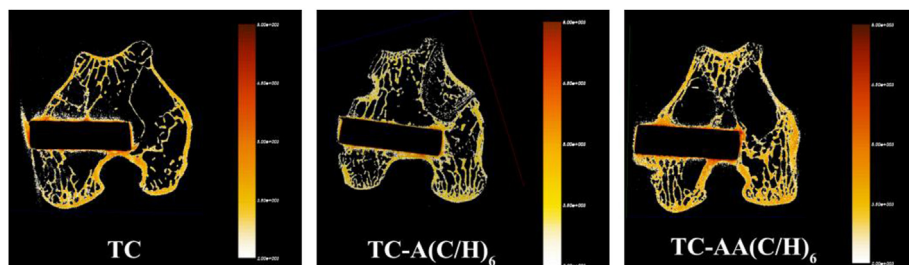


Fig. 4. Micro-CT images along the midsagittal planes in the region around implants after 3 months of implantation.

Trabecular bone around the biomacromolecule modified TCs (TC-A(C/H)₆ and TC-AA(C/H)₆) was more than that around non-modified TCs, according with our previous studies described [16,17]. However, what is interesting is that trabecular bone around TC-AA(C/H)₆ was more abundant than that around TC-A(C/H)₆. The results indicated that the stability of Col/HA multilayer could promote osteogenesis around implants.

To further investigate the growth of trabecular bone, the four parameters of trabecular volume of interest (VOI, Φ 3.5 mm) in the axial direction were studied. The BV/TV, Tb.Th, Tb.N of TC-AA(C/H)₆ were $9.805 \pm 0.481\%$, 0.0561 ± 0.0029 mm, and 1.746 ± 0.061 mm⁻¹ respectively. While BV/TV, Tb.Th, Tb.N of TC-A(C/H)₆ were $8.255 \pm 0.415\%$, 0.0514 ± 0.0021 mm, and 1.607 ± 0.049 mm⁻¹, all of which were significantly lower than those of TC-AA(C/H)₆. The results displayed that the trabecular bone around TC-AA(C/H)₆ grew better than that around TC-A(C/H)₆, which may be attribute to the favorable stability of Col/HA multilayer on TC-AA(C/H)₆ (see Fig. 5).

3.2. Scanning electron microscopy of material-bone interface

The excellent combination between new bone and the surface of material is very important to implant stability. To compare the osseointegration on the surface of TC-A(C/H)₆ and TC-AA(C/H)₆, SEM was used to observe the material-bone interface, results were showed as Fig. 4. Because of bioinert of Ti, there was few new bone

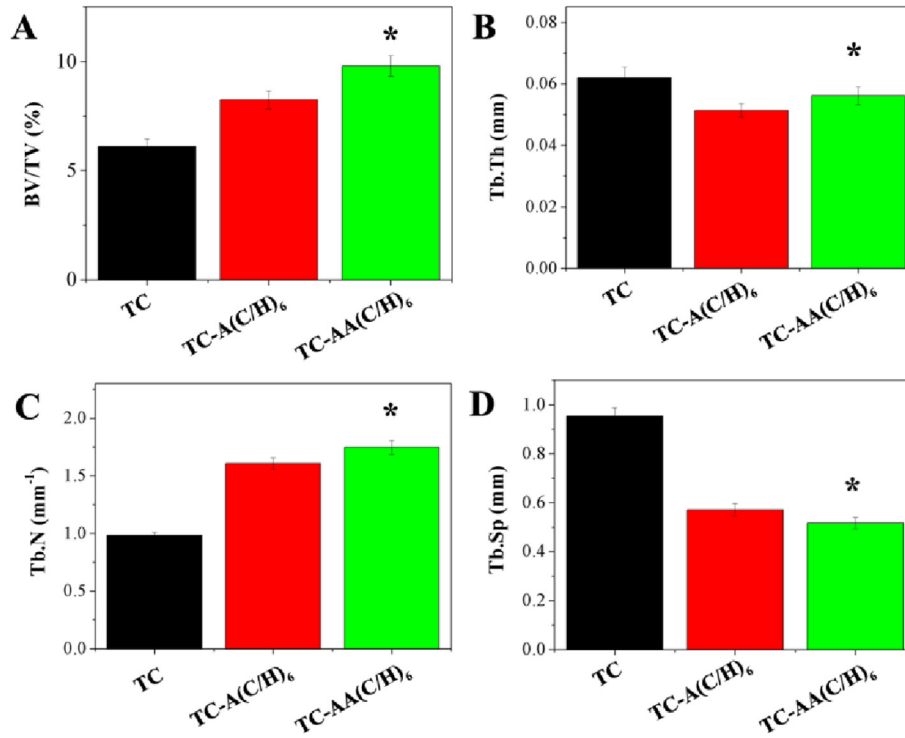


Fig. 5. The four kinds of microstructural parameters of the trabecular volume of interest (VOI) in the axial direction: BV/TV (A), Tb.Th (B), Tb.N (C), and Tb.Sp (D). * $p < 0.05$ denotes differences compared between TC-AA(C/H)₆ and TC-A(C/H)₆.

