Experimental analysis of invitro biocompatibility of nano modified titanium alloys and stainless steel

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Abstract. The aim of this paper is to conduct an experimental analysis in invitro biocompatibility of nano modified titanium alloys. The titanium alloy was etched with sulfuric acid and hydrochloric acid above 200°C. The biocompatibility study was carried on the modified titanium plate. The cell culture was carried out the results are expressed as the number of attached cells to the unit surface of the material of the plastic culture plate. SEM analysis and DNA isolation and electrophoresis were carried out. Modified titanium shows more cell attachment. Innovation carried out in this research is the fabrication of porous material using white gold alloy. White gold alloy was prepared by nanoscale modification of surface by acid etching. Nonporous are seen on the surface. The SEM, EDAX analysis confirms the modifications of the surface by acid etching.

Key words: Biocompatibility, Titanium, SEM

1. Introduction

Millions of people all over the world are affected by trauma and accidents many lose their legs due to infection and non-union of the bones. In this research 316 L stainless steel and Titanium were experimentally analysed. In this Experimental analysis of invitro Biocompatibility of nanomodified Titanium alloys and 316L stainless steel were carried out in this research [1]. Titanium was found to be the best biomaterial, which stands out with its extraordinary properties as compared to 316 L stainless steel. The commercially pure titanium and its alloys showed better corrosion resistance than any other material when in contact with human bone, body fluids, and soft tissue [2]. Medical grade titanium Gr. (IV) was used in this research due to excellent corrosion resistance properties. 316L SS was used in this research due to its corrosion resistance properties. From the literature it is found that the titanium is 40% lower in density than stainless steel; at the same time has good fracture and wear resistant properties [3]. However no material even titanium has been completely free of adverse reactions on humans body fluids.

The search of the best biomaterial for implantation is incomplete without the material selection. So materials are selected based on the application such as biomedical implants etc., Implant materials are rejected due to biocompatibility between the bone and the metal
implants [4]. This is basically due to the difference in the properties of the human bone and the metal implants. The most important metal property is the young's modulus. The young's modulus of the metals is not equal to the metals young's modulus. So in this research the surface of the metal is modified into a nanostructure. Nanoporous are formed on the surface of the implant surface [5]. From the literature review it is found that the cell interactions are highly dependent on surface topography (micro/nanoporous structure) and surface chemistry, as experimented [6]. It seems is does not have any direct relationship between these two parameters is not found. It can be advantageous to learn such physio-chemical characteristics of surfaces, which would help in developing osteoblast friendly biomaterials. That is new bone cells are grown on the surface of the implants and entangles to the nanoporous materials. This helps to reduce corrosion on the implant [7]. The stability of the nanoporous implants is more compared to the normal implants.

Commercially pure titanium and its major medical alloy, Ti-6Al-4V and 316L SS are widely used in clinical dentistry and orthopaedics because of their biocompatibility and mechanical properties [8-9]. In this research both the titanium and 316L SS plates are made into a nanoporous structure and used for the MG63 osteoblast-like cells (osteosarcoma cells) to be cultured on titanium and 316L SS metals. To determine whether the cells are sensitive to submicron scale features. In this research osteoblast cells were cultured on smooth and nanoporous surface [10-12]. To attain the nanoporous surface, the surface is chemically etched using strong acids. As Morphology of the cells remained similar on smooth surface, but showed elongated structure on etched surfaces. The innovation in this research is nanoporous material was formed using a noble metal gold. By this method of acid etching on white gold process (Gold 80% and Copper 20%). The copper is mixed with the gold in a ratio of 20:80 is treated by the acid etching. The copper is dissolved in the acid and the surface of the gold is formed with porous holes.

2. Objective
The objective of the research is to develop in invitro biocompatibility nanosurface modified titanium and modified 316 L SS. The second objective is to find the cell attachment of the nanosurface modified titanium and modified 316 L SS. The third objective is the fabrication of white gold by chemical nanoetching to convert white gold to nanostructured white gold.

3. Methodology
The methodology of nanoscale modification of Titanium and 316L SS metal surface. Titanium metal is heated in H$_2$SO$_4$ and HCL acid at 200°C (dual etching) for 10 minutes and then polished [13]. Etching done using acid produces pits of micro size ranging from 2 to 5 micrometer in diameter on titanium surfaces. Acid etching enhances osseointegration, it is carried out by immersing titanium implants for 10 minutes in a mixture of concentrated sulphuric acid and Hydrocholric acid. The mixture is heated above 200°C (dual acid etching) and it is carried out to produce a rough micro surface. The same methodology was adopted for 316L SS.

The methodology of producing gold nanoporous material is similar to the method adopted for the titanium metal. In this acid etching process the copper is dissolved in the concentrated acid at high temperatures. Where ever copper is present in the gold sample; the copper gets dissolved resulting in nonoporous holes.

