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Effective inhibition of nickel release by tantalum-implanted TiNi alloy and its cyto-compatibility evaluation in vitro

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Abstract Titanium–nickel (TiNi) shape memory alloy is modified with tantalum (Ta) plasma immersion ion implantation (PIII). Scanning electron microscope (SEM), atomic force microscopy (AFM), inductively coupled plasma mass spectrometry (ICP-MS), methyl-thiazol-tetrazolium (MTT), and cell culture are adopted to evaluate the surface morphology, roughness, Ni release, in vitro cytotoxicity, and cell behavior of TiNi and Ta-implanted TiNi (Ta–TiNi) alloys. Results showed that the surface became rougher and was covered by ordered and uniform grains after Ta implantation. Ni release was averagely inhibited by Ta–TiNi to up to 60% of that in unmodified TiNi alloy within 30 days. MTT assays demonstrated that Ta–TiNi sample allowed greater degree of cell proliferation for both smooth muscle cell and osteoblasts, indicating excellent protection and cyto-compatibility. A negative correlation was observed between Ni release and cell proliferation. Analysis of the cell morphology revealed healthy cells extending on the alloy surface, which indicated that TiNi alloy had good cyto-compatibility despite the initial Ni dissolution, but the implanted Ta would endow traditional TiNi alloy much lower Ni release, improved cyto-compatibility and other potential merits.

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Introduction

Over the last two decades, titanium–nickel (TiNi) alloy has been gaining increasing importance for its potential as an implantation material in many minimal invasive surgeries, diagnostic modalities, and other applications. TiNi alloys are known for their properties of unique shape-memory effect, superelasticity, and high damping [1, 2]. The evaluation of the biocompatibility of these materials is essential for medical applications. The results of most of the biocompatibility studies on TiNi alloys suggest that these alloys have low cytotoxicity and genotoxicity [2–5]. However, the release and accumulation of Ni ions are considered as a potential risk, in terms of toxicity to cells, tissues, and genes and causing allergy and carcinogenicity [6–9]. A recent study reported that Ni(II) influences gene expression and cholesterol metabolism [8]. The key to increase the biocompatibility of TiNi alloys is to suppress the dissolution of Ni ions, which is closely associated with the corrosion resistance of the metal.

Various methods [10] have been employed to inhibit the dissolution of Ni ions and thus enhance the biocompatibility of these metals: thermal oxidation [11], calcium phosphate coating [12, 13], hydrothermal synthesis [14], advanced plasma immersion ion implantation and deposition (PIII&D) [15], chemical and electrochemical passivation [16], plasma source ion implantation (argon, oxygen) [17], arc ion plating [18, 19]. Plasma immersion ion implantation (PIII) is an effective technique to improve the corrosion resistance of the biomedical materials without adversely affecting their bulk properties. This technique has been successfully used for implanting non-metallic elements, such as N, O, and C, so that the near-surface Ni concentration can be significantly suppressed [20, 21]. Tantalum (Ta) has been widely used in

surgical appliances and implants to improve the radiopacity of the substrate. It is known to have excellent fracture toughness, workability, corrosion resistance, and intrinsic radiopacity and is almost completely immune to body fluids [22–25]. Tantalum PIII technology is known to change the surface morphology and chemical composition of the TiNi alloy and provides superior corrosion resistance [26, 27]. However, very few studies have been reported on the evaluation of cytotoxicity of Ta–TiNi in particular.

The aim of our study is to evaluate the cyto-compatibility and Ni release from Ta–TiNi alloy by cell culture and ions release during 1 month. The influence on cell growth and proliferation by implanted Ta–TiNi alloy have been studied through methyl-thiazol-tetrazolium (MTT) testing, cell morphology observation, and release kinetic analysis. These findings may provide useful information for improving the cyto-compatibility of implantable medical devices.

