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Original Article

The novel toxic free titanium-based amorphous alloy for biomedical application

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ABSTRACT

Ti based amorphous alloy exhibits excellent properties for biomedical applications. In general, it has high strength, low elastic modulus, good corrosion resistance and satisfactory biocompatibility. This work reported on a systematic study of a novel $Ti_{44}Zr_{10}Pd_{10}Cu_6Co_{23}Ta_7$, $Ti_{44}Zr_{10}Pd_{10}Cu_{10}Co_{19}Ta_7$ and $Ti_{44}Zr_{10}Pd_{10}Cu_{14}Co_{15}Ta_7$ metallic glass. Cylindrical rod samples with diameter of 5 mm and 20 mm length were fabricated by induction melting and casting into copper mold. The cast rod was then used as plasma cathode in filtered cathodic vacuum arc (FCVA) deposition chamber. The Ti-based metallic glass (MG) thin film was produced and tested for subsequent cell culture investigation to understand the biocompatibility nature of the new alloy. X-ray photoelectron spectrometry (XPS) was employed to characterize the surface chemistry. The Ti-6Al-4V alloy was studied in parallel as a control material. This novel Ti-based MG composition has shown promising osteoblast biocompatible characteristics and no cytotoxicity on human osteoblast-like cells (SaOS-2). Moreover, cells on Ti-based MG thin film exhibited greater levels of calcium deposition using Alizarin red staining technique to those of the control. All results point out that the novel Ti-based amorphous alloy has potential for using as a new coating for biomedical application and deserve further study.

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1. Introduction

Metallic glass or amorphous alloys are metal with an amorphous microstructure. The component atoms of amorphous alloys are randomly packed, instead of arranged in usual crystalline structures. This microstructure leads to many excellent

mechanical properties and high processability, which has inspired great interest in their biomedical applications [1–3].

The unique properties of amorphous alloys have a potential to solve problems encountered by current biomedical materials for example elastic moduli closer to bone which leading to reduction in stress shielding problem, better hardness and corrosion resistance [4,5]. Amorphous alloy can be manufactured in a form of metal foam to gain further Young's modulus matching to human bone [6,7]. Amorphous alloy generally demonstrates excellent wear resistance comparing with

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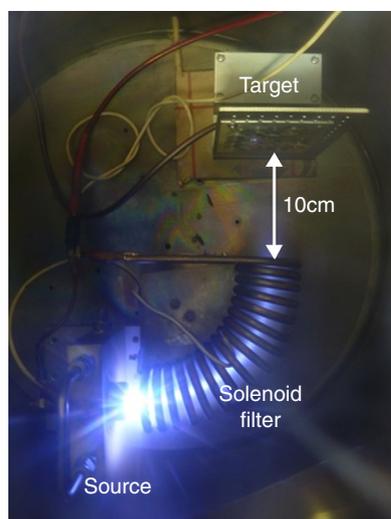


Fig. 1 – Setting for filtered cathodic vacuum arc coating.

traditional alloy [8], then it has potential to prevent wear debris and its sequelae. Without crystalline structure, the amorphous alloy is considered free of crystalline defects. These lead to superior corrosion resistance to crystalline alloys in previous reports [9,10]. Finally, the high processability of the amorphous alloy is a huge advantage to their clinical application potential.

Many of current amorphous alloys contain toxic elements like Beryllium, Aluminum or Nickel that had been reported about causing cancer or allergy. These toxic ions limit the use of amorphous alloy as biomaterial. To create a new type of biomedical amorphous alloy, the safety of use is the first concerning issue. The purposes of this work are to synthesize the new type of amorphous alloys for without toxic elements for using as a new coating for biomedical application.

2. Materials and methods

Ti-based alloy ingots with a nominal composition of the $\text{Ti}_{44}\text{Zr}_{10}\text{Pd}_{10}\text{Cu}_{6+x}\text{Co}_{23-x}\text{Ta}_7$ ($x=0, 4, 8$) was synthesized using arc-melting 6 elements of 99.9% or better purity in a titanium-gettered argon atmosphere. The Ti-6Al-4V alloy was used as a reference material. Cylindrical rod samples with a diameter of 5 mm and length of 20 mm were fabricated by copper mold casting technique. The cylindrical rods of 3 new alloy formula and Ti6-Al-4V as a reference material were then used as plasma cathode in filtered cathodic vacuum arc (FCVA) deposition technique to make Ti-based metallic glass thin film (Fig. 1).

