The Effect of Collagen Coating on Surface Biocompatibility of the Titanium Alloys

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ABSTRACT

Titanium alloys coated with collagen have been widely used to improve their biocompatibility. In this study, the titanium alloys were coated with type I collagen (Col I) and type II collagen (Col II), respectively. The coating amount was quantified with HPLC/MS. Their biocompatibility was evaluated and the effects of collagen types on cell differentiation were compared by cellular proteins identification and quantification. The quantity of Col I and Col II coated on titanium alloy were 0.813 ± 0.015 mg and 0.774 ± 0.013 mg, respectively. Mouse Col I and fibronectin were abundantly expressed within Col I group. The most abundant proteins in Col II group were osteopontin and prelamin-A/C. In conclusion, the

collagen coating could improve the biocompatibility of titanium alloy materials, and different types of collagen could induce cell to express proteins with various functions.

KEYWORDS

Titanium alloy, collagen coating, surface biocompatibility, cell differentiation, HPLC/MS

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1 INTRODUCTION

Titanium alloys are one kind of natural superplastic materials and have been widely used for replacing hard tissues, such as the bone, teeth and orthopedic implants due to their superior physical, mechanical and biological properties [1-3]. Titanium alloys are far

superior to other metals or alloys in resistance to corrosion. They have light weight and superior tribological properties, and have become an important candidate in cardiovascular, orthopaedic and dental application [4-5]. The native titanium alloy surfaces are covered by oxide films which compromise their biological properties [6]. Surface modification is the effective mean to change the alloying surface properties [7-8]. In order to improve the bioactivity and biocompatibility of the titanium alloy surfaces, the physical, chemical, biological and physicochemical methods were investigated [8]. Biochemical approach could result the increased surface features, however, most biomolecules had no affinity to the titanium alloys [9-12]. Coupling agents are required to bind with titanium alloy surfaces and biomolecules. Silanes, carboxylates and phosphates are usually used for this purpose [13-15]. As one of the most widely used crosslinkers, silane can provide stable covalent linkage between titanium alloy surfaces and proteins or other biopolymers [15-19].

The proteins, such as collagen, fibronectin, laminin, RGD peptide etc, coupled on the titanium alloy surfaces are the main determinant of the cytocompatibility, and they play an important role in hard tissue repair [20-22]. Collagen is the main component of the extracellular matrix. Due to its low antigenicity, good biocompatibility, biodegradability and non-toxicity, it has been widely used in tissue engineering [23-25]. It can induce cellular processes such as signaling, differentiation, migration, and apoptosis [26]. The arginine-glycine-aspartate (RGD) amino acid sequence in collagen can promote cell growth, attachment, and interact with integrin [27]. Type I collagen (Col I) is widely present in bone, skin, tendon, ligament and so on. Type II collagen (Col II) is present in cartilage and vitreous humor [28]. Col I or Col II products are used for bone grafting and bone filling [29]. Col I has been exploited to enhance osteoblast adhesion and differentiation in vitro, and in vivo osseointegration of titanium alloys [30-32]. Nagai reported that Ti6Al4V alloy surfaces coated with Col I could promote fibroblast adhesion [33]. Kado proved that Col I coated titanium surfaces improved the adhesion and spreading of human periodontal ligament cells significantly [34]. Col II is more beneficial to chondrocyte phenotype formation and structure maintenance than Col I [35-38]. However, the difference comparison of Col I and Col II coated titanium alloys on biocompatibility and cell differentiation has not been studied thus far.

In this study, we coated Col I and Col II on titanium alloys using KH-550 as a mediator for crosslinking. The amount of Col I and Col II coated on the titanium alloys was quantified using HPLC/MS. The biocompatibilities of these two materials were evaluated by culturing mouse pre-osteoblasts. Effects of Col I and Col II on cell differentiation were compared by cell protein recognition and quantification with HPLC-MS.

2 MATERIALS AND METHODS

2.1 Chemicals and Materials

Titanium alloys were provided by Peking University Dental Hospital. KH550 was provided by Sinopharm Group. Trypsin (sequence grade) was purchased from Promega (Madison, WI, USA). Type I and II collagens were purchased from Hebei Collatech Co Ltd.