Materials and methods

Sample preparation

The material tested in this study was a TiNi (Ti–50.9 at.%Ni) metal sheet purchased from Beijing GEE Technology & Trade Company. The metal sheet was cut to $8 \times 8 \times 1$ mm^3 standard samples and subjected to heat treatment at 600 °C for 0.5 h. The TiNi plates were then mechanically polished progressively with wetted metallographic polishing SiC emery papers (grade 80, 200, 500, 800, 1200, and 2000) and mirror-polished with diamond paste, as needed. Prior to Ta implantation, samples were ultrasonically cleaned in acetone, anhydrous ethanol, and deionized water for 10 min each, and placed into PIII chamber. The vacuum chamber was evacuated to a base pressure of 1×10^{-4} Pa, and then the samples were bombarded with energetic argon ions at −5 kV to remove residual surface contaminants. Pure Ta (99.99%), purchased from General Research Institute for Nonferrous Metals, was used for implantation. The surface modification process employing Ta metal vapor vacuum arc (MEVVA) plasma source was carried out at −45 kV bias voltage with an ion current of 2 mA. Ta was implanted in the samples with a moderate incident ion dose of 1.5×10^{17} ions/cm^2, which affords the best corrosion resistance due to its relatively slippery surface morphology according to our previous work [26]. Detailed analysis of surface chemistry has also been demonstrated in that work and a composite TiO_2/Ta_2O_5 layer formed on the surface as compact aggregates of nano-grains after Ta implantation. The samples after Ta implantation were denominated as Ta–TiNi. All the samples were degreased before the test. Before immersion in extract medium, the samples were autoclaved at 120 °C for 20 min and sterilized by UV.

Surface morphology and roughness

To determine the surface topography of the samples, scanning electron microscope (SEM) images are obtained using JSM-5800 (JEOL, Japan). The roughness of TiNi and Ta–TiNi samples was obtained by atomic force microscope (AFM; Veeco Instruments, USA) analysis. Several different areas are chosen random for each sample.

Preparation of leaching liquor

Leaching liquor was prepared using culture medium with the samples for 12 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 14 days, 18 days, 23 days, and 30 days with the ratio of 3 cm^2/mL [28] culture medium—Dulbecco's modified Eagle's medium (DMEM) (the composition and content were list in Table 1) (GIBCO) at 37 °C in 5% CO_2 cell incubator (SANYO, Japan). Three samples were used separately for leaching liquor preparation at each time point. The liquor obtained was sterilized by filtration with 0.22-μm disposable filter membrane (Millipore), and then preserved in 4 °C before use. The extract medium without alloys obtained at each of the time intervals was chosen as blank control for all the tests.

Ni release

The leaching liquor of all the samples was acidified with nitric acid (analytical grade). The amount of Ni ions released was determined by inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer, Elan 6000). The results for each immersion time were mean values of three measurements.

Table 1 The main component of extraction medium (Dulbecco's modified Eagle's medium)

Component	Content
Glucose	450 mg/L
Glutamate	584 mg/L
Sodium pyruvate	110 mg/L
Bicarbonate	3.70 mg/L
Serum	10%
Double-antibody	100 U of penicillin/mL, 100 mg of streptomycin/mL
pH	7.2
Endotoxin	<0.1 ng/mL

Cell culture

Rat embryo osteoblasts (MC3T3-E1) and rabbit aortic smooth muscle cells (CCC-SMC-1) (provided by Graduate School of Basic Medical Science, Chinese Academy of Medical Sciences, China) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO), antibiotics (100 U of penicillin/mL, 100 mg of streptomycin/mL), and L-glutamine (2 mM), at pH = 7.2 ± 0.2 and 37 °C in CO_2 incubator (5% CO_2). The cells were allowed to reach confluency before subculturing into culture flasks (Corning costar, America). The cells were washed with warm phosphate-buffered solution (PBS, GIBCO), and then the adherent cells were detached by treating with 0.25% trypsin–EDTA (GIBCO) for 1–2 min. The cell suspension was made uniform, seeded onto culture flasks in 1:2 ratio during subculturing, and allowed to attach for 3 h under standard conditions.

Methyl-thiazol-tetrazolium (MTT) assay

The cells were seeded in 96-well plates with 100 μL/well at a density of 6×10^4 cells/mL. After seeding, the cells were allowed to adhere for 4–6 h. The original solution was removed and 100 μL leaching liquor of Ta–TiNi and TiNi (during 1 month) aforementioned was added separately. Four parallel leaching liquors of each sample were used at each time point. Cells with only culture medium obtained at each of the time intervals were used as negative controls. After culturing for 72 h, MTT reagent (Sigma) was added at a final concentration of 5 mg/mL (20 μL/well). After 4 h incubation, the medium was aspirated and the dark-blue crystals were rinsed with PBS twice. The crystals were dissolved in 150 μL dimethyl sulfoxide (DMSO) (Amresco). The optical density (OD) was recorded at 492 nm using an enzyme-link meter (Thermo, Multiskan MK3).

