

Cell Viability and Growth on Metallic Surfaces: *in vitro* Studies

A. Gupta^a, P. Majumdar^b, J. Amit^a, A. Rajesh^a, S.B. Singh^{b*} and M. Chakraborty^b

^a School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India.

^b Department of Metallurgical and Materials Engineering, Indian Institute of Technology, Kharagpur, India.

Materials to be used as permanent implants in human body must be biocompatible, corrosion resistant, tissue compatible, vital and elastic, in order to serve for a longer period. Titanium and its alloys have become one of the most attractive classes of biomedical implant materials and are generally preferred to stainless steels and Co-Cr alloys because they are light-weight, have superior biocompatibility and corrosion resistance, good mechanical properties and low elastic modulus. Biocompatibility depends upon different material factors such as substrate mechanical origin, surface structure and chemical composition, as well as, on implant design and other factors. The biocompatibility of implant materials is investigated in animal experiments (*in vivo* test) and cytocompatibility using cells (*in vitro* test). Implant loosening in bone fixation using bone cement is an unresolved complication associated with internal fixation in orthopedics. In this regard, titanium and titanium-based alloys are promising biomaterials for development of orthopedic implants suitable for cementless fixation. It is generally accepted that the problem of fixation can be overcome by modifying the implant/bone interface for improved osseointegration. Cell attachment and adherence to a surface is the first step that marks the beginning of the biological process. Cell attachment and spreading on alloy surfaces are thus major parameters in cementless orthopedic implant technology. Though titanium and its alloys are already in wide use as dental and orthopedic implants, the effects of the surface characteristics of these materials, including roughness, on the response of target tissues *in vivo* are not well understood. It has already been proved that cell attachment and biological bonding of a cell and the viability of an adherent cell is highly influenced by the surface characteristics of a biomaterial, especially its roughness, which can be engineered to improve the biocompatibility of an implant, of a given composition.

In the present *in vitro* study, fibroblast viability and growth has been evaluated using quantitative method. Fibroblast cell line AH-927, a feline fibroblast cell line, was used for the study. The substrates used were 316L stainless steel, Cp-titanium (Grade-IV), Ti-6Al-4V, Ti-13Nb-13Zr, and Ti-35.11Nb-5.7Ta-7.18Zr. For a given composition, different surface preparations were used, they are: sand blasted (600 μm SiC particles), coarse (belt) ground (SiC abrasive paper of grade 80), paper polished (SiC abrasive paper of grade 600) and diamond polished (0.25 μm diamond particles). After culturing the cells with the substrates, their viability and growth were assessed after 24 and 48 hours using MTT assay and the results were compared with respect to different compositions with surface modifications and also with a control (cell grown without any sample in the culture plate). It has been found that sand blasted Ti-13Zr-13Nb alloy is most suitable in terms of supporting the cell growth and viability.

Introduction

The basic technique used by an orthopedician to fix an implant for arthroplasty is to use bone cement (polymethylmethacrylate; PMMA) as an interface between the metal implant surface and the bone.. Local tissue effects of bone cement is related to: (i) heat of polymerization that may exceed the coagulation temperature of the tissue proteins (about 67°C), (ii) occlusion of the metaphyseal arteries that may produce area of bone necrosis

and (iii) cytotoxic and lipolytic effects of the unpolymerised monomer. Moreover, its flexural modulus is low leading to failure on excessive loading. It has been reported that during the first three weeks of post operative period tissue damage occurs (3mm layer of fibrin and soft tissue become necrotic). During third post operative week a process of repair begins and lasts up to two years. The bed of the implant is organized by in-growth of fibrous tissues and capillaries that replace the

necrotic bone and contains some fragments of MMA with foreign body reaction [1, 2]. Loosening of the implant bed leads to crevice corrosion. Despite all these consequences, arthroplasty using the bone cement as an interface continues to be the most preferred method in the absence of more advanced methodology and materials.

All these have led the biomaterial scientist to search for new materials with ideal mechanical properties that in turn induce the direct bone cell growth over the implant surfaces so that the use of bone cement as an interface can be omitted. In this regard titanium (Ti) or titanium-based alloys are one of the promising class of materials that can be used with many advantages over the most commonly used stainless steel (SS) and Co-Cr alloys. The resistance of Ti or its alloys to corrosion in chloride environment is excellent and better than SS and Co alloys [3]. Its protective oxide surface is highly inert and reforms easily after the damage. The modulus of elasticity of Ti / Ti alloys is about half of SS and Co alloys, which is an advantage in load transfer to bone [1, 2]. In order to use Ti alloys, it is important to study the cell behavior (Adhesion, Viability and Growth) in relation to surface properties so that it can be developed as cementless prosthesis. For bone in-growth to occur on metallic surface, three criteria have to be fulfilled (i) pores on the surface should be greater than 40 μm in size (ii) micro motion should be absent ($> 150 \mu\text{m}$), leads to fibrous in-growth leading to weak interface and (iii) porous surface should be in intimate contact with the bone [4]. As far as mesenchymal origin cells or any other adherent cells are concerned, the first and the foremost requirement for the differentiation and growth is to find a suitable substrate for attachment. The intercellular adhesion is also of some importance [5-7]. In many tissues including bone, cell progeny are prevented from wandering away by being attached to the extra-cellular-matrix, to other cells or both. The accumulating cells do not just remain passively stuck together; instead, the tissue architecture is generated and actively maintained by selective adhesions that the cells make and progressively adjust. Selective adhesion is even more essential for the development of the tissues that have more Complex origins involving cell migration. In these tissues one population of cells invades another and assembles within it, and perhaps with other migrating cell, to form an orderly structure. The