Figure 1: The process of collagen coating on the titanium alloys.

Cell line: Mouse embryonic osteoblast precursor cells (MC-3T3-E1) were purchased from Cell Resource Center, Peking Union Medical College of China. α -MEM culture medium was purchased from Thermo Fisher (San Jose, CA, USA). Glucose oxidase kit was purchased from Shanghai rsbio biopharmaceutical Co Ltd (Shanghai, China). MinuteTM animal cell total protein extraction kit was purchased from Invent Biotechnologies Inc (Taiwan, China). All other chemicals were commercial products of analytical grade. In a Word 2010 document, insert a picture.

2.2 Chemicals and Materials

2.2.1 Surface activation of titanium alloys. The titanium alloys ($\Phi 10$ cm) were washed with acetone, ethanol and ultra-pure water for 10 min, respectively. They were dried, immersed in the Piranha solution, incubated at 90 °C for 2 h and dried at 150 °C for 4 h. Then they were put in 5% KH-550 toluene solution and incubated for 1 h. After that, the titanium alloys were immersed in toluene and ultrasonic cleaned for 10 min. Finally, they were dried in the fume hood.

2.2.2 Glutaraldehyde coupling. After surface activated, the titanium alloys were immersed in 2.5% glutaraldehyde which contained 0.01% sodium borohydride at 40°C for 3 h. Then they were washed with deionized water for five times, and each time was for 10 min. At last, they were dried at room temperature for 2 h.

2.2.3 Collagen coating. Bovine Col I was dissolved in 0.01 M sodium acetate buffer (pH 8.0). The surface-activated titanium alloys were placed in Col I solutions and reacted for 24 h. Then they were washed by deionized water and placed in lysine solution (10 mg/mL) for 2 h. Then the titanium alloys were washed again by deionized water, dried and stored at 4 °C. The coating process of Col II was same as above. The process was shown in figure 1.

2.3 Characterization of modified Titanium alloys

2.3.1 Analysis of surface element. X-ray photoelectron spectroscopy (XPS) spectrometer (ESCALAB 250Xi, Thermo Fisher Scientific, USA) was used to analyze the elementary of the modified titanium alloys. The samples were performed by XPS using an Al/K ($h\nu 1486.6$ eV) anode mono-X-ray source at power of 225W with emission current 15 mA and operating voltage 15 kV. The XPS bonding energy (BE) values were charge corrected with respect to those of adventitious carbon at 284.8 eV. The minimum energy resolution is 0.48 eV (Ag3d5/2). The diameter of XPS analysis region is 15 μ m. The data are analyzed by Avantage data system software. The spectra of C1s, N1s, O1s and Si2p signals were collected. The concentrations of the elements were calculated by the peak areas.

2.3.2 Protein quantification. The titanium alloys coated with collagen were placed in ammonium bicarbonate solution (0.05 M, pH 8.0) and incubated at 60 °C for 30 min. Then they were cooled to room temperature, mixed with 20 µg trypsin, and incubated at 37 °C for 18 h. The supernatant was freeze-dried and resolved with 100 µl ammonium bicarbonate solution (0.05 M, pH 8.0). Then the samples were centrifuged for 10 min (4 °C, 10,000 g), and supernatants were collected for analysis [28].

2.4 Cell experiment

2.4.1 Mouse pre-osteoblast cell culture. The titanium alloys were divided into three groups. The blank group was the titanium alloys only treated with Piranha solution. The Col I and Col II groups were the titanium alloys coated with Col I and Col II, respectively. All of the titanium alloys were cut into small pieces (1×1 cm) and washed with 75% ethanol, irradiated with UV for 30 min, then placed into the 24-well plate. The mouse pre-osteoblast cells at the logarithmic growth phase were digested with 0.25% trypsin and counted. The cell suspensions (1×10⁵ / mL) were added into the well and the plate was incubated in 5% CO₂ saturated humidity incubator at 37 °C. The DMEM/High Glucose was replaced every day.

2.4.2 Cell attachment status. After cell culture for 24 h, 3 pieces titanium alloys of each group were taken and washed with phosphate buffer for 3 times. They were fixed with 2.5% glutaraldehyde for 1 h and washed with phosphate buffer for 3 times. Then they were dehydrated with gradual ethanol, dried with CO₂ critical point and sprayed with gold. Scanning electron microscope (JSM 6700F, JEOL, Japan) was used to observe the state of cell adhesion on the surface of the material.