Observation of cell morphology by SEM

The samples of Ta–TiNi and TiNi were placed in 24-well plates with two parallel samples at each time interval, after which cell suspension of 2×10^4 cells/mL was added to each well (other wells were blank contrast-cover glass). After incubation at 37 °C for 12, 24, 48, and 72 h, the liquid was removed and cells were rinsed in PBS thrice. The cells were then fixed with 2% glutaraldehyde in PBS for 1.5 h. After washing with the same buffer, the cells were dehydrated in sequential concentrations of ethanol of 30%, 50%, 70%, 90%, and 100% (each concentration for 10 min), and air dried. The specimens were coated with gold–palladium and then examined under an S-530 SEM (Hitachi, Japan).

Results and discussion

Surface morphology and roughness

Figure 1 shows the surface topographies for TiNi and Ta–TiNi samples (magnification ×10000). The difference is clearly seen from Fig. 1. TiNi sample behaviors are smoother with some slight grooves on the surface, as shown in Fig. 1a. For Ta–TiNi sample, the surface becomes rougher and is covered by ordered and uniform grains with some connecting together into laminated structure. This is supported by the theory that PIII treatment often roughens a surface by sputtering [29].

The average surface roughness (R_a) of the samples is shown here. TiNi and Ta–TiNi samples possess the roughness of 3.2 ± 1.3 nm and 8.5 ± 0.9 nm, respectively. After Ta implantation, it appears that surface of the Ta–TiNi becomes rougher than the TiNi sample, which is consistent with Fig. 1. The roughness of Ta–TiNi sample increases only slightly but can have a significant effect on the properties of the surface.

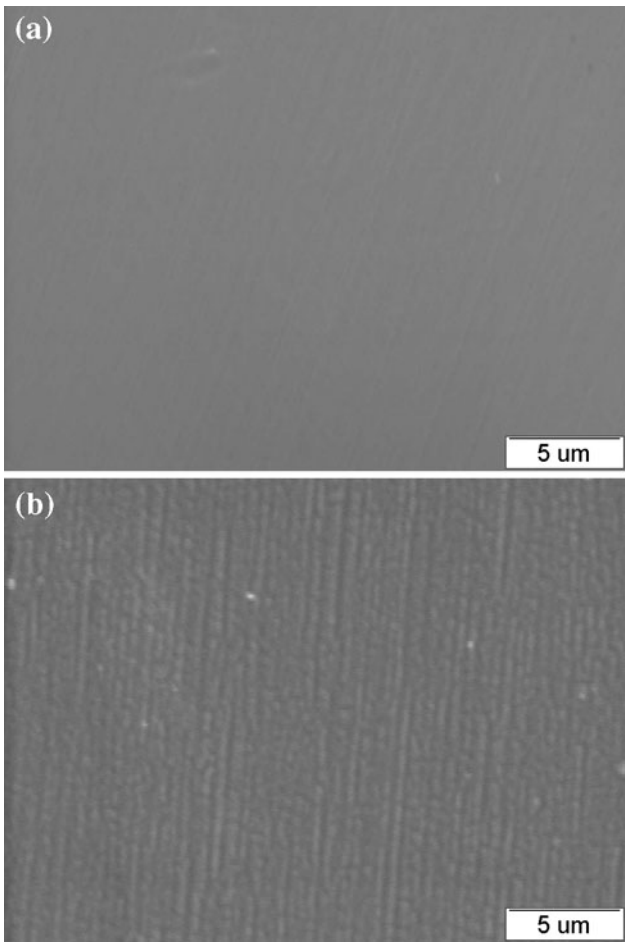

Fig. 1 SEM images for the surface of **a** TiNi and **b** Ta–TiNi

Ni release

As shown in Fig. 2, after immersing the samples for a few hours, Ni ions could be detected and its concentration quickly increased within 12 h. This stage is the first stage (or log phase) of release since the concentration of Ni increased exponentially. The dissolution rate constants within 12 h were in the order of TiNi > Ta–TiNi, which indicated that the release rate constant of TiNi is 26.4 μg L^{-1} day^{-1}, two-fold higher than that of Ta–TiNi (11.8 μg L^{-1} day^{-1}). Ta implantation resulted in significant inhibition of Ni release as much as 60% of that of TiNi during 24 h. The release rates became obviously slow after 24 h, but the accumulation of Ni kept increasing steadily during the experimental period.