contacting with TC implant but lot of gap (deep colour) between bone tissue (light colour, with many bone whirlpools in it) and TC implant (Fig. 6(a1) and (b1)), although TC implant was surround by bone tissue. Lot of new bone contacted with TC-A(C/H)₆ implant (Fig. 6(a2)). However, after observed carefully, much gap also can be found between bone tissue and TC-A(C/H)₆ implant (Fig. 4(b2)). By contrast, there was no gap between bone tissue and TC-AA(C/H)₆ implants. New bone grew into the hole of TC-AA(C/H)₆ implants and contacted closely with the surface of implants (Fig. 6(a3) and (b3)). The results indicated that TC-AA(C/H)₆ implants with favourable stability possess better osseointegration compared with TC-A(C/H)₆ implants.

3.3. Histological examination

Fig. 7 shows the histological appearance of the implant and bone after 3 months of implantation. Although compared with TC implant, new bone could come into the porous structure of TC-A(C/H)₆ implant, there had some gap between bone tissue and TC-A(C/H)₆ implant. Contrarily, new bone contacted with TC-AA(C/H)₆ implant closely and no gap between them. The result of histological examination was consistent with that of SEM, and confirmed that the favourable stability of multilayer could improve the osseointegration of multilayer modified titanium coating implant.

The morphology and chemical composition of surface is very important to osseointegration of implant. Biochemical modification

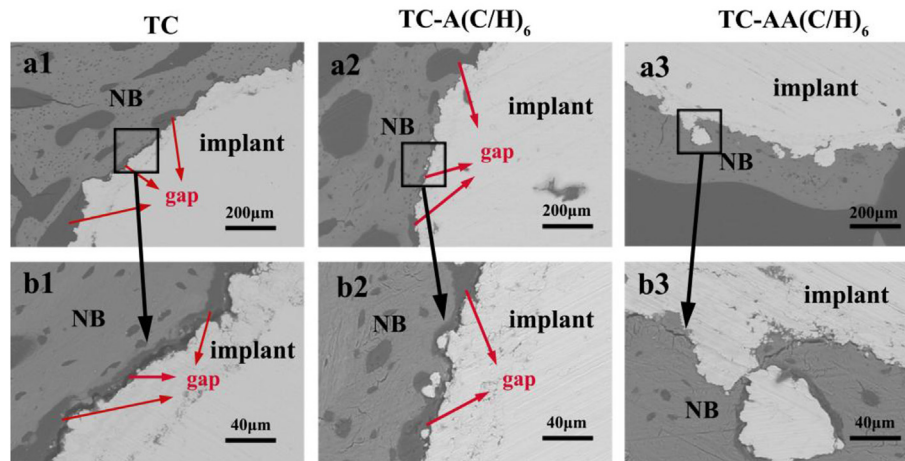


Fig. 6. Scanning electron micrograph of the interface between implants and bone tissue in different groups. The gap with deep colour is the red arrow pointed. A: $\times 100$ magnification; B: $\times 500$ magnification. NB: new bone.



Fig. 7. Histological morphology of the interface between implants and bone tissue after 3 months implantation in rabbit femur condyles.

could change the chemical composition of implant surface through introducing biomacromolecule, such as collagen I and hyaluronic acid, which possess biological properties and improve cell attachment, spreading, proliferation and differentiation. However, because electrostatic interaction is not unstable, the Col/HA multilayer absorbed on TCs was easy to be desorbed in vivo. Meanwhile, the chemical composition of implant surface changed, which led to reduced biological properties of TCs. After implantation, the Col/HA multilayer by covalent immobilization was stable and could maintain the bioactive surface. Therefore, the Col/HA multilayer covalently immobilized TC implants showed favourable osseointegration performance. New bone could grow on the surface of implants and connected with them closely, which may reduce aseptic loosening of implants.

4. Conclusions

In this study, osseointegration of two kinds of Col/HA multilayer modified TCs were compared through in vivo study. The findings indicated that stability of Col/HA multilayer was critical to the osteogenesis, osteoinduction and osseointegration of Col/HA multilayer modified TC implants. In addition, the Col/HA multilayer covalently immobilized TCs may reduce aseptic loosening of implants.

Acknowledgements

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