2.4.3 Cell counting. Three titanium alloys per group per day were used for cell counting. The lysis buffer (Reagent A100, Chemometec, Danmark) was used to elute the cells which attached onto the titanium alloys. In order to allow the nuclei to be stained by trypan blue, the lysis buffer was used to permeate the plasma membrane. Furthermore, cell clusters were disaggregated with the lysis buffer. Then, cell nucleus was counted with a blood counting chamber.

2.5 Protein mapping using HPLC-MS

2.5.1 Tryptic digestion. After cultured for 3 days on the titanium alloys, the mouse pre-osteoblast cells were digested with 0.25% trypsin and washed by phosphate buffer for 3 times to remove the culture media. The cell protein was extracted by cell total protein extraction kit. The cellular protein solution was replaced by ammonium bicarbonate solution (0.05 M, pH 8.0). The concentration of cellular protein was determined by BCA protein assay kit (Thermo, USA). Then the protein concentration was adjusted to 1 mg/mL with ammonium bicarbonate solution (0.05 M, pH 8.0). Dithiothreitol was added to the protein solution with a final concentration of 10 mM. The mixed solution was incubated at 37 °C for 1 h. When the samples were cooled to room temperature, the iodoacetamide was added to the solution to a final concentration of 55 mM. The mixtures were incubated at room temperature for 30 min. Each sample was mixed with 20 µg trypsin and incubated at 37 °C for 18 h. The digested solution was dried with a vacuum centrifuge. Then it was redissolved with 0.1% formic acid and centrifuged at

10,000×g for 10 min. The supernatant was collected for HPLC-MS analysis.

2.5.2 HPLC-MS analysis. A C18 column (75 µm ×15 cm, 3 µm, Dr. Maisch GmbH) was used for peptide separation with an EASY-nLC 1000. Solvent A with water-FA (100:0.1, v/v) and solvent B with acetonitrile -FA (100:0.1, v/v) were used for the analysis. A gradient of elution program was as following: 0-5min, 3%-8% B; 5-45min, 8%-20% B; 45-55min, 20%-35% B; 55-58min, 35%-80% B; 58-60min, 80% B. Flow rate: 400 nL/min. Online peptide identification was performed on an Orbitrap Fusion mass spectrometer (Orbitrap Fusion Tribrid, Thermo Fisher, San Jose, CA, USA), with a Nanospray Flex ion source. The MS scan range was m/z 300 – 1800 with a resolution setting of 120,000, followed by Orbitrap HCD-MS/MS. The MS² spectra resolution was 30,000.

2.5.3 Protein mapping. MS/MS data was processed using Mascot Daemon (v.2.5.1, 2014, Matrix Science, Boston, USA). Bovine Col I and Col II database were used for bovine collagen marker peptide identification. Tandem mass spectra were used for collagen quantification. And mouse pre-osteoblasts databases were used for protein difference analysis. Two missing cleavages were allowed for trypsin cleavage. The mass error was set to 10 ppm and 0.02 Da for precursor and fragment ions, respectively.

3 RESULTS

3.1 Element Analysis of KH550 modified titanium alloy

To explore the modification process of titanium alloy surface, the XPS was used to detect all elements except for hydrogen and helium. The surface element composition of the samples before and after modification with KH550 was analyzed. The spectra were shown in figure 2. The surface atomic compositions and the ratio of Ti, Al, V, N, O, Si, C on the blank and modified titanium alloys were listed in Table 1. The binding energy that N1s signal peak appeared in the XPS spectrum of the titanium alloys modified with KH-550 was 399.4 eV. It corresponded to the binding energy of N1s in -NH₂, and the results indicated that the titanium alloy surface was modified with aminosilane. The modified C1s signal peak appeared at the binding energy of 284.8 eV, which corresponded to the C-C bond in KH-550. It indicated that the modified KH-550 was successfully coupled to the surface of the titanium alloys. The Si2p signal peak appeared at 102.2 eV, indicating that Si-O bonds were formed between Si-OH and KH-550. There was 0.81% N on the surface of unmodified titanium alloys. It may be the non-specific adsorption of N₂ in the air.

