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In Vitro Cytocompatibility Study of a Medical β-Type Ti-35.5Nb-5.7Ta Titanium Alloy

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This research was part of a European Commission funded research project for the development of a new generation of titanium based orthopaedic biomaterials. In particular, hip implants of a β-type Ti-35.5Nb-5.7Ta alloy were fabricated by the powder metallurgy route. An advanced surface treatment technology of Laser Engineered Net Shaping (LENS) was used to apply a ZrO_2/Zr coating on the implant surfaces where relative movement takes place between the implant and the bone, in order to enhance the wear resistance. The present work was conducted to evaluate the cytotoxicity of the uncoated and ZrO_2/Zr coated β-type Ti-35.5Nb-5.7Ta alloy in both indirect and direct contact of human embryonic kidney 293T (HEK293T) cells. The cellular response was quantified by cell viability assessments using Alamar Blue assay and cell attachment. Cytotoxicity experiments showed that the responses of the uncoated and ZrO_2/Zr coated alloy on surviving cells were non-toxic. HEK293T cells displayed appropriate survival rates, good cell adhesion and cell spreading. These results confirm that the β-type Ti-35.5Nb-5.7Ta alloy, in both coated and uncoated conditions possesses good cytocompatibility, which allowed adhesion and proliferation of HEK293T cells to occur in both indirect and direct contact cell culture methods by Alamar Blue assay.

Keywords: β-Type Titanium Alloy, Biomaterials, Cytotoxicity, Cell Attachment, HEK293T Cell, Alamar Blue Assay.

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1. INTRODUCTION

Common metallic biomaterials are stainless steels, cobalt–chromium alloys and titanium (Ti) alloys.[1–4] In comparison with stainless steels and cobalt–chromium alloys, Ti alloys have higher biocompatibility, corrosion resistance and specific strength.[5–7] The most common Ti alloys are α-type, $(\alpha+\beta)$-type, and β-type alloys.[8,9] Pure Ti and $(\alpha+\beta)$-type Ti-6Al-4V alloys are extensively employed for hard tissue replacement devices, such as artificial hip joints and dental implants.[8,10] In fact, most of the hip and knee implants are made of Ti-6Al-4V alloy. This alloy contains Al element, which can result in neurological problems such as Alzheimer disease and V element, which has cytotoxicity. Additionally, this alloy has a substantially higher elastic modulus than human bones, leading to the "stress shielding phenomenon."[6] Therefore, the development of β-type Ti alloys with a lower elastic modulus and higher ductility than the Ti-6Al-4V alloy has recently attracted extensive interests.[8] One of the research focuses is on allergic and toxic element free β-type Ti alloys.[11,12] The most common alloying elements in the β-type Ti alloys are Nb, Zr, Mo and Ta.[13] Among them, Ta is immune to all acid environments and reduces metal release in the blood[14] and Nb improves the passivation film by decreasing the concentration of anion vacancies. More importantly, the addition of Ta and Nb into Ti changes the alloy from alpha to beta (bcc) allotropic form with a reduced elastic modulus and excellent ductility. Therefore, these two elements were selected in our alloy development.

Friction occurs between the surfaces of moving implant parts and the bone surface in contact with the implants. For implants with low wear resistance, the friction can lead to the release of metal shavings in the blood and the premature fracture of the implants.[13] Low wear resistance is a critical problem for commercial Ti implants and for the newly developed β-Ti alloy as well. In order to overcome the problem, a biocompatible wear resistant ZrO_2/Zr coating[15,16] was employed on the implant surfaces where wear control is important.

The production of a new metallic biomaterial contains new alloying elements or coating material may have a considerable influence on the biological characteristics.[17] The release of metal ions from Ti alloy implants may generate an adverse biological effect or elicit allergic reactions.[18] Thus, testing the effects of compounds on the viability of cells is important in order to predict the potential

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toxic effects of implant for the future use in the body. Among several available cytotoxicity assays, MTT assay has been broadly utilized for the cytotoxicity evaluation of Ti implants.[19–21] A new method for measuring the cytotoxicity has recently become available, which uses Alamar Blue. Alamar Blue is soluble, stable in culture medium and is non-toxic. The continuous monitoring of cells in culture is therefore permitted. Specifically, Alamar Blue does not alter the viability of cells cultured for various times as monitored by Trypan Blue exclusion.[22] General cytotoxicity tests can be carried out on many cell types (e.g., human mesenchymal stem cells, human osteoblast cells or fibroblasts).[23–25] *In vitro* cytocompatibility testing using human cells is desirable for biomaterials intended for human applications. In this work, the *in vitro* cytotoxicity of a newly developed β-Ti alloy was investigated by both indirect and direct contact cell culture methods, with human embryonic kidney 293T (HEK293T) cells grown on the samples. HEK 293T is a cell line which is routinely used in our laboratory. HEK293T cells are easy to grow and have been widely used in cell biology researches. One thing to consider is that HEK293T cells are highly proliferative. This cell line is also commonly reported for cytotoxicity assays of biomaterials and pharmaceutical agents, because kidney is the key organ that filters blood, and hence is exposed to higher doses of any metal ions leaching from the implant.[26–28] In this *in vitro* investigation, the cellular behaviour of HEK293T cells grown on the samples of a new β-type Ti-35.5Nb-5.7Ta alloy using Alamar Blue assay was analysed to verify the cytocompatibility of the alloy.