After log phase, the Ni release entered stationary phase since the release became very slow and the accumulation effect emerged. Ni level for TiNi and Ta–TiNi is the highest at around 48 h of elution testing, which was in agreement with the findings of the previous studies [30, 31]. After 30 days of immersion, the amount of dissolved Ni ions accumulated in the leaching liquor, with the amount being 42 μg/L for TiNi and 12 μg/L from Ta–TiNi. Release of Ni ions was inhibited in the case of Ta–TiNi than TiNi. The roughness of the alloy surface would be an important factor for controlling metal ion release since its surface microstructure affects the release rate. The relatively inhomogenous surface induced larger contact area in ambient liquid, which may lead to more release of Ni ions. However, Ta–TiNi sample with higher roughness behaves much lower release rate, indicating the effectiveness of Ta implantation on inhibition of Ni ions release. The implantation of Ta into TiNi alloy improved the corrosion resistance of TiNi [26], decreasing the release of Ni ions into culture media. Yeung et al. [32] obtained similar results that enhanced corrosion resistance of N, C, and O implanted TiNi reduced Ni ions released after electrochemical test.

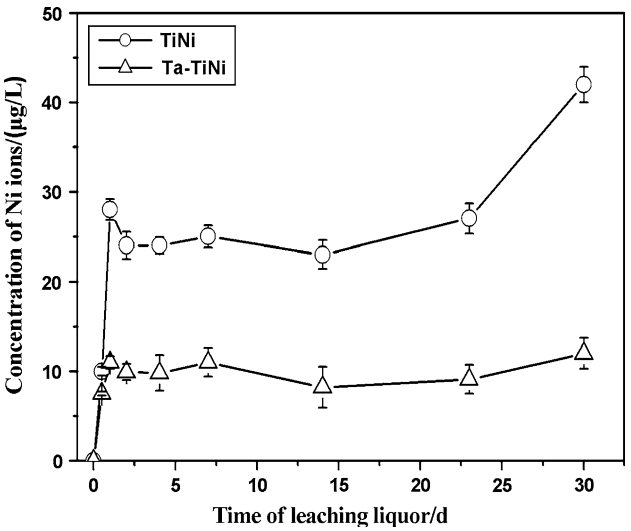

Fig. 2 The concentration of Ni ions in leaching liquor (culture medium) from TiNi and Ta–TiNi during 1 month

The released Ni kept increasing in TiNi during the entire experimental period and reached 42 μg/L in the 4th week, but after implantation with Ta, the increasing trend was obviously inhibited and the total amount of Ni ions released from Ta–TiNi was only 29% of TiNi. Ta–TiNi and the PIII technique for Ta-implantation have exhibited significant benefits on the inhibition of Ni ion release in medical application of TiNi alloy, especially in the first 24 h, which can effectively prevent acute cytotoxicity and allergic reactions. The trace release of Ni ions also reduced the risk of the aggregation of the toxic Ni and its compound in the nucleolus and thus decreased carcinogenicity [33].

The reason for the inhibition of Ni-ion release from Ta–TiNi is possibly attributed to two points. One is the declined Ni concentration in the surface layer with PIII treatment [26]. A more recent study of Chu et al. [34] confirmed that lower concentration of Ni in the modified surface layer resulted in a lower release of Ni ions. The other is a composite TiO_2/Ta_2O_5 layer formed on the surface as compact aggregates of nano-grains by PIII technology which has been shown in our previous work [26]. This layer is more stable than a pure TiO_2 passive layer on the surface of unmodified TiNi, and therefore enhances the cyto-compatibility of the material [26, 35].