The round glass substrate with a diameter of 1.5 cm and thickness of 0.5 mm were attached to the aluminum plate with an aluminum tape then posted at 10 cm away from the solenoid filter as a target for FCVA coating (Fig. 1). The parameter for FCVA is shown in Table 1. The Ti-based thin film on glass substrate was further employed for alloy characterization tests and biocompatibility tests.

Table 1 – Parameters for filtered cathodic vacuum arc (FCVA) deposition.

Parameters for FCVA	
Resistivity	<2 k Ω
Distance from solenoid to target	10 cm
Time	60 min
Pressure	<5.0 $\times 10^{-5}$
Pulse	2.1 Hz
Voltage	600 V
Bias target	1 kV

2.1. Thin film characterization

2.1.1. X-ray photoelectron spectrometer (XPS)

X-ray photoelectron spectroscopy (XPS) is an essentially non-destructive technique that used to analytical chemical bonding, element composition, chemical and electronic state of every element in the material.

In this work, the XPS spectra were studied at Synchrotron Light Research Institute (Public Organization, Thailand) BL5.2: SUT-NANOTEC-SLRI by using a ULVAC-PHI Versa-Probe II XPS at energy step 0.05 eV, pass energy 46.95.

2.2. Biocompatibility testing

We performed biocompatibility test with osteoblast like cell (SaOS-2) In Vitro. Before every test, the coated discs were sterilized by autoclaving.

2.2.1. Culture condition

Osteoblast cell line, osteoblast-like cell (SaOS-2), was used for biocompatibility test in the present study. The SaOS-2 was cultured in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin and 0.25 $\mu\text{g mL}^{-1}$ amphotericin B. Cells were maintained at 37 °C in 100% humidity and 5% CO_2 . Confluent cells were detached using 0.25% trypsin with ethylene diamine tetraacetic acid and re-suspended in fresh culture medium. The media were changed every 2–3 days.

2.2.2. Cell proliferation

Cell proliferation was determined by Methylthiazol Tetrazolium (MTT) assay. Cells were seeds on triplicate samples discs ($n=3$) with a concentration of 50,000 cells/well in a 24-well plate. The assay was performed at 3, 5 and 7 days. After each culture period, the media was gently removed and the specimens were rinsed with phosphate-buffered saline (PBS) to remove unattached cells and to avoid the effects of media on the biochemical assays. Then MTT solution (300 μL ; 0.5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide in culture medium without phenol red) was added. After 30 min of incubation, MTT solution was discarded and then the formazan crystals were dissolved in dimethylsulfoxide (DMSO) (900 μL /well) and glycine buffer (pH = 10) (125 μL /well). The absorbance was read at a wavelength of 570 nm by Thermospectronic Genesis 10 UV-vis spectrometer.

2.2.3. Cell differentiation

For the osteoblast differentiation, cells were grown on triplicate sample disc ($n=3$) with a concentration of 60,000 cells/well in a 24-well plate. After 2 days of cultured, the media was changed to differentiation medium (normal culture medium supplemented with $50 \mu\text{g mL}^{-1}$ ascorbic acid, 5 mM β -glycerophosphate and 250 nM Dexamethasone). Cells were cultured in differentiation medium for 1, 5 and 10 days. Cell differentiation behavior was characterized by Alizarin red staining for calcium deposition analysis.

2.2.3.1. Alizarin red staining. Alizarin is an organic compound that could react with calcium ions. Alizarin red S, a dye that stains calcium salts selectively and is widely used for mineral histochemistry of calcium, served to analyze the mineralization level of cells. In this study, SaOS-2 100,000 cells were seeded onto the disc. The osteogenic inductions were induced after 24 h. On day 28, the medium was discarded then the cells on the discs were fixed with 95% ethanol for 10 min then rinsed several times with distilled water. 0.1% Alizarin red was added onto the disc then incubated at 37°C for 30 min and rinse several times with distilled water before proceeded to light microscope evaluation under $10\times$ magnification.