3.2 Analysis of collagen content on titanium alloy surface

Standard Col I and Col II were digested by trypsin and analyzed with HPLC-MS. The enzymatic peptide information was searched with Mascot software against the bovine Col I and Col II database. GFSGLDGAK and GEAGAQGPMGPAGPAGAR were confirmed as the marker peptides of Col I and Col II, respectively. And the MS/MS quantitative ions were m/z 417.03 and 980.87, respectively (Figure 3). GLAGMA was selected as the internal standard. The

Figure 2: The XPS scan spectra of titanium alloys before (a) and after (b) modified by KH550.

Table 1: The surface elementary composition of the titanium alloys.

	Ti/%	Al/%	V/%	N/%	O/%	Si/%	C/%
Bare	29.7	3.47	0.42	0.81	64.53	1.06	28.65
KH550	0.65	0	0	8.25	23.09	12.92	55.08

concentration of marker peptide was the abscissa. The ratio of internal standard to marker peptide peak area was the ordinate. The linear regression equations were plotted: $A=0.615+0.018\times C$, $R^2=0.997$ (GFSGLDGAK) and $A=-0.924+18.205\times C$, $R^2=0.998$ (GEAQAQGPMGPAGPAGAR). The amount of Col I and Col II coated on the titanium alloys were 0.813 ± 0.015 mg and 0.774 ± 0.013 mg, respectively.

3.3 Cell attachment morphology

SEM scan spectra of mouse pre-osteoblast cells cultured on three groups of titanium alloys for 24 h were shown in figure 4. It indicated that the cells could attach and grow normally on the three groups of titanium alloys. The cell extension area on the surface of the blank group was small and most of the area formed a bulge to appear as strip-shaped, and the flat or polygonal cells were less, also were the pseudopodias of the cell. While the cells attached to the surface of Col I and Col II groups had a larger extension area, most were flat or polygonal, and the pseudopodia of cells were more than the blank group.

3.4 Cell counting

Nucleus counting was used to evaluate the proliferation of mouse pre-osteoblast cells growing on titanium alloys after 1, 2 and 3 days (figure 5). It was found that cells cultured within Col I group was the most abundant. The number of cells in the blank group was the least. From the process of the cell proliferation, the proliferation rate of Col I group was the fastest, and that of the blank group was the slowest. The result showed that titanium alloys coated with Col I and Col II could promote the proliferation of mouse pre-osteoblast cells.

3.5 Cell protein expression

After cultured for 3 days, three groups of mouse pre-osteoblasts protein were digested and analyzed by HPLC-MS. Using all mouse pre-osteoblasts total protein as a database, three groups of enzymatic peptide information were searched with Mascot software. We found that there were 6 kinds of protein with significant differences among the three groups. Firstly, we identified the marker peptides of the 6 proteins. Then the marker peptides were synthesized, and the amount of proteins was quantified with the content of marker peptides (figure 6).

The peptide DGEAGAQGAPGAPGAGER was identified in $\alpha 1$ chain of mouse Col I, but not presented in bovine Col I and other proteins. It was used as the marker peptide to quantify the mouse Col I in the cells. We found that the mouse Col I was abundantly expressed in the Col I group, and its relative content in Col I group was 18.9%. The marker peptide of fibronectin was CDPIDQCQD-SETR. The relative content of fibronectin was 23.0% in Col I group. These two proteins took the majority in Col I group. Other proteins were quantified with the same method. The marker peptides of osteopontin and prelamin-A/C were ESQESADQSDVIDSQASSK and EAAISTAISEK, respectively. Their contents in Col II group were higher than the blank and Col I group, which were 20.0% and 21.3%, respectively. The marker peptide of alkaline phosphatase was QQAQETIK, whose content were 18.1% and 17.1% in the blank group and Col II group, and higher than Col I group. The content of nidogen-2 was 23.9% in the blank group, which was higher than the other groups.

4 DISCUSSION

In recent years, titanium alloys have been widely used as biomaterials in dental implants and artificial bone implants. Previous study

Figure 3: MS/MS extracted ion chromatograms of (a) GFSGLDGAK (a1(I)) and (b) GEAGAQQGPMGPAGPAGAR (a1(II)) collagen peptides and their MS/MS spectras.