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2. MATERIALS AND METHODS

2.1. Sample Preparation

Powder metallurgy route was employed for the production of alloy samples. Ti, Nb, and Ta powders were blended together with different particle sizes such as (a) smaller than 40 μm, (b) 40–80 μm and (c) 80–125 μm. Mixing time of 30 min, 1 h, 2 h and 3 h were used. Then the mixtures of Ti-35.5Nb-5.7Ta were compacted in rubber molds by cold isostatic pressing technique using different pressures in the range of 200–450 MPa for several compaction times of up to 1 minute. The sintering temperatures were 900, 950, 1000, 1050 and 1100 °C with the sintering time of 3–10 h. The produced samples with around 23% porosity were selected for experiments. After the implants were sintered, ZrO_2/Zr coating was applied on the surface of the samples using Laser Engineered Net Shaping (LENS) technology. During the coating process, Zr was deposited on the surface of Ti-35.5Nb-5.7Ta alloy and then oxidized using LENS technology. Table I presents the list of the produced β-type Ti-35.5Nb-5.7Ta alloy samples, including information of sample ID, dimensions and processing conditions such as mixing time, compaction pressure, and sintering temperature and time. The samples were prepared by changing the particle sizes of Ti, Nb, and Ta powders including small (W group: Ti < 45 μm, Nb < 45 μm, Ta < 45 μm), medium (M group: Ti < 74 μm, Nb < 75 μm, Ta < 74 μm), and large (L group: Ti < 149 μm, Nb < 75 μm, Ta < 149 μm) with (Y) and without (N) ZrO_2/Zr coating.

2.2. Cell Culture

All standard GIBCO cell culture media, GIBCO AlamarBlue®, reagents and consumables were purchased from Fisher UK unless otherwise mentioned. The used cell line for the cytotoxicity assays in this study was human embryonic kidney 293T (HEK293T) cells. HEK293T Cells were maintained in 80 cm^2 tissue culture flasks under standard culture conditions of 5% CO_2 in air at 37 °C with medium renewal for every 2–3 days. The used culture medium was Dulbecco's Modified Eagle Medium (DMEM), low glucose supplemented with Glutamate (GlutaMAX), containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Sigma, UK) and 10% Fetal Calf Serum (FCS), referred to as DMEM complete medium in the experiments. When the cells reached >70–80% cell confluence, they were split with a 1:5 ratio. Cells with 23 times passages were used for the experiments.

2.3. Cytotoxicity Assay

The cytotoxicity of the disk metal samples as described in Table I, with the dimensions of 12 to 13 mm diameter, 0.5 mm thickness and surface area of $\sim$4.15 cm^2 was evaluated by two methods including (1) indirect and (2) direct contact cell culture. For the indirect cytotoxicity assay, the extracts were prepared from leachable materials of the samples and then the HEK293T cells were treated with the extracts under aseptic conditions. For the direct contact cytotoxicity assay, HEK 293T cells were seeded directly on the surface of samples.

2.3.1. Indirect Contact

The extraction of leachable moieties from the samples was performed in the sterile cell culture dishes using aseptic techniques in a standard 24 well tissue culture dish in a CO_2 incubator at 37 °C and 5% CO_2 in air under static conditions, in accordance to the ISO 10993-12 standard guidelines.[29] Accordingly, the samples were incubated in the DMEM complete medium (extraction medium) at 37 °C under 5% CO_2 in air for 7 days in a 24 well Nunc cell culture dish. Each sample was immersed in 1.5 ml of extraction medium. The samples were sterilized by washing with ultrapure deionised water for 2 h, 0.9% saline for 2 h and 70% ethanol for 24 h on a shaker with a rotation speed of 150 rpm. Then, under aseptic conditions, the samples from 70% ethanol were rinsed thoroughly in sterile PBS before the addition of extraction medium. Passage 23 HEK 293T cells were

Table I. The list of produced β-type Ti-35.5Nb-5.7Ta alloy with different production details including sample ID, manufacturing conditions (mixing time, compaction pressure, sintering temperature, and sintering time) and dimensions prepared by varying the particle sizes including small (W group: Ti < 45 μm, Nb < 45 μm, Ta < 45 μm), medium (M group: Ti < 74 μm, Nb < 75 μm, Ta < 74 μm), and large (L group: Ti < 149 μm, Nb < 75 μm, Ta < 149 μm) with (Y) and without (N) ZrO_2/Zr coatings.