2.2.3.2. Alkaline phosphatase. The differentiation of osteoblasts cells was evaluated via a function of alkaline phosphatase activity. After each culture period, the media were gently removed and rinsed with PBS. The extract buffer ($100 \mu\text{L}$) was added to each well. The aliquot was incubated with the substrate of p-Nitrophenyl Phosphate (pNPP; $110 \mu\text{L}$) containing 0.1M aminopropanol. After incubating at 37°C for 15 min, NaOH (0.1M; $900 \mu\text{L}$) was added to stop the reaction. ALP activity was measured by monitoring the color change of pNPP. The absorbance was measured at 410 nm wavelength using Thermospectronic Genesis 10 UV-vis spectrometer. Total protein was determined using BCA assay. ALP enzymatic activity was normalized to total protein.

3. Results

3.1. Thin film characterization results

3.1.1. X-ray photoelectron spectrometer (XPS)

We found that the binding energies of Ti 2p, O 1s, Cu 2p, Co 2p, Pd 3d and Ta 4d shift from the reference [11]. Figs. 2 and 3 represent the XPS spectrum of Ti 2p at binding energy 457.55 eV and 463.23 eV and O 1s at 528.81 eV corresponds to binding energies for TiO_2 [12,13].

3.2. Biocompatibility test results

After the SaOS-2 cells were thawed from cryopreserve vial, the cells were resuspended in fresh medium then incubated in the cell culture incubator supplied with 5% CO_2 , 95% O_2 at 37°C . At 5 days after thawing process, the photo was taking at confluency around 90%. It is shown epithelial like cell morphology with adherent on plastic surface (culture container) (Fig. 4).

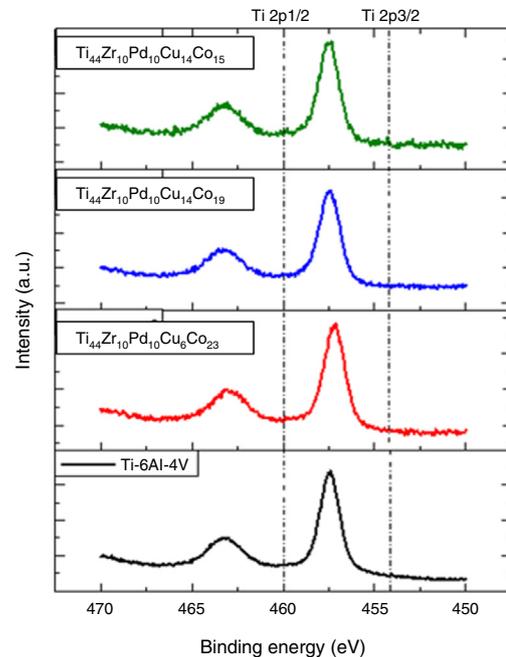


Fig. 2 – XPS spectrum of Ti 2p at binding energy at 457.55 eV and 463.23 eV.

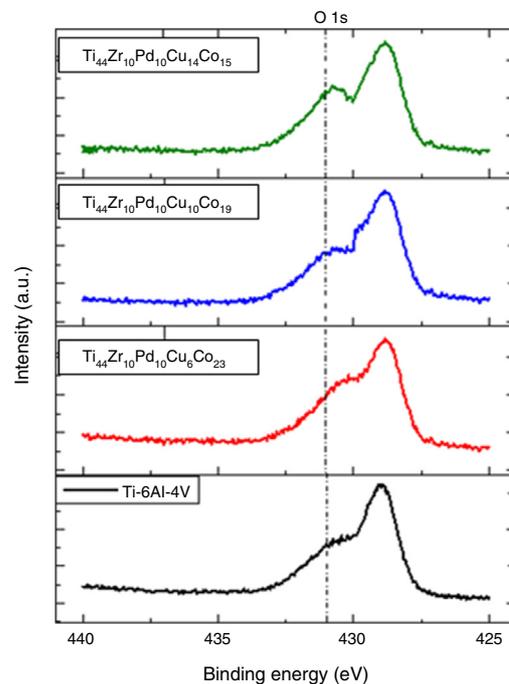


Fig. 3 – XPS spectrum of O 1s at 528.81 eV.