Cytotoxicity test

Methyl-thiazol-tetrazolium (MTT) assay was adopted to evaluate the cytotoxicity of Ta–TiNi and TiNi. The cell proliferation percentage, P, was defined as follows:

$$P = \mathrm{OD}_{\mathrm{test-material}}/\mathrm{OD}_{\mathrm{negative}} \times 100\%$$

The value of P was used to evaluate the cytotoxicity grade of each according to the 5-level (0–4) definition [8]. Toxicity grade 0 means $P \in [81\%, 100\%]$, grade 1 means $P \in [61\%, 80\%]$, grade 2 means $P \in [41\%, 60\%]$, grade 3 means $P \in [21\%, 40\%]$ and grade 4 means $P \in [0,20\%]$.

The MTT results, as shown in Fig. 3, indicate that the proliferation of smooth muscle cells (SMC) was inhibited with extending the days of leaching liquor, indicating that the released Ni ions in leaching liquor had obvious toxic effects to smooth muscle cells. In the first 48 h, the cell proliferation even surpassed 100% for Ta–TiNi. This was possibly because of the implanted Ta with the concentration of 0.01 mg/L in leaching liquor, which affords excellent biocompatibility and osteoconductivity [22]. An obvious decrease in the cell proliferation was found during the 3rd and 4th day, which may due to the accumulation of

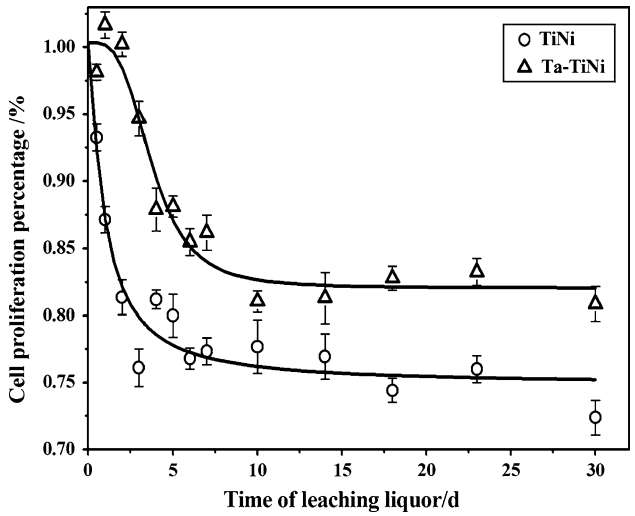

Fig. 3 Cell proliferation evaluation using MTT assay (optical density) at 72 h of cell culture; SMC was cultured with leaching liquor (30 days) of TiNi and Ta–TiNi

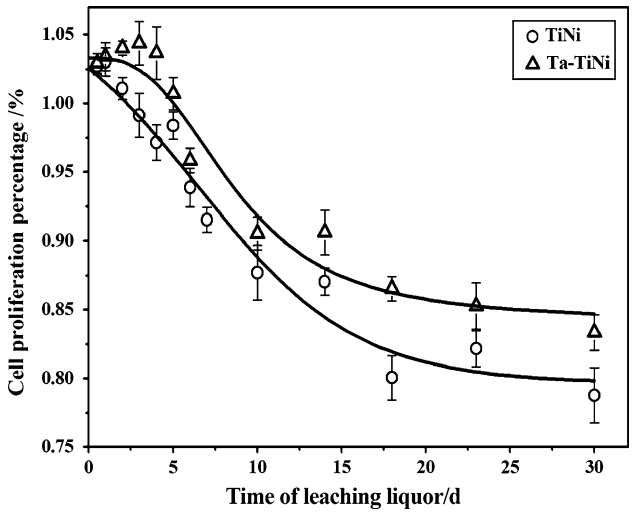

Fig. 4 Cell proliferation evaluation using MTT assay (optical density) at 72 h of cell culture; osteoblasts were cultured with leaching liquor (30 days) of TiNi and Ta–TiNi

Ni ions. The rate constants of decrease in proliferation were calculated to be 77%/day with TiNi and 47%/day of Ta–TiNi. Eventually, SMC proliferation reduced to 80–85% with toxicity grade 0 in the following 3 weeks for Ta–TiNi. There was lower cell proliferation—about 75% with toxicity grade 1—with TiNi. This indicates that implanted Ta is safe for cell growth and duplication. For osteoblasts (Fig. 4), the cell proliferation decreased to 79% with TiNi exhibiting toxicity grade 1 while Ta–TiNi sample displays 83% in 30 days with toxicity grade 0.

