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# Application of novel anodized titanium for enhanced recruitment of H9C2 cardiac myoblast

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ABSTRACT

***Objective(s):*** Anodized treated titanium surfaces, have been proposed as potential surfaces with better cell attachment capacities. We have investigated the adhesion and proliferation properties of H9C2 cardiac myoblasts on anodized treated titanium surface.
***Materials and Methods:*** Surface topography and anodized tubules were examined by high-resolution scanning electron microscopy (SEM). Control and test substrates were inserted to the bottom of 24-well tissue culture plates. Culture media including H9C2 cells were loaded on the surface of substrate and control wells at the second passage. Evaluation of cell growth, proliferation, viability and surface cytotoxicity was performed using MTT test. After 48 hr, some samples were inspected by SEM. DAPI-staining was used to count attached cells.
***Results:*** MTT results for cells cultured on anodized titanium and unanodized titanium surfaces was equal to 1.56 and 0.55 fold change compared to tissue culture polystyrene (TCPS). The surface had no cytotoxic effects on cells. The average cell attachment to TCPS, unanodized and anodized titanium surface was 2497±40.16, 1250±20.11 and 4859.5±54.173, respectively. Cell adhesion to anodized titanium was showed 1.95 and 3.89 fold increase compared to TCPS and unanodized titanium, respectively ($P<0.05$).
***Conclusion:*** Anodized titanium surfaces can be potentially applied for enhanced recruitment of H9C2 cells. This unique property makes these inexpensive anodized surfaces as a candidate surface for attachment of cardiac cells and consequently for cardiac regeneration purposes.

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## Introduction

Cardiac regenerative medicine is rapidly growing in order to treat the patients suffering from heart diseases through supporting or replacing non-functional cardiac tissues (1-4). Various tissue engineering methods have been proposed so far such as proper induction of cell differentiation, usage of highly efficient scaffolds, etc., (5, 6). Ultimately, high-yield tissue engineering involves construction of tissue equivalents from cells seeded within 3-D biomaterials followed by subsequent culturing and implantation of cell-seeded scaffolds (1, 7).

In terms of enhanced cell attachment to the applied scaffold, search for optimal biomaterial surfaces for myocardial tissue is still going on. Like other cells, an indicated substrate for this purpose might show biocompatibility without any toxic leaching and permitting cells with close contact with these substrates to do their natural activities (8-11).

Among substrates used in tissue engineering, titanium has been widely employed due to its biocompatibility, elastic shape memory as a smart biomaterial, excellent corrosion resistance and suitable mechanical properties (12-16).

Some surface treatment strategies have shown to be more cell-friendly with enhanced cell attachment as well as enhanced cell adhesion. Search for finding the most effective treatment of titanium is still continuing. Among surface modification processes, anodization (anodic oxidation), facilitates formation of $TiO_2$ nano-textured and nano-tubular on the metal surface (4, 17-19).

The altered metal topography by anodization prohibits uncontrolled metal oxidation, chemical reactions and corrosion while provides a natural environment for cell growth. Lack of elicited inflammatory reactions and macrophage adhesion has also been demonstrated previously (20). Therefore, we hypothesized that these anodized titanium surface might be a suitable substrate for

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for particular cardiac cell-culture and tissue-engineering applications. The mere drawback for application of introduced anodization technique is its high cost due to the expensive platinum used for serving as a cathode pole for anodization. Hereby, we present an inexpensive anodization method by using stainless steel to serve as the cathode pole. Thus, in this study, the biocompatibility of modified anodized titanium was evaluated by assessing the adhesion and proliferation of H9c2 rat cardiac myoblasts.