Sample ID	Mixing time (h)	Compaction pressure (MPa)	Sintering temperature (°C)	Sintering time (h)	Thickness (mm)	Diameter (mm)	ZrO_2/Zr coating
W9	2	200	1000	10	5.478	12.341	N
W15	2	200	1100	6	5.349	11.951	N
W16	2	450	1100	6	4.78	12.6	N
W17	2	200	1100	10	5.522	12.035	N
W18	2	450	1100	10	4.742	12.261	N
W7c	2	200	1000	6	5.221	11.939	Y
W15c	2	450	1100	6	5.023	12.163	Y
M15	2	200	1100	6	5.504	12.518	N
M16	2	450	1100	6	4.814	12.567	N
M17	2	200	1100	10	5.298	12.008	N
M18	2	450	1100	10	4.695	12.494	N
L16	2	450	1100	6	4.821	13.192	N
L17	2	200	1100	10	5.335	13.146	N
L18	2	450	1100	10	4.784	13.062	N

trypsinized from subconfluent culture by adding 5 ml of 0.5× trypsin (Trypsin-EDTA (10×) liquid [0.5% Trypsin 5.3 mM EDTA.4Na]) solution to the cells in the 80 cm^2 tissue culture flask. The flask was incubated at 37 °C with 5% CO_2 in air. The trypsin reaction was stopped by adding 5 ml of DMEM complete medium. The cell suspension was then centrifuged at 200 g for 5 min. The cell pellet was suspended in 10 ml of DMEM complete medium. Cells were then counted with a hemocytometer and brought the concentration to 100,000 cells per ml. The cells were seeded at either 2000 or 5000 cells in 200 μl per well in a 96 well Nunc cell culture dish. The cells were allowed to attach in the wells for 24 h by incubating at 37 °C under 5% CO_2 in air. After 24 h of culture, 100 μl of culture medium was removed from each well and replaced with the prepared extracts from the samples. The extracts were added at dilutions of 100%, 75%, 50%, 25%, 10% and 0% in DMEM complete medium to make up 200 μl per well containing cells. Then the cells were exposed to the prepared extracts and were cultured for a further 24 h before adding the Alamar Blue. DMEM complete medium in wells without cells was considered as the negative control. Each test and negative control treatments were triplicated.

2.3.2. Direct Contact

For the cleaning and sterilization process, the samples were sequentially washed under aseptic conditions with deionized (DI) water, phosphate-buffered saline (PBS), 0.25% trypsin solution in PBS at 37 °C in 5% CO_2 incubator, PBS, 70% ethanol, PBS and DMEM complete medium containing Penicillin-Streptomycin and amphotericin B at 37 °C in 5% CO_2 incubator. Thereafter, HEK293T cells were seeded directly on the washed and sterilized samples in a 24 well Nunc cell culture dish. Cells were seeded at 10,000 cells per well in 1 ml of DMEM complete medium. The cells were allowed to attach in the wells for 24 h at 37 °C in 5% CO_2 incubator.

2.3.3. Alamar Blue Assay

Following 24 h cell incubation, 20 μl and 100 μl Alamar Blue solution was added to each well containing test extracts (indirect contact) and the samples (direct contact), respectively to obtain a 10% Alamar Blue solution. As negative control, Alamar Blue solution was added to the medium without cells. The plate was further incubated for about 24 h at 37 °C. The absorbances of test and control wells were read at 570 and 630 nm with a standard spectrophotometer. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction.[30] The calculation of the percentage of Alamar Blue reduction (%AB reduction) was performed as Eq. (1) according to the manufacturer's protocol:

$$\%\ \text{AB Reduction} = [((\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)) / ((\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1))] \times 100 \quad (1)$$

In the formula (Eq. (1)), $\varepsilon\lambda_1$ and $\varepsilon\lambda_2$ are constants representing the molar extinction coefficient of AB at 570 and 630 nm, respectively, in the oxidized (ε_{ox}) and reduced (ε_{red}) forms. $A\lambda_1$ and $A\lambda_2$ are the absorbance of test wells at 570 and 630 nm, respectively and $A'\lambda_1$ and $A'\lambda_2$ are the absorbance of negative control wells at 570 and 630 nm, respectively. The values of %AB reduction were corrected for the background values of negative controls containing medium without cells.