4. Experiments and results
4.1 Testing for Biocompatibility
Samples: Titanium (Gr.IV) sample is used to modify the Surface [14], All the Samples are cut to 1 cm$^2$ size.

a. Cell culture
Cell Culture was carried out to determine the Cell number. To isolate DNA from the adherent cells. Balb/3T3 cells are maintained at 37°C in humid/ 5% CO/95% air. Culture media is RPMI 1640 containing 10% fetal serum.

4.2 Reagents
MEM, foetal and trypsin reagents are used in this experiment.

4.3 Biocompatibility studies
The biocompatibility studies are carried out on 316L SS and Titanium plate nanostructure modified surface. As a first step cells are implanted on the acid etched nanostructure modified plate. After the cells are adherent on the modified titanium and 316 L SS plates. The number of cells is counted [5]. This number cells is more the biocompatibility is high when the number of cells are less the biocompatibility is less. First the plate is modified with acid etching. The SEM analysis is carried out on the 316L SS and Titanium (Gr.IV ).

The SEM analysis of 316L SS plate is shown in the figure 1. The image shows microns level holes on the plate. The nanoporous holes on the plate helps in improving the biocompatibility of the plate. The tissues grow into the plate in to the microporous holes. The stability of the plate improves.

![SEM analysis on nanoporous holes 316L SS plate](image1)

**Figure: 1** SEM analysis on nanoporous holes 316L SS plate

![SEM Micrograph Morphology of modified surface of Titanium plate treated with and polished at 100μm](image2)

**Figure: 2** SEM Micrograph Morphology of modified surface of Titanium plate treated with and polished at 100 μm. The flakes are shown on the surface. The grain boundaries are seen on the plate.

![SEM Micrograph Morphology of modified surface of Titanium plate treated with acid and polished at 20μm](image3)

**Figure: 3** SEM Micrograph Morphology of modified surface of Titanium plate treated with acid and polished at 20μm
The SEM Micrograph Morphology of modified surface of Titanium plate treated with acid and polished at 20μm is shown in the figure 3. The magnification was carried out with a sample of 20μm. The SEM image shows flakes of material on the surfaces it is the grain boundaries [15].

SEM image of the characteristic nanometric sponge like structure is achieved by treatment of Titanium with $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$.

The roughness developed due to the acid treatment and polishing of the titanium plate, which can be viewed from the following SEM analysis at different magnification, is shown in figure 3, 4 and 5.

**Figure: 4 SEM Micrograph Morphology of modified surface of Titanium plate treated with acid and polished at 2μm.**

The figure 4 shows the SEM Micrograph Morphology of modified surface of Titanium plate treated with acid and polished at 2μm. In this magnification at 2μm it is found that the white patches shows that the acid has etched the surface. The dark spots shows the unetched surface [16]. From this SEM figures 1 to 4 we can conclude that the etching has taken place due to acid treatment of the Titanium (Gr.IV) and 316L SS metal sample plates.

**Figure: 5 EDAX Picture of Titanium after modification**

The abscissa of the EDAX spectrum indicates the ionization energy and ordinate indicates the counts. Higher the counts of a particular element, higher will be its presence at that point or area of interest. The figure 5 shows that the ionization energy and the count for the titanium is high compared to other elements [17]. From this EDAX picture we conclude the chemical composition of the sample being tested. We find that the Titanium is predominant in the sample after etching.

4.4 Cell attachment

4.4.1 Cell number

Cell attachment on each nanoporous titanium (Gr. IV) substratum and 316L SS was evaluated. The evaluation is carried out for attachment of number of cells and it was recorded after 4 and 18h of incubation as follows. The cells are seeded onto the titanium and 316L SS materials and incubated. Subsequently the specimens are washed with PBS to discard the unattached cells. The adherent cells were removed from the substratum after incubation with trypsin 0.25% in EDTA [18]. The resulting cell suspension was counted using a particle counter. The results are expressed as the number of adherent cells per surface unit of the material of the plastic culture plate.
Table: 1  Percent cell attachment

|                | Percent Cell Attachment |
|----------------|-------------------------|
|                | 4 h         | 48 h         |
| Control        | 50          | 90           |
| 316L           | 20          | 35           |
| 316L(M)        | 25          | 41           |
| Titanium       | 30          | 45           |
| Titanium(M)    | 35          | 60           |

The number of cells attached on each substratum as a percentage of the initially seeded number of cells. Statistically important is the differences in the numbers of adherent cells. The time interval between 4h and 48h of incubation were found only in culture plates [19]. On substrata modified Titanium, the cells adhered more readily. The number of adherent cells per surface unit of Titanium was increased with increased surface roughness of the plate.