## Materials and Methods

### *Sample preparation*

A sheet of Commercially Pure Titanium Grade 1 ASTM B 265 (William Gregory ltd, UK) with thickness of 1 mm was cutted into an 8 × 5 cm pieces using an automatic metal cutter machine. Titanium pieces were cleaned with liquid soap and 70% ethanol for 10 min in an ultrasonic bath. Substances were dried in an oven at about 65 ˚C for 30 min in order to prepare them for anodization. After anodization, substrates were ultrasonically washed in an ultrasonic bath with acetone for 20 min and 70% ethanol for 20 min. To remove naturally formed oxide layers, substrates were immersed in an acid mixture containing 2 ml of 48% Hydrofluoric acid, 3 ml of 70% HNO3 and 100 ml deionized (DI) water for 5 min prior to anodization. Some of acid-polished substrates were treated immediately by anodization. Titanium substrates were used as the anode in anodization process while a stainless steel sheet served as the cathode. The novelty of this method is application of stainless steel instead of platinum as the cathode pole, which makes it cost-saving method (Patent no. V12011A000166). Anode and cathode poles were connected by copper wires and linked to positive and negative port of a 30 V/3 A power supply, respectively. While processing, anode and cathode poles were kept in parallel at a distance about 2 cm and submerged into an electrolyte solution of diluted HF (1.5 wt %) in a Teflon beaker. A constant potential of 20 V between anode and cathode pole was applied. After accomplishment of anodization process within 20 min, substrates were rinsed thoroughly with DI and dried in an oven at about 65 ˚C for 30 min. Anodized nano- tubular titanium was then cutted into small pieces of 1×1 $cm^2$ with automatic cutter machine. Then, sheets were cleaned serially with tetra chloro ethylene for 20 min in acetone and 20 min in 70% Ethanol in ultrasonic bath. Treatment with UV for 30 min was applied for sterilization of anodized nano-tubular titanium pieces. A piece of unanodized 1×1 $cm^2$ titanium sample was used as a control after cleaning through above-mentioned serial procedure. Indeed, one tissue culture polystyrene plate was also served as a control surface (21).

### *Cell culture*

H9C2 cells, an immortalized cell line derived from fetal rat hearts were purchased from National Cell bank of Iran (NCBI). Cells at a density of $10^5/cm^2$ were cultured as monolayers in Dulbecco's Minimal Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were counted under invert microscopy using improved hemo-cytometer neobar. Culture medium was replaced by fresh medium every two days. Control and test substrates were inserted to the bottom of 24-well tissue culture plates. Before loading cells on the surface of substrates, PBS used in protein coating step was completely removed from wells and 1 ml of the culture media including cells was added to the wells including substrates. Plates were incubated at standard cell culture conditions (temperature 37 ˚C and $CO_2$ 5%). Incubated plates were used for evaluation of cell attachment and cell growth and proliferation. For each evaluation, all experiments were done in triplicate.

### *Cell growth, proliferation, viability and surface cytotoxicity*

About $2\times10^4$ cells were cultured on the substrate surface in 12 well plates for 48 hr. Then, MTT (5 mg/ml) was added to the culture media and plates were kept in incubator. After 4 hr, culture media was removed and acidic isopropanol (100 ml) was added (kept for 10 min at 37 ˚C). Cells were centrifuged for 5 min at 14000 rpm and supernatant was used for analysis by ELISA reader at 570 nm. Reference wave length was 630 nm.

### *Cell attachment assay*

To evaluate cell attachment, $5\times10^4$ cells/ml were incubated at 37 ˚C in 12-well plates with substrates. After 4 hr, culture media was removed and cells were washed twice with PBS. Then, cells were treated with paraformaldehyde 4% for 30 min at 4 ˚C. After washing with PBS, cells were treated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) in dark room for 15 min. Subsequently, cells were washed twice with PBS and attached cells to the substrates were counted by fluorescence microscope. Untreated tissue culture polystyrene was used as a negative control.

### *Electron microscopic evaluation*

About $5\times10^4$ cells/ml were incubated at 37 ˚C in 12-well plates with substrates. After 4 hr, culture media was removed and cells were washed twice with PBS. Cells were treated with gluthardehyde for 2 hr at room temperature. Then, dehydration step was performed by alcohols in increasing concentrations of 50, 60, 70, 80, 90 and 100%. Finally, substrates were dried at room temperature. Untreated substrate was used as negative control.