2.4. Cell Attachment

The attachment and morphology of cells on the samples in direct contact cell culture method was visualised

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using scanning electron microscope (SEM: JEOL JCM-6000 NeoScope benchtop). First, the cells on the samples were fixed by adding 100 μl of 10% neutral buffered formalin (NBF) (4% formaldehyde) (Sigma, UK). After ten minutes, the media plus NBF solution was replaced with 1 ml NBF and left over night to fix the cells on the samples. The samples were then rinsed thrice with PBS and were treated with 3% glutaraldehyde solution in PBS for 1 h. Thereafter, the samples were washed twice with PBS followed by dehydration of the cells on the samples through 0.5 h treatments in 30%, 50%, 70%, 95% and 100% ethanol solutions. The samples were then dried for 1 h at 60 °C in an oven. The fixed, dehydrated and dried samples were transferred into a vacuum desiccator. For SEM examination, the samples were mounted on the stubs and coated with gold using an AGAR high resolution sputter coater for 90 seconds. SEM images of the cells on the samples were recorded at 10 kV in the secondary electrons (SE) mode.

2.5. Statistical Analysis

Statistical analysis was carried out using statistical software (SPSS v.20). Statistical variances between groups were determined by one-way analysis of variance (ANOVA). Tukey's honestly significant difference test was used for post hoc evaluation of differences between groups. A p value of <0.05 was considered to be statistically significant. All data represented are expressed as mean value $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

The results of Alamar Blue cell cytotoxicity assays are presented in Figures 1 and 2. Figure 1 shows the percent reduction of Alamar Blue reagent incubated with HEK 293T cells exposed to the prepared extracts from samples and control groups in 24 h with cell seeding densities of approximately 2000 cells per well (Figs. 1(a, c)) and 5000 cells per well (Fig. 1(b)). The test extracts were from β-type Ti-35.5Nb-5.7Ta alloy samples (as summarised in Table I), prepared using metal particle sizes in three categories including small (W group: Ti $<$ 45 μm, Nb $<$ 45 μm, Ta $<$ 45 μm) in Figure 1(a); medium (M group: Ti $<$ 74 μm, Nb $<$ 75 μm, Ta $<$ 74 μm) in Figure 1(b); and large (L group: Ti $<$ 149 μm, Nb $<$ 75 μm, Ta $<$ 149 μm) in Figure 1(c). A comparison between W group (Fig. 1(a)) and L group (Fig. 1(c)) showed that by moving from W to L group, the percent reduction of Alamar Blue decreased, indicating that increasing the particle size had adverse effect on the cytocompatibility. It may be due to the corrosion behaviour of samples. Releasing ions into the cell culture media due to the electrochemical corrosion process has cytotoxicity effect for the cells. Although the corrosion occurs even for the samples with smaller particle sizes, the amount of corrosion in this case is less than that of the larger particle sizes since the corrosion is more uniform for a sample with smaller particle sizes. It has also been indicated that decreasing the grain size resulted in an improved biocompatibility of the commercially pure Ti.[31]