Since Ta–TiNi sample had a favorable influence on SMC and osteoblasts proliferation in vitro than TiNi, we speculated that there is a negative correlation between the cell proliferation and the concentration of Ni ions in the culture medium. However, cells cultured with 2 days leaching liquor displayed higher proliferation than that cultured with 5 days leaching liquor (Figs. 3, 4) although with a relatively higher Ni ions concentration (Fig. 2), suggesting that Ni ions concentration is not the only factor that influences cell proliferation. The trace amounts of dissolved Ta ions and the corrosion product during immersion may also affect the cell proliferation during cell culture. Similar results have also been observed by Lu et al. [8] who shows that Ni (II) may inhibit the proliferation of L-929 mouse fibroblast cells through the induction of apoptosis. Ta implantation inhibits the release of Ni ions at each immersing time, resulting in higher cell proliferation.

The proliferation rate of smooth muscle cells decreased rapidly during the first 3 days due to acute toxicity compared to the rapid decrease of osteoblasts from 4 to10 days. Meanwhile, lower cell proliferation (the difference of toxicity grade) of smooth muscle cell than that of osteoblasts indicated that they were more sensitive and accurate in the Ni toxicity detection. The significant growth inhibition by Ni release may be the main reason [9, 30].

Cell morphology

The morphology of the osteoblasts cultured on Ta–TiNi and TiNi for 12 h, 24 h, and 48 h is shown in Fig. 5. Cell density increased gradually with the culture time. The osteoblasts attached and extended well on the alloy surface, and no abnormalities were found. However, distinctly higher proliferation rate and better cell morphology were observed with Ta–TiNi after 48 h of incubation than with TiNi, with the former showing lesser cell injury and cell debris (Fig. 5c). Implantation of Ta ions increased surface roughness of Ta–TiNi sample that may be responsible for the higher proliferation rate compared with untreated TiNi alloy. Rough surface morphology provides more adhesion sites resulting in more cell adhesion, which may lead to higher proliferation.

Figure 6 shows clearly the surface morphology of one cell that extended fully, and normal microstructure, excellent growth status and good organization of the cytoskeleton in cases of both Ta–TiNi and TiNi after incubation for 72 h are observed. The results suggest that TiNi alloy showed good cyto-compatibility, despite the initial Ni dissolution. This result is consistent with Ryhanen's study, where it was reported that TiNi did not induce toxic effects such as decrease in the cell proliferation or inhibition of cell growth in contact with the substrate [2, 30, 36, 37]. Ta implantation is well tolerated by cells. This is supported by the point that plasma-treated surfaces are well tolerated by osteoblasts [32]. However, the integrity of the cell morphology and non-toxicity by Ta-implantation,