Figure: 6 cell attachment of samples

The Table 1 shows the percent cell attachment of various samples at 4th and 48th hr. The percentage of Cell attachment of samples of titanium (Gr.IV) and 316L SS are compared for the 4th hr. and 48th hr. are compared as shown in the form of bar chart in figure 6. The cell attachment for the titanium (Gr. IV) and 316L SS bar charts are compared [20]. From the above comparison of the bar charts it is found that the cell attachment of titanium sample is high for the 4th hr. and also for the 48th hr. Showing the values of the percent cell attachment at 4h, 48h of the various samples. DNA isolation and electrophoresis shown is figure 7.

4.4.2 DNA isolation and electrophoresis

The procedure for DNA isolation and electrophoresis is follows. For isolation of adherent tissue cultured cells, the tissue culture media is first decanted. Then the cells are removed from the wells by repeated treatment with trypsin (5%). The cells are pelleted out and DNA is obtained was subjected to Gel Electrophoresis along with control DNA from normal cells.

Figure: 7 DNA Gel Electrophoresis

4.4.3 DNA Gel Electrophoresis

Gel electrophoresis is method used to separate mixtures of DNA, RNA, or proteins with respect to the molecular size. In gel electrophoresis, the molecules to be separated were
pushed through a gel that contains small pores with the help of an electrical field. The figure 7 shows the DNA Gel Electrophoresis conducted for separating the DNA, RNA and proteins.

The DNA from cells has migrated to the same distance as the control, which indicates that there is no change in the Genomic composition before and after the treatment.

DNA isolated control and Electrophoresis cells from plastic wells, Ti cells from Titanium surface, Ti(M)-cells from Titanium modified surface, SS-cells from 316L plate, SS(M)-Cells from 316L modified surface.

In this study, titanium and stainless steel is treated with solutions to enable surface. Treated titanium surface and stainless steel effects on Balb/3T3 cells are investigated. The results are found and were summarized below:

1. All the surface modification procedures adopted in this study is found to be non–toxic to cells.
2. Polished titanium surface is exhibited to appreciable initial cell attachment and good cell proliferation. Similarly modified stainless steel plate shows better cell attachment when compare with non-modified surface.
3. Acid treatment improves cell attachment when compared with ordinary titanium.
4. Acid treated titanium shows the highest cell attachment in the study (other than the positive control of tissue culture plastic), though cell numbers are found to be on par with others after the period of 48 hrs. Incubation period, this feature of Acid treated titanium helps in developing better biocompatible implant.
5. The Genotoxicity of the samples are nil as seen from the Agarose gel Electrophoresis. So all these samples are Biocompatible.

The special feature of cells anchoring with the surface of Acid treated titanium, may address the issue of implant loosening in orthopedic surgeries [13]. From the 4 hr cell attachment study and 48hr proliferation study, it can be concluded that acid treated titanium provides support in initial hours of implantation as well as bolster proliferation and at the same time helps the bone tissue to have a tight bonding with the surface [8].

Modifying the surface of metals based on etching with combination of strong acids, and oxidants can generate nanotopography, concentrated sulphuric and nitric acid to reproducibility yield networks of sized pits and 20nm in diameter.

Nanometer diameter on 316LSS and Titanium alloys surface morphology can be controlled by adjusting the exposure to etching solution. The metals are dipped in the sodium hydroxide solution. The OH groups on the metal surface influences the cell activity.

5. Fabrication of white gold for nano modifications of surfaces

**Figure 8** White Gold Porous Material

The fabrication of porous white gold was carried out in the laboratory. The gold is a noble metal it does not corrode in the body fluids. The gold is taken as 80% and copper is taken as 20% in ratio [14]. The gold and copper are mixed 80:20 proportions and annealed to get white gold. After The figure 8 shows the white gold nanoporous material sample.

When gold and copper mixed in 80:20 proportion immersed in nitric acid, small nano sized porous pits are formed [15]. It is evident from the percentage of chemical composition. EDAX image is shown in figure 9. The presence of gold is high as evident from the EDAX Picture, the presence of copper is low as evident from the EDAX picture.
Figure: 9 EDAX Pictures of Gold and Copper

The figure 10 shows the percentage weight of gold and copper. The EDAX analysis shows the percentage of gold and copper present in the white gold sample before etching was carried out [16]. This helps us to understand that proper mixture of gold and copper has taken place.

Figure: 10 Bar chart white gold chemical composition %

Figure: 11 SEM image of White Gold

SEM image is shown in figure 11. It shows the presence of Gold and Copper. The SEM shows the surface modification on the white gold sample due acid treatment. Nanoporous are found on the surface of the sample due to the acid treatment on the white gold sample. When implants are produced using noble metals [17], The noble metals do not corrode when used as an implants material. The noble metal do not corrode in the human body fluid resulting in increased biocompatibility between the bone and the Implants.