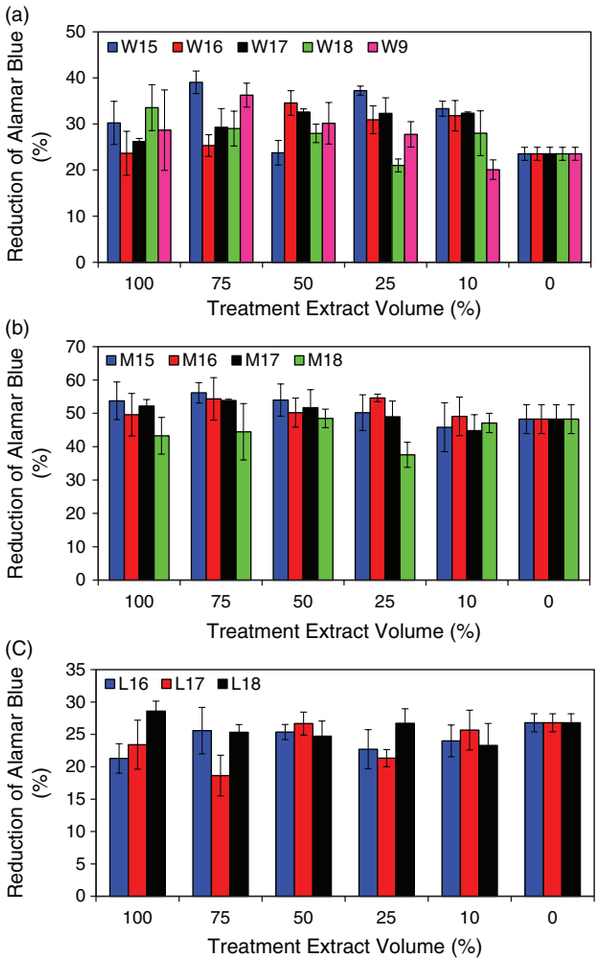

Fig. 1. Percent reduction of Alamar Blue reagent incubated with HEK 293T cells exposed to the prepared extracts from samples and control groups in 24 h with cell seeding densities of approximately 2000 cells per well (Figs. 1(a, c)) and 5000 cells per well (Fig. 1(b)). Test extracts were from β-type Ti-35.5Nb-5.7Ta alloy samples (as summarised in Table I) prepared using metal particle sizes of three categories including small (W group: Ti $<$ 45 μm, Nb $<$ 45 μm, Ta $<$ 45 μm) in Figure 1(a); medium (M group: Ti $<$ 74 μm, Nb $<$ 75 μm, Ta $<$ 74 μm) in Figure 1(b); and large (L group: Ti $<$ 149 μm, Nb $<$ 75 μm, Ta $<$ 149 μm) in Figure 1(c).

Figure 2 shows the percent reduction of Alamar Blue reagent incubated with HEK 293T cells exposed to the prepared extracts from samples and control group in 24 h with cell seeding densities of approximately 5000 cells per well (Fig. 2(a)) and 2000 cells per well (Fig. 2(b)). Test extracts were from ZrO_2/Zr coated β-type Ti-35.5Nb-5.7Ta alloy samples (as summarised in Table I) prepared using small particle sizes (W group: Ti $<$ 45 μm, Nb $<$ 45 μm, Ta $<$ 45 μm). According to the results of the indirect cell

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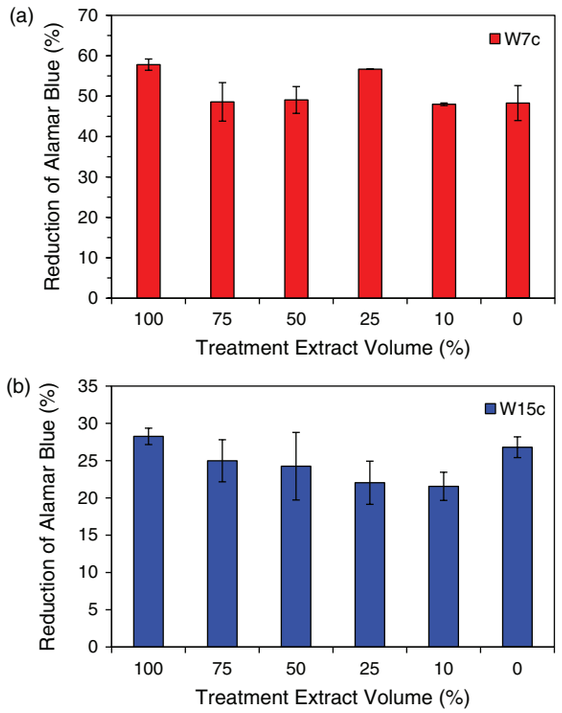

Fig. 2. Percent reduction of Alamar Blue reagent incubated with HEK 293T cells exposed to the prepared extracts from samples and control group in 24 h with cell seeding densities of approximately 5000 cells per well (Fig. 2(a)) and 2000 cells per well (Fig. 2(b)). Test extract were from ZrO_2/Zr coated β-type Ti-35.5Nb-5.7Ta alloy samples (as summarised in Table I) prepared using small particle sizes (W group: Ti < 45 μm, Nb < 45 μm, Ta < 45 μm).

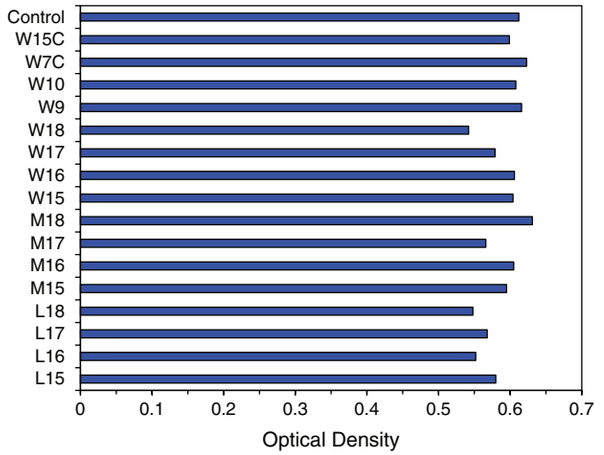

Fig. 3. Absorbance (optical density) of 10% Alamar Blue at 570 nm with reference to that at 630 nm after 24 h cell incubation indicating the reduction of Alamar Blue reagent incubated for 24 h with cells seeded at 10,000 cells per well on the different types of samples. Control wells did not have the samples, but were seeded with cells at the same density as the test wells.