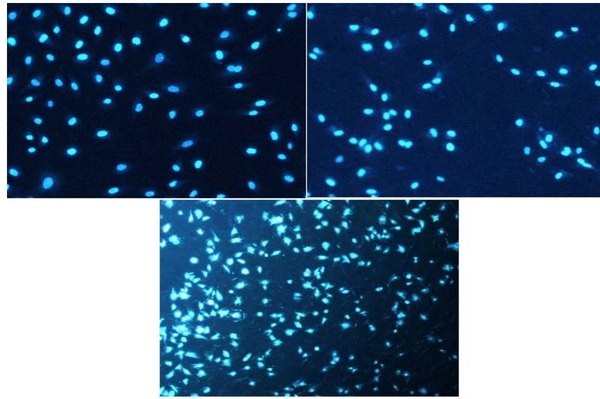

**Figure 1.** Fluorescent images of increased H9C2 cell adhesion on tissue culture polystyrene, unanodized and anodized titanium with nanotube structures (upper, middle and lower panels, respectively). Cell nuclei are stained with DAPI

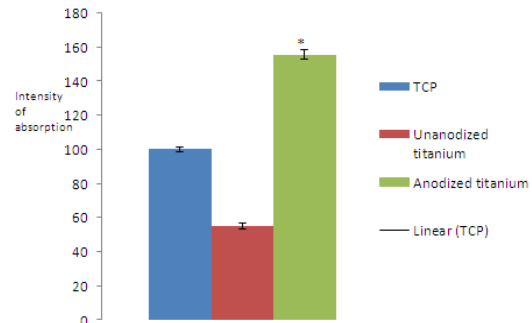


**Figure 2.** Cell survival and proliferation on tissue culture polystyrene, unanodized titanium and anodized titanium with nanotube structures (%). Cell survival and proliferation were evaluated using MTT test. * denotes $P$<0.05. Data are expressed as mean±SEM (n=3)

### *Statistical analysis*

Numerical data were analyzed through analysis of variance (ANOVA) followed by student t-test using SPSS software. *P-values* less than 0.05 were considered statistically significant.

## Results

### *H9C2 Cell viability and attachment after exposure with surfaces*

Cell viability and proliferation of cells co-cultured with substrates were assessed using the MTT colorimetric assay. Conversion of MTT to purple formazan crystals by mitochondrial dehydrogenases of viable cells is the basis of MTT assay. MTT conversion was significantly increased in cells co-cultured with anodized titanium substrates. MTT results for cells cultured on anodized titanium and unanodized titanium surfaces was equal to 1.56 and 0.55 fold change compared to tissue culture polystyrene (TCPS). This reflects a higher cell growth, proliferation and viability on this anodized titanium ared to control samples ($P$<0.05). The average surface as comp cell attachment results obtained from triplicate DAPI staining showed that 2497±40.16, 1250±20.11 and 4859.5±54.173 H9C2 cells were attached to TCPS, unanodized and anodized titanium surface, respectively. Cell adhesion to anodized titanium Cell adhesion to anodized titanium was showed 1.95 and 3.89 fold increase compared to TCPS and unanodized titanium, respectively ($P$<0.05). Figure 1 represents DAPI-stained H9C2 cells. The surface had no cytotoxic effects on cells. Data in Figures 2 and 3 represent measures of cell survival, proliferation and adhesion on different investigated surfaces in this experiment. High magnification (30000X) SEM picture of anodized titanium surface is shown in Figure 4. High magnification SEM picture of flattened H9C2 cells, after 48 hr of culture is also represented in Figure 5 (2000X).

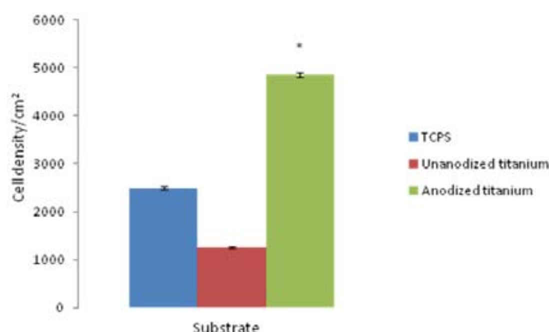


**Figure 3.** The density of attached H9C2 cells to tissue culture polystyrene (n=18), unanodized (n=3) and anodized titanium with nanotube structures (n=21). Cell adhesion was evaluated by counting stained nuclei of attached cell/cm² of substance surface. * denotes $P$<0.05. Data are expressed as mean±SEM