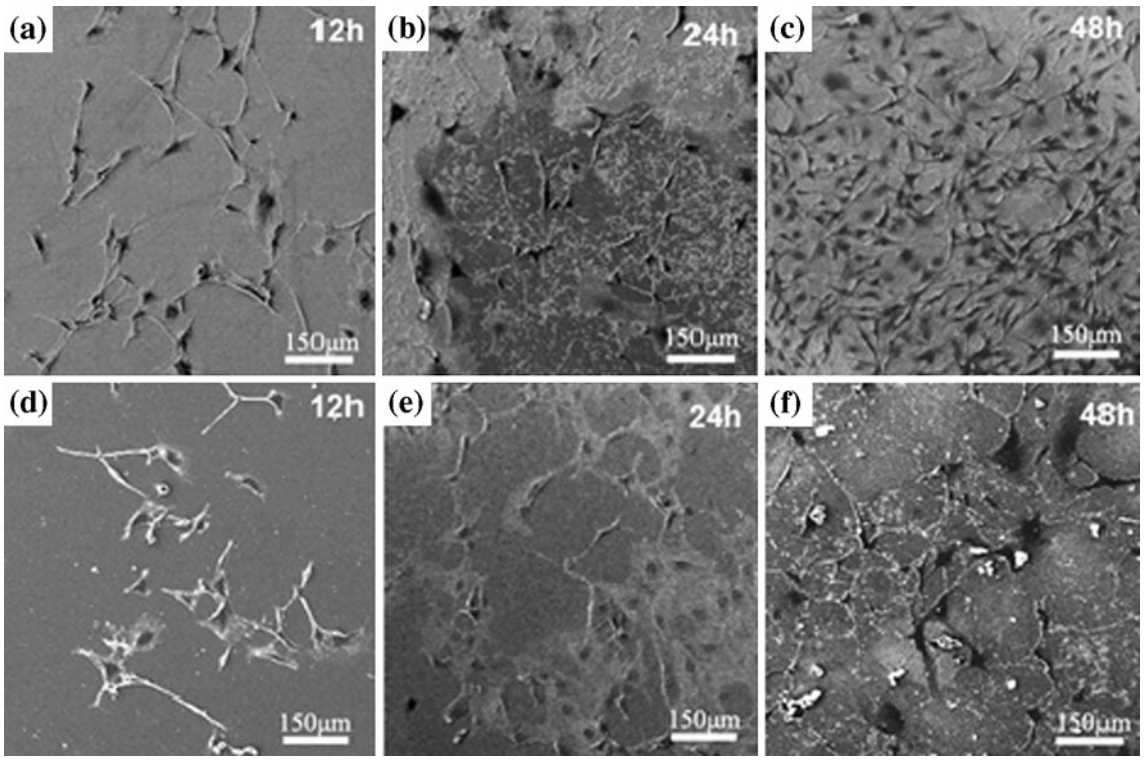

Fig. 5 SEM images (×100) of attached osteoblasts after 12, 24, and 48 h of adhesion onto Ta–TiNi (**a–c**) and TiNi (**d–f**)

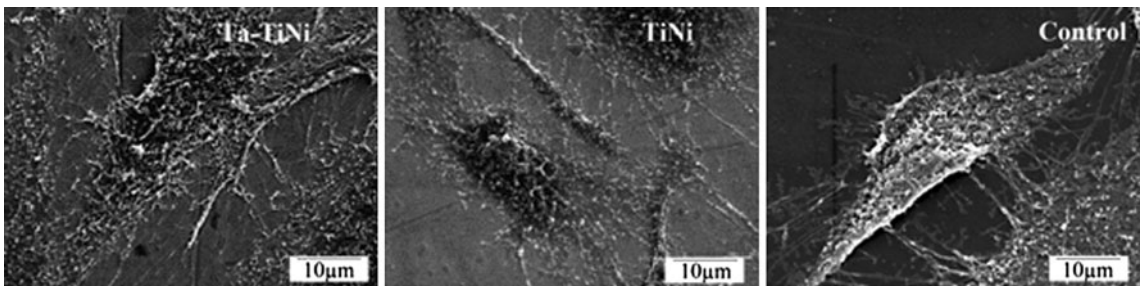

Fig. 6 SEM images (×1000) of attached osteoblasts after 72 h of adhesion onto Ta–TiNi and TiNi

as seen in MTT test, cannot indicate the level of gene expression, especially when there is a high release of Ni ions [8]. The coating of tantalum in the Ta-implanted alloy enhances the cyto-compatibility of traditional TiNi alloy and affords other advantages [22].

Conclusions

The present study demonstrates that Ta-implanted TiNi alloy by plasma immersion ion implantation (PIII) suppresses Ni release, decreases cytotoxicity, and increases cell proliferation than unmodified TiNi alloy. Ta implantation resulted in significant inhibition of Ni release as much as 60% of that of TiNi during the immersion time. The implanted Ta may stimulate short-term cell growth and proliferation, and also gives better cyto-compatibility. There is a negative correlation between Ni release and cell proliferation. Ta–TiNi sample displays good cell adhesion and stimulates cell proliferation, especially after 48 h of culture. The smooth muscle cell is more suitable as a test sample for detection of Ni release due to its sensitivity and lower proliferation rate.

Generally, observation of cell proliferation and cell morphology can provide strong support information for evaluating alloy cyto-compatibility. However, since the

implantable medical device is placed in complex in vivo environment, other novel and refined evaluation methods are urgently required.

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