culture (Figs. 1 and 2), none of the test extracts caused any statistically significant decrease in percent reduction of Alamar Blue reagent in comparison to the cells that were not exposed to any extracts (control group). Similarly, no morphological differences were observed for the cells exposed to any of the test extracts under the optical microscope according to our visual inspections during the indirect contact test. These results confirm that any ionic, chemical moieties or particles leaching from the samples are not cytotoxic. These results are in agreement with the observation made by Niinomi,[11] who evaluated the cytocompatibility of Ti-based implant including Nb, Ta and Zr elements.

Further cytotoxicity evaluation involved direct contact method, wherein the cells were seeded directly on the β-type Ti-35.5Nb-5.7Ta alloy samples. Figure 3 shows the absorbance (optical density) of 10% Alamar Blue at 570 nm with reference to that at 630 nm after 24 h cell incubation, indicating the reduction of Alamar Blue reagent incubated for 24 h with cells seeded at 10,000 cells per well on different types of samples. Control wells did not have the samples, but were seeded with the cells at the same density as the test wells. The optical densities indicating Alamar Blue reduction for the cells on different test variants were comparable to that observed for control wells seeded with cells at the same density.

Further investigation was made in this direction, paying particular attention to cell attachment. Cell adhesion is another factor that reflects cell/material interaction, mainly in applications that integrate the prosthesis to the bone in early stages. The adhesion of cells to substrate is essential for cell proliferation, differentiation and the formation of neo-tissue.[32] The morphology of attached cells on the samples was observed under SEM as illustrated in Figures 4 and 5. Figure 4 shows SEM images from (a) L15, (b) L16, (c) L17, (d) L18, (e) M15, (f) M16, (g) M17, (h) M18, (i) W15, (j) W16, (k) W17, (l) W18, (m) W9, (n) W10, (o) W7C, and (p) W15C samples (sample designation as in Table I), from which the morphology of cells on the samples can be seen. According to Figure 4, cells attached and well-spread cells were observed on the surfaces. However, there was no significant difference in cell spreading on any of the samples.

The SEM images in Figure 5 show the cell attachment and spreading on uncoated or porous (Figs. 5(a, b)) and ZrO_2/Zr coated or non-porous (Figs. 5(c, d)) samples. The uncoated surfaces have been designed to be in contact with the bone tissues without relative movement in real applications. For this purpose, the uncoated samples have around 23% porosity in order to encourage the biological fixation by providing sites for tissue in-growth. Existence of porosity in the structure gave rise to a high surface roughness for the uncoated samples. However, the moving parts between metal (implant)—bone and metal–metal (friction between the acetabular cell and the ball etc.) cause the release of metal shavings in the blood. That is why in this research a coating of ZrO_2/Zr has been applied on the surface to provide high wear resistance in these areas. Applying the