## Discussion

Permanent H9C2 cardiac cell line, a sub-clone derived from embryonic BDIX rat heart tissue has gained great importance for *in vitro* studies on cardiac cell biology (23-28). Cells seeded on this surface, remained viable and proliferated in an accelerated manner compared to untreated substrates. Search for finding optimal biomaterial surface is a currently

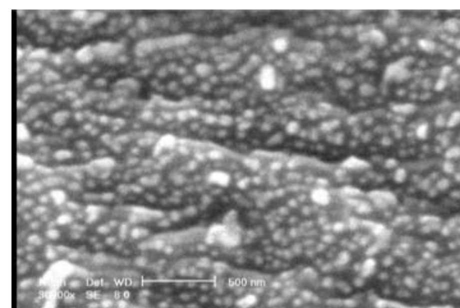

**Figure 4.** High magnification SEM picture of anodized titanium surface. Photo was taken at high magnification (3000X). Bar 500 nm

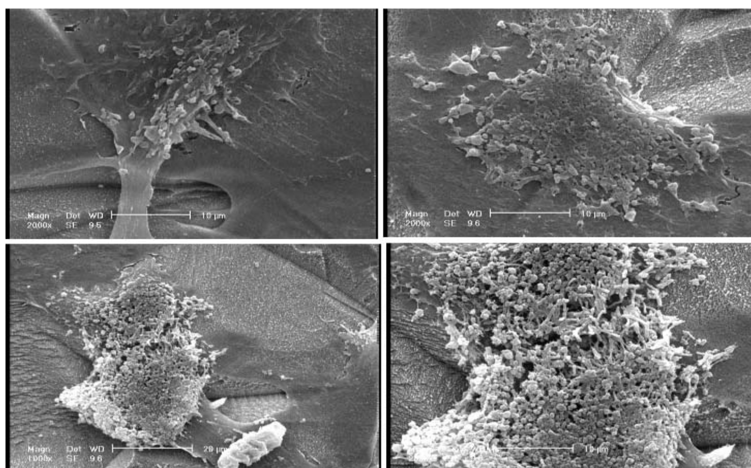

**Figure 5.** High magnification SEM pictures of flattened H9C2 cells, after 48 hr of culture, covered anodized titanium surface. Photo was taken at high magnification (1000X, 2000X). Bar 500 nm

focused issue in cardiac tissue engineering. Anodized treated titanium surface in this study was promising for this purpose through enhancement of cell attachment. Altered surface topography after titanium anodization might influence H9C2 cardiac myoblast adhesion. Formed $TiO_2$ nanotubes on the surface of substrate contribute to promoted cell attachment and proliferation through mimicking natural nano-scale features of these cells. The provided surface area and reactive sites for enhanced cell adhesion by the unique nanotube structure in this treated substance as demonstrated in SEM photograph would partly explain the observed enhanced cell adhesion. Our data implicates that anodized nano-tubular surface topography of titanium leads to greater cardiac myoblast adhesion and proliferation.

Inexpensive technology used for designing this surface, makes this substrates a candidate scaffold for cardiac cell growth, delivery and engraftment. Enhanced H9C2 adhesion onto this surface will facilitate cardiac regeneration for clinical application. Anodized titanium treated surface is a biocompatible and bio-inert surface which is not associated with elicitation of inflammatory reactions (20). Attenuated macrophage density on the surface of anodized treated titanium surface has been shown by Rajyalakshmi *et al*, which guarantees safe application of this surface for regeneration purposes (20). Inflammatory reactions arisen from the interaction between medical device and host tissue lead to failure of cell retention and survival.

Thus, our data are in favor of the application of anodized titanium surface created through this novel method for cardiac regeneration purposes. This unbelievably inexpensive method which replaces platinum with stainless steel serving as the cathode pole in anodization process, avoids chemical modification of surface which might be harmful to cells.

## Conclusion

Anodized titanium surfaces can be potentially applied for enhanced recruitment of H9C2 cells. This unique property makes these inexpensive anodized surfaces as candidate scaffold for attachment of cardiac cells and consequently for cardiac regeneration.

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## Conflict of interest

Authors declare conflict of interest.

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