whole process involves the cell motility and cell adhesion, which in turn is guided by "the phenomenon of pathway guidance". Adhesion of the cells to artificial substrate is determined by the ability of the surface to adsorb the proteins, charge of the surface, roughness, porosity, wettability etc [8]. In the light of the above discussion, the present research study was undertaken to investigate the effect of surface roughness of different metal/alloys substrate on the adhesion of fibroblasts. The range of surface roughness for different substrate was taken into the consideration and cell viability was estimated.

Materials and Methods

The substrates used were 316L Stainless Steel, Cp- Titanium (Grade-IV), Ti-6Al-4V, Ti-13Zr-13Nb (TZN), and Ti-35.11 Nb-5.7Ta-7.18Zr (TNTZ) (all compositions are in wt %). For a given composition, four different surface preparations were used; they are: Sand Blasted - SB (600 μm SiC particles), Belt Ground - BG (SiC abrasive paper of grade 80), Paper Polished - PP (SiC abrasive paper of grade 600) and Diamond Polished - DP (0.25 μm diamond particles). The samples were cut into 10 mm x 6 mm x 1.5 mm sheets for cell culture studies.

Surface roughness was measured using a contact profilometer on five samples randomly chosen from all the alloy groups. Prior to *in vitro* experiment, samples were ultrasonically cleaned in soap water, acetone and distilled water, respectively and then sterilized in an autoclave for 30 minutes.

Cell Culture

Stock culture of AH-927, a feline fibroblast cell line was recovered at -80°C and plated in 25 cm^2 tissue culture flask in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) with antibiotics (Penicillin - 100 IU/ml and Streptomycin - 100 $\mu\text{g}/\text{ml}$) and maintained in the culture medium. The cells were recovered using 0.25% trypsin and 0.02% ethylene diamine tetra acetate (EDT A) solution. The cells were recovered, rinsed and cultured on plates of size 10mm x 6mm x 1.5mm of different materials as mentioned above in 24 well culture plates. Cells were inoculated at a density of 30000 cells per ml in each well of the culture plate containing the samples. Well containing no sample was taken as control.

Cell Viability Assay: 'The MTT Assay'

5 mg per ml stock solution of MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was made in sterile phosphated buffered saline (PBS). Lysis solution was made by adding 99.4 ml of di-methyl sulphoxide (DMSO) and 0.6 ml of acetic acid. After incubating the cells with the samples for 24 and 48 hours, the consumed medium was removed carefully from all the wells of the culture plate and was replaced with the fresh complete DMEM. 100 μ l per ml of stock solution of the MTT dye was added to each well and the 24 well culture plates were incubated for three hours in CO₂ chamber (cell lysis didn't occur in this time). The supernatant was aspirated carefully, taking care not to remove Formazan crystals formed with in the cells. Subsequently, lysis solution in amounts equal to that of the DMEM added before incubation was added and the cells were lysated over 5 minutes and mixed well. 200 μ l of the lysate from each well of 24 well culture plate was transferred to the pre-marked 96 well ELISA plate and then the optical density (OD) of the lysate was measured at 595nm using ELISA reader. The percentage of OD value (OD for metal specimen/OD for control group x 100%) was calculated. A larger OD value represented higher cell viability and thus adhesion.

Results

The average surface roughness, R_a , of five randomly selected specimens from all the alloy groups is given as; Sand blasted 4.78-6.63 μ m; Belt ground 2.21-3.63 μ m; Paper polished 0.58-0.97 μ m and Diamond polished 0.27-0.46 μ m.

The viability of cultured fibroblasts on the different surfaces is shown in Fig.1 to Fig.10 at 24 and 48 hours respectively as measured by the MTT assay. Cells cultured on the Ti-13Zr-13Nb showed higher viability (Fig. 4 and 9) in both the cases, after 24 hours as well as after 48 hours. In almost all the cases it has been found that there is an increasing trend in cell viability with an increase in the roughness for a given substrate.