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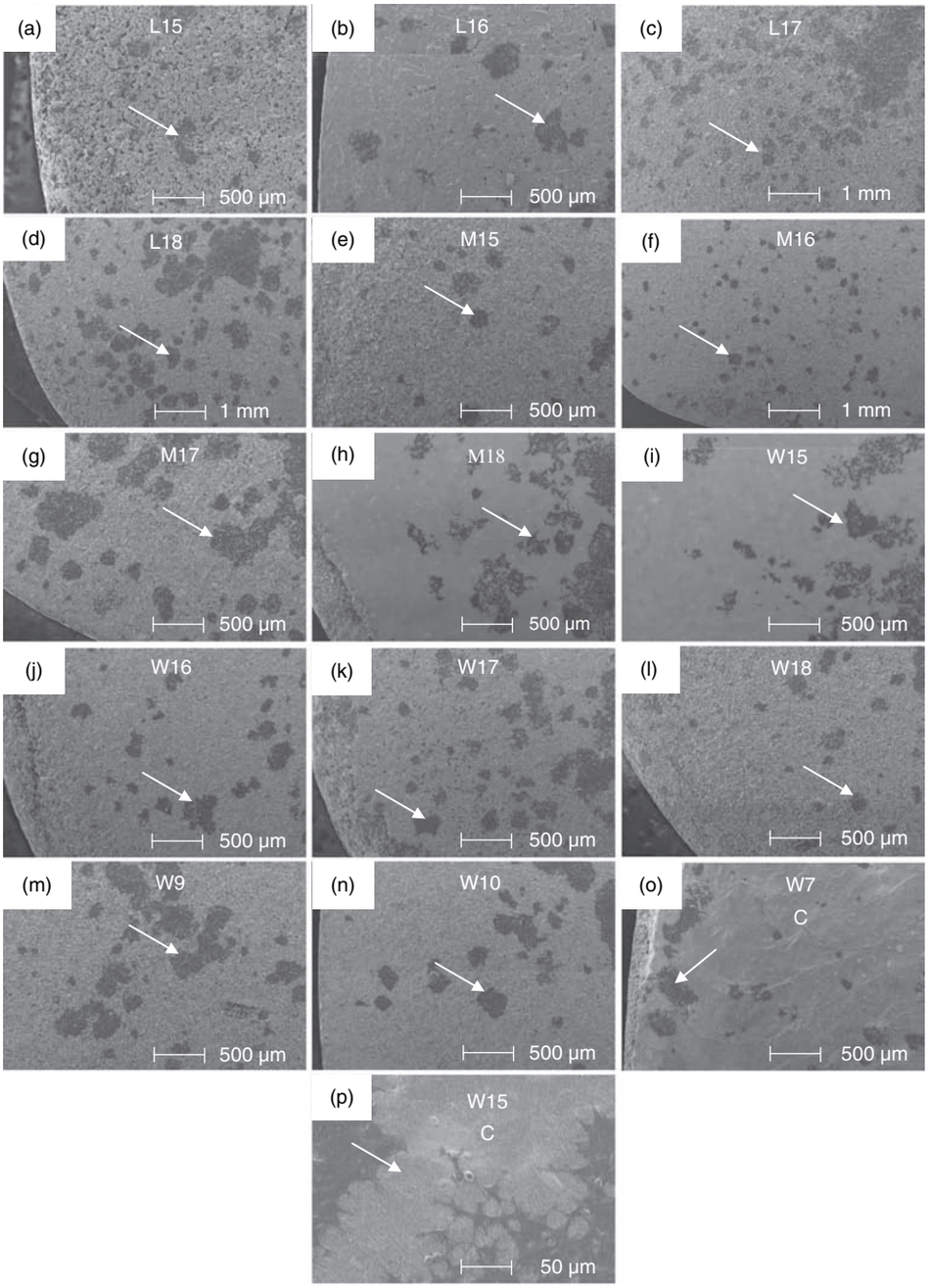

Fig. 4. SEM images from (a) L15, (b) L16, (c) L17, (d) L18, (e) M15, (f) M16, (g) M17, (h) M18, (i) W15, (j) W16, (k) W17, (l) W18, (m) W9, (n) W10, (o) W7C, and (p) W15C samples (sample designation as in Table I) showing the morphology of cells on the samples.

ZrO_2/Zr coating on the surface filled the surface pores results in a decrease in the surface roughness compared to uncoated samples. Accordingly, the surface roughness of uncoated sample was much more than that of the ZrO_2/Zr coated one due to the existence of porosity in their structure. Surface properties of the samples such as surface roughness would have crucial effect on the cell attachment and proliferation in direct contact cytotoxicity assay. It can be seen from Figure 5 that the cells are well spread on the samples with a few filopodia at the cell periphery. For the uncoated samples with a rough surface (Figs. 5(a, b)), the cells appeared to extend cytoplasmic processes (pseudopodia) into the pores, while on ZrO_2/Zr coated or non-porous (Figs. 5(c, d)) samples with lesser roughness, the cytoplasmic processes was observed spreading on the surface. Multiple colonies of cells were seen on all samples, suggesting that the cells readily attached, and proliferated and spread on the surface of samples (Figs. 4 and 5).