3.2.1. Cell proliferation

The MTT assay is a colorimetric assay technique utilizing the ability of metabolically active cells that reduce a yellow tetrazolium-based compound to a purple formazan product. The number of living cells in the culture is directly related to the quantity of formazan product, which is measured by the absorbance at 570 nm of wavelength. After cells were exposed to Ti-based MG thin film for 3 days, 5 days and 7 days, there was no significant difference compared to the glass substrate

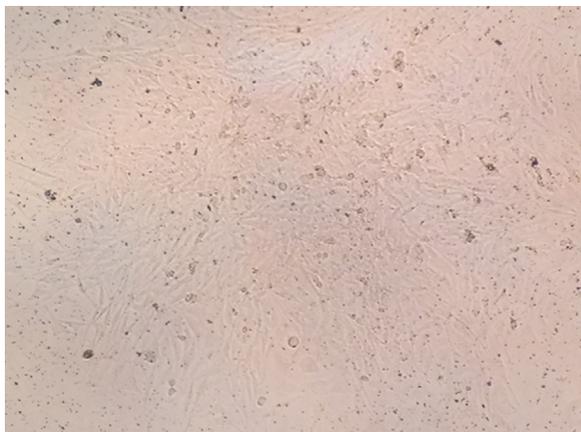


Fig. 4 – The SaOS-2 cells morphology and adherent at 5 days after thawing process.

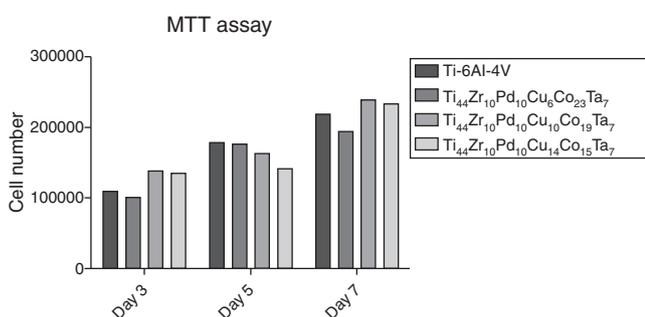


Fig. 5 – The MTT assay of SaOS-2 cells on Ti-based MG thin film.



Fig. 6 – The Alizarin red staining result in negative control samples.

and Ti-6Al-4V control, suggesting that Ti-based MG thin film was not toxic to SaOS-2 (Fig. 5).

3.2.2. Cell differentiation

3.2.2.1. Alizarin red staining. The Alizarin red staining result in negative control samples clearly revealed that no calcium mineralization had been detected (Fig. 6).

Alizarin red staining in Ti-6Al-4V and 3 novel metallic glass alloys, the results are shown in Fig. 7. The alkaline phosphatase study results are shown in Fig. 8.

Alizarin red staining results was analyzed using Image J program (set color threshold as RGB: 200, 120, 80) to quantify the calcium mineralization. The results are demonstrated in Tables 2 and 3. The Ti₄₄Zr₁₀Pd₁₀Cu₆Co₂₃Ta₇ and Ti₄₄Zr₁₀Pd₁₀Cu₆Co₂₃Ta₇ shown significantly more Ca deposit area and also the bigger size of calcium deposit comparing with Ti-6Al-4V.

4. Discussion

In the previous report, the new Ti-based amorphous alloy composite without toxic elements has been synthesized in Ti-Zr-Cu-Pd alloy system such as Ti₄₀Zr₁₀Cu₃₆Pd₁₄, which exhibit high corrosion resistance and good combination of strength and ductility, implying a high potential as biomaterials [14]. We developed our metallic glass based on this combination. We decided to decrease Copper due to the reported cytotoxicity of Cu as released from Zr-based BMG during 3T3 fibroblast cell line tests [15]. We had decided on many potential element to replace Cu. Finally, the additional elements that we chose were Ta and Co. We selected Ta because it is believed to be a more effective element to increase corrosion resistance and biocompatibility of metal materials. Qin et al. added Ta into Ti-Zr-Cu-Pd alloy and found higher strength and better plastic deformation comparing with Ti-Zr-Cu-Pd base alloy [16]. Another element that we chose is Cobalt. Louzguine et al. described the effect of the addition of 5 at.% Cobalt to replace Copper on the mechanical properties and stability of the supercooled liquid of Cu₆₀Zr₃₀Ti₁₀ amorphous alloy. They concluded that the addition of Copper to Cu-based metallic glass improved the mechanical properties, stabilizes the supercooled liquid, increased Young's modulus and compressive strength of the alloy [17].