A.Nanci., [10] pointed out Roughening of the surface with strong acids such as HCL, H$_2$SO$_4$, HNO$_3$ and HF on titanium implants, forms micropits on titanium surfaces ranging from 0.5 to 2 μm in diameter [9]. In his research he showcased the etching of metal surfaces by chemical approach to modify the surface of metals with combinations of strong acids and oxidants generated nanotopography [18]. Mixture of sulphuric acids (H$_2$SO$_4$) and hydrogen peroxide (H$_2$O$_2$) was used to yield around 20nm in diameter on Titanium metal samples.

Experimental studied the immersion of titanium implants in a mixture of concentrated HCL and H$_2$SO$_4$ heated above 100°C for several minutes (dual acid etching) to provide a microrough surface.

The work highlighted the reduced healing time on ITI Implants using a sand blasted acid etched surface on ITI SLA implant Clinical ORAL Implants. Research are being carried to improve the increase in cell adhesion and bone formation. High temperature acid etching produced a homogeneous micro porous surface with high bone to
impact contact [19]. TPS surface showed low microporous surface in in vitro studies. The Invitro biocompatibility of nanoscale modification surface of 316L SS SEM Analysis shows nanoporous on the surface. Modified titanium shows more cell attachment.

White gold alloy was used to prepare nanoscale modified surface. Nanoporous are seen on the surface of the white gold. The SEM, EDAX analysis confirms the modifications of the surface of the white gold.

6. Discussion

6.1 Biocompatibility studies

The biocompatibility test was conducted on the titanium and 316L SS it was found the titanium (Gr.IV) showed more biocompatibility than the 316L SS it is due to adherent of cells on the modified titanium is more compared to the modified 316L SS. The percentage of Cell attachment for 4h in modified is 30 and for 48h is 60 so is modified titanium percentage of cell attachment is more than the modified 316L SS.

6.2 SEM and EDAX of Titanium and 316L SS

The SEM and EDAX of Titanium and 316L SS shows nanoholes have formed on the sample. The nanoholes are formed due to acid etching, the nanoholes are responsible for the entanglement of tissue on the titanium and 316L SS metal samples.

6.3 Nano topography generation

Surface modification is key to the bone growth. Modifying the surface of metals based on etching with combination of strong acids, and oxidants can generate nanotopography, concentrated sulphuric and nitric acid to reproducibility yield networks of sized pits and 20nm in diameter. In this research it is found that the modified titanium showed more nanopits than the modified 316L SS surface.

Acid treated on nano titanium implants provide initial hours of implantation and bolster proliferation. This helps the bone tissue to have a tight bonding with the implant metal surface. Titanium is stronger and lighter in weight compared to stainless steel. Titanium is fatigue resistant and it is applied to making of an implant. Titanium has greater superior strength under fatigue, and it is capable of withstanding strain during internal fixation.

From the experiments conducted by the researcher it was found titanium is more corrosion resistant than the 316L SS. Titanium is considered the most biocompatible metal – not harmful or toxic to living tissue – due to its resistance to corrosion from bodily fluids. This ability of the titanium is to withstand the harsh bodily environment. Protective oxide film that forms naturally in the presence of oxygen reduces corrosion in the implants. Titanium is considered the most biocompatible metal – not harmful or toxic to living tissue – due to its resistance to corrosion from bodily fluids.

Its ability to physically bond with bone also gives titanium an advantage over other materials that require the use of an adhesive to remain attached. Titanium implants last longer, and much larger forces are required to break the bonds that join them to the body compared with their alternatives.

Titanium alloys commonly used in load-bearing implants are significantly less stiff – and closer in performance to human bone – than stainless steel or cobalt-based alloys.

6.4 Fabrication and nano modification of surfaces of white gold

Noble metals are used in the manufacture of implants due to their corrosion resistance properties. These noble metals do not corrode in acids as evident from the acid etching process. The gold and copper are mixed in 80: 20 proportion. After etching copper evaporates the nanoholes are formed on the gold sample. This nanoholes helps the tissue to grow inside the nanoholes of the implants. This helps to reduce corrosion in the implants.

The stability of the implants is also good; this helps to improve the biocompatibility of the implants.
7. Conclusion

Invitro biocompatibility nanoscale modification of surface, Titanium SEM Analysis shows nanoporous on the surface. Modified titanium shows more cell attachment. The novelty of the research is the fabrication of white gold by chemical etching process. White gold alloy was prepared by nanoscale modification of surface. Nanoporous are seen on the surface. The SEM picture as EDAX picture confirms the modifications of the surface. It can be concluded that acid treated nano titanium provides good support for initial hours of implantation as well as bolster proliferation. Nano titanium helps the bone tissue to have a tight bonding with the nano structured surface of the titanium.

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