Figure 4: SEM scan spectra of blank group (A), Col I group (B) and Col II group (C) with the magnification 300.

has reported that Col I coated on the titanium alloys could promote cell adhesion [3]. No publications have reported the coating of Col II on titanium alloys. In this report, we coated Col I and Col II on titanium alloys and compared the differences in biocompatibility and cell differentiation between them.

Titanium alloys were modified with KH550. The element composition of the titanium alloys surfaces was analyzed by XPS. As shown in figure 1, the N1s peak at 399.4 eV in Figure 1 (b) belonged to amidogen. The C1s peak at 284.8 eV belonged to C–C (284.6 eV) in KH550. It indicated that KH-550 was successfully cultured onto

Figure 5: Cell counting results of blank, Col I and Col II groups.

Figure 6: The relative content of different proteins expressed in blank, Col I and Col II groups.

the titanium alloys surface. The Si2p peak at 102.2 eV belonged to Si-O bonds which were formed between Si-OH and KH-550. These results indicated that KH550 was immobilized onto the titanium alloys surface and there was amidogen on the surface of the titanium alloys, which provided a basis for further collagen coupling.

Collagen coating is usually used to improve surface hydrophilicity and cell compatibility of titanium alloys. The influence of different collagen types coated on the titanium alloys to the cell compatibility and differentiation was different. In this research, Col I and Col II were coated on titanium alloys, respectively. The marker peptides and the contents of Col I and Col II were identified and quantified by HPLC-MS. The results showed that there were no significant differences between the coated amounts of Col I and Col II, and the collagen type had no effect on the coating amount to the titanium alloys. Thus, the collagen type may be the main factor affecting the cell proliferation and differentiation.

It was reported that collagen was suitable for cell adhesion and growth. For example, the Phe-Hyp-Gly sequence in collagen was the ligands for the integrin family receptors of cell surface. It could also promote cell attachment and proliferation by activating specific biological signals [39, 40]. The cell attachment morphology and amount of the mouse pre-osteoblast cells were analyzed by SEM and nucleus counting. Compared with the blank group, the titanium alloys coated with collagen were more suitable for cell attachment

(Figure 3). The cell counting results showed that the number of cells in Col I and Col II group were higher than the blank group. And the blank group was $2.5 \times 10^5/\text{mL}$ on day 3, which was much lower than the Col I ($3.9 \times 10^5/\text{mL}$) and Col II group ($2.9 \times 10^5/\text{mL}$). Not only the cell proliferation rate, but the spreading area, pseudopodia of the collagen group were also better than the blank group. The cell protein expression results of mouse pre-osteoblast cells were analyzed by HPLC-MS.

Six kinds of proteins were identified. It showed that mouse Col I and fibronectin were abundantly expressed in Col I group. Fibronectin binds to various proteins on the cell surface, participates in cell adhesion and osteoblast fibril synthesis, and regulates Col I deposition [41, 42]. The most abundant proteins in Col II group were osteopontin and prelam-A/C. Osteopontin is expressed in a large amount during the cell mineralization phase, which can promote the deposition of hydroxyapatite [43]. Prelamin-A/C plays a role in osteogenesis and bone formation. Nidogen-2, the most abundant protein in the blank group, was a kind of glycoproteins. And it can be involved in extracellular matrix interactions [44]. The results showed that cells cultured on Col I coating material mainly expressed proteins related to matrix synthesis, but cells cultured on Col II coating material mostly expressed proteins related to mineralization and calcium salt deposition. This indicated that collagen coating could improve the biocompatibility of the material, and different types of collagen could regulate cells to express different functional proteins.

5 CONCLUSIONS

The titanium alloys were modified by KH550 and coated with Col I and Col II, respectively. The coated amount of Col I and Col II on the titanium alloys surface were similar. Titanium alloys coated with collagen exhibited better state of cell adhesion and faster proliferation rate than the blank group. The cells cultured on Col I and Col II group expressed proteins associated with matrix synthesis and mineralization, calcium salt deposition, respectively. Collagen coating can improve the biocompatibility of titanium alloys, and different types of collagen can induce cell to express proteins with different functions.

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