XPS spectra suggested that on the surface of all samples (Ti-6Al-4V and series of novel amorphous alloy) demonstrated oxide of Ti. The oxidation of each element depends on "the Standard Electrode Potential (SEP), which is evaluated relative to the standard oxidation of hydrogen gas, measures the ability of the metal atoms to get oxidized" [18]. A lower SEP corresponds to an easier oxidation reaction. The mechanism for oxidation is still being explored in the field of bulk metallic glass due to the fact that the oxidation behaviors are combinations of various factors. For instance, Oh et al. [19] reported the phase separation that occurred in Cu₄₃Zr₄₃Al₇Ag₇ bulk metallic glass in which gold and silver formed nanometer level localized cluster. The copper cluster would readily oxidize in ambient according to SEP. However, the standard potential could not directly relate to the oxide observation that was created during the synthesis because of elevated temperature and environment. In this report, the detection of oxides could not be directly related to SEP. The thin oxidized layer was created at high temperatures during the synthesis, and consequently the film became protective film at room temperature after the synthesis. When Ti-6Al-4V and Ti₄₄Zr₁₀Pd₁₀Cu_{6+x}Co_{23-x}Ta₇ (x = 0, 4, 8) were exposed to air and investment mold material during casting, oxidation layer formed. Similar XPS results were

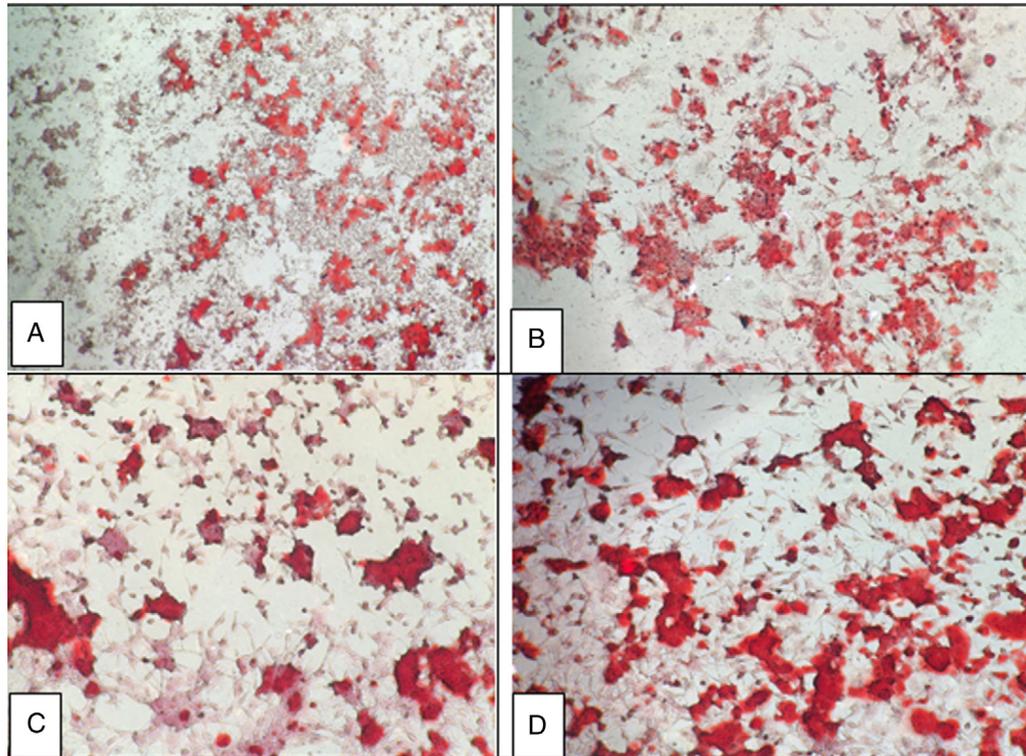


Fig. 7 – The Alizarin red staining results of SaOS-2 cells on Ti-based MG thin film.

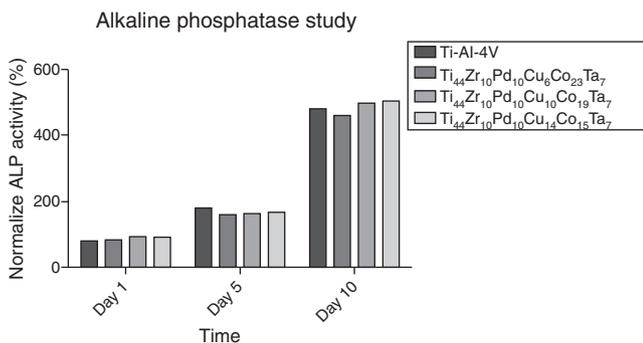


Fig. 8 – The alkaline phosphatase study results.

previously reported on Ti-Zr-Pd-Cu-Sn BMG systems [20]. The XPS also confirmed that the thin film created by FCAV technique contained all elements the same as their novel alloy ingots.