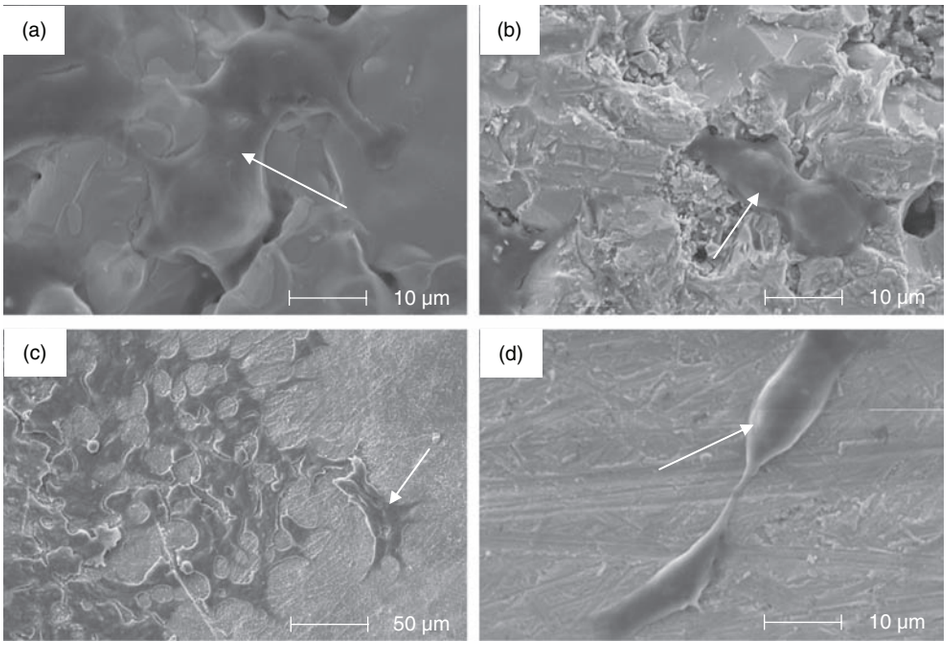

Fig. 5. SEM images showing the cell attachment and spreading on uncoated or porous (Figs. 5(a, b)) and ZrO_2/Zr coated or non-porous (Figs. 5(c, d)) samples.

The broad aim of this project was to develop a new generation of biomaterials by modifying the used raw materials, manufacturing procedures and applying a coating that would provide orthopaedic implants. Poor biocompatibility can cause

(a) thrombosis, which involves blood coagulation and adhesion of blood platelets to the implant surface, and
(b) the fibrous tissue encapsulation of the implants.[33]

Another issue relevant with the biocompatibility is the release of metal ions (shavings) from the implants into the tissue and circulatory system. The release of metal shavings can be avoided if materials with excellent wear resistance are used in the moving part of the implant where the friction is high. The ZrO_2/Zr coating was applied in order to reduce friction and wear. The LENS technology, in combination with the following heat treatment in the present development, allowed the ZrO_2 layer to be formed gradually on top of a pure Zr layer, without introducing severe residual stress. Hence the possibility of de-bonding for the ZrO_2/Zr coating is diminished. With this coating, the wear is expected to be largely reduced and so is the possibility of osteolysis. ISO10993-5 standard[34] provides guidelines for the *in vitro* biocompatibility testing of biomaterials. This testing can be done either by direct contact of cells with the material, or by indirect contact with the extracts obtained from the material by immersing the samples in an appropriate extract vehicle (preferably, the corresponding cell culture medium used for the cells of choice) to the cells. Furthermore, *in vitro* biocompatibility testing using human cells is desirable for biomaterials intended for human applications. HEK 293T cells when seeded at 2000 cells per well in a 96 well plate takes 5–7 days to reach confluence. Accordingly, approximately 2000 or 5000 cells per well were seeded for this pilot cytotoxicity study using Alamar Blue assay to ensure that they don't become confluent (complete coverage of the available culture well surface by cells) for the period of three days. In Alamar Blue assay, the oxidised form of Alamar Blue molecules are readily taken up by the cells and are reduced intracellularly by oxidoreductases and the mitochondrial electron transport chain, with a corresponding shift in its absorbance and fluorescence.[35] The reduced form diffuses back from the cells into the culture medium, where it can be sampled or measured directly in the microplate by fluorometery or dual wavelength spectrophotometry.[35] Unlike other common cytotoxicity assays, e.g., MTT and Neutral red, Alamar Blue reagent is not cytotoxic, and hence allows monitoring of cell viability for several days without killing the cells.[36] Cells, in general, can be incubated in Alamar Blue reagent for durations ranging from 1 to 24 h or more, depending on the metabolic rate of the cells.[36] However, with the HEK 293T cells we observed that the rate of Alamar Blue reduction was slow and required more than 4 h for colour change from blue to pink for naked eye. Hence we incubated the cells for another 24 h before reading the absorbance at 570 nm and 630 nm.

4. CONCLUSION

The present work focused on the cytotoxicity of a newly developed uncoated and ZrO_2/Zr coated β-type Ti-35.5Nb-5.7Ta alloy by studying the behaviour of human embryonic

kidney 293T cells (HEK293T) through the indirect and direct contact cell culture methods. Based on the results obtained from the cytotoxicity assessment by the Alamar Blue assay, it can be concluded that, the uncoated and ZrO_2/Zr coated β-type Ti-35.5Nb-5.7Ta alloy examined here do not cause toxic effects and show good cell adhesion, indicating *in vitro* cytocompatibility to human HEK293T cells. Future researches are focused on the cytocompatibility studies of the developed biomaterial using other cell types such as human mesenchymal stem cells and human osteoblast cells.

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