Copper containing crystalline alloy and amorphous alloy has been reported about cell toxicity. Buzzi et al. [15] reported cytotoxicity to fibroblast cell line from Zr based amorphous alloy containing Cu ($Zr_{58}Cu_{22}Fe_8Al_{12}$). Elshahawy et al. [21] stated that Cu released from gold alloys, which are commonly

used as fixed prosthodontic restorations, show evidence of a high cytotoxic effect on fibroblast cells. In contrast, some previous research did not demonstrated negative effects of copper containing alloys to the cells [22] and there were compatible with results of our study. The explanation of this issue is the formation of TiO_2 that developed on the surface of novel Ti based amorphous alloy which contain Ti 44 at.%. The TiO_2 has been reported about ability to provide good biocompatibility and bactericidal effect [23]. It may conceal the copper from direct contact to the cells or decrease the copper ion release into the cell growth medium to the optimum level. In addition, our novel alloy compositions contain lower amount of copper than the alloy previously reported, then may lead to less toxicity from copper.

Some of previous study reported about biocompatibility and mechanical property of the toxic free amorphous alloy. Zhu et al. [24] have developed amorphous alloy with component of $Ti_{40}Zr_{10}Cu_{40-x}Pd_{10+x}$ (with $x=0, 2, 4, 6, 8$ and 10). They found good glass forming ability, compressive strength, Young's modulus and an elastic elongation. They suggested that their series of new alloy were suitable for application to biomaterials. Oak conducted human cell test (SaOS-2) on $Ti_{45}Zr_{10}Pd_{10}Cu_{31}Sn_4$. They found results of good biocompatibility and glass forming ability [25,26]. However, due to the

Table 2 – Quantitative analysis of Alizarin red staining using Image J program.

	Uncoated cover glass	Ti-6Al-4V	$Ti_{44}Zr_{10}Pd_{10}-Cu_6Co_{23}Ta_7$	$Ti_{44}Zr_{10}Pd_{10}-Cu_{10}Co_{19}Ta_7$	$Ti_{44}Zr_{10}Pd_{10}-Cu_{14}Co_{15}Ta_7$
Total area (pixels)	0	41,533 ± 25,726	63,936 ± 12,569	78,721 ± 23,853	188,047 ± 34,563
Average size (pixels)	0	96.3 ± 18.6	103.7 ± 5.3	310.5 ± 27.6	398.9 ± 106.5

Table 3 – The comparison of area and size of calcium deposit between Ti–6Al–4V and the novel alloys.

	Ti ₄₄ Zr ₁₀ Pd ₁₀ –Cu ₆ Co ₂₃ Ta ₇	Ti ₄₄ Zr ₁₀ Pd ₁₀ –Cu ₁₀ Co ₁₉ Ta ₇	Ti ₄₄ Zr ₁₀ Pd ₁₀ –Cu ₁₄ Co ₁₅ Ta ₇
Ti–6Al–4V			
Total area	P 0.03	P 0.006*	P < 0.0001*
Average size	P 0.27	P 0.0001*	P < 0.0001*

* Significant, *p* value < 0.05.

nature of novel material, it is impossible to find a previously match amorphous alloy study to compare with current study results. In our study, after cells were exposed to all novel amorphous alloy samples for 3 days, 5 days and 7 days, there was no significant difference compared to the glass substrate and Ti–6Al–4V control, suggesting that Ti-based MG thin film was not toxic to SaOS-2.

5. Conclusion

A novel Ti-based amorphous alloy Ti₄₄Zr₁₀Pd₁₀Cu_{6+x}Co_{23–x}Ta₇ (*x* = 0, 4, 8) demonstrated biocompatible characteristic to osteoblast like cells (SaOS-2). No cytotoxic was found during the cell culture and proliferation. A novel amorphous alloy thin film shown supporting of the osteogenic differentiation process of SaOS-2 cells and the differentiation was even better than control Ti–6Al–4V. All results point out that the novel Ti based amorphous alloy has potential for using as a new coating for biomedical application.

Conflicts of interest

The authors declare no conflicts of interest.

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