In general, for the first 24 hours, the viability of the cell on sand blasted surfaces of all the samples is comparable or higher to that on the polystyrene surface normally used for the cell culture purpose. Though 316L SS and cp-Ti did not follow this trend, cells grown on the rough surfaces (all substrates with the exception of DP surface) of the TZN and

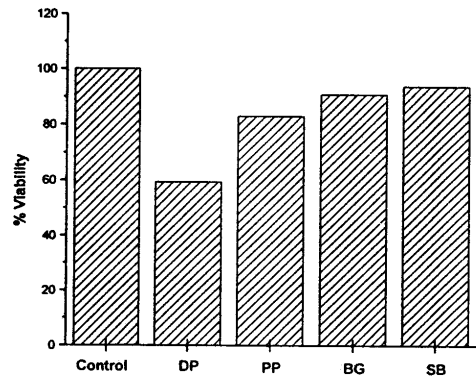


Fig 1: Cell viability of 316L SS with different surface modification at 24 hours

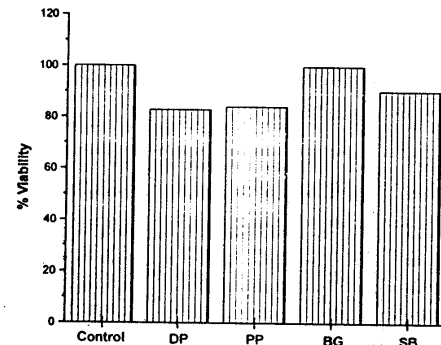


Fig 2: Cell viability of cp-titanium with different surface modification at 24 hours

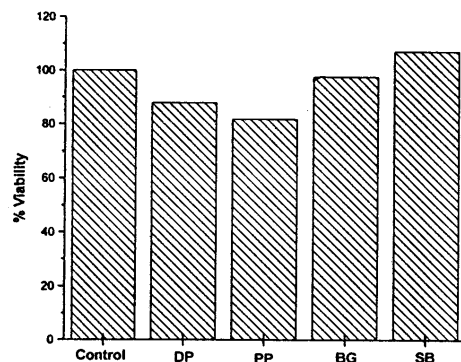


Fig 3: Cell viability of Ti-6Al-4V with different surface modification at 24 hours

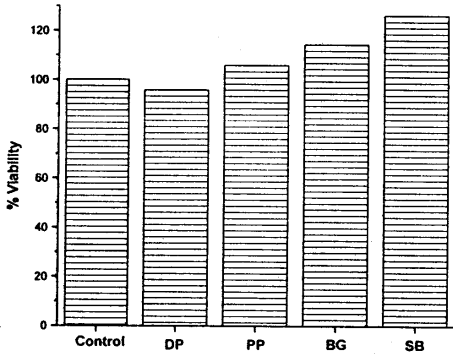


Fig 4: Cell viability of Ti-13Zr-13Nb with different surface modification at 24 hours

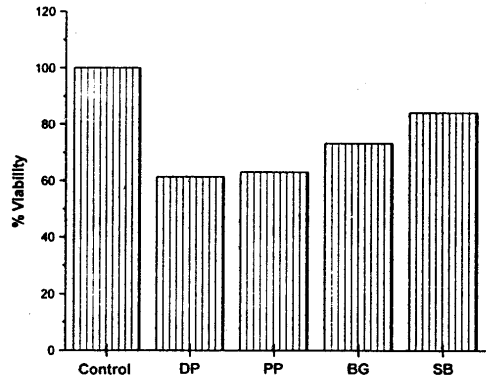


Fig 7: Cell viability of cp-titanium with different surface modification at 48 hours

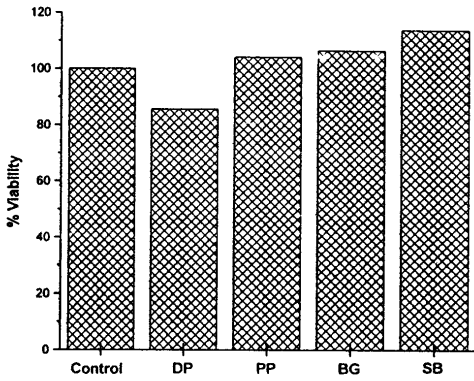


Fig 5: Cell viability of Ti-35.11 Nb-5.7Ta-7.18Zr with different surface modification at 24 hours

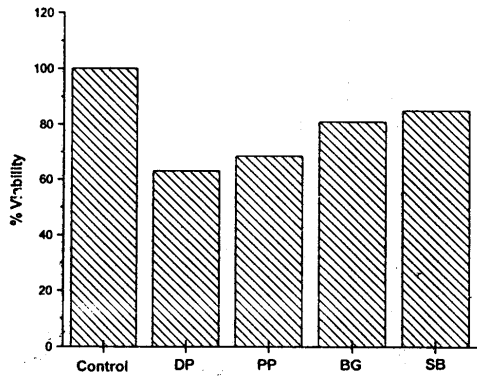


Fig 8: Cell viability of Ti-6Al-4V with different surface modification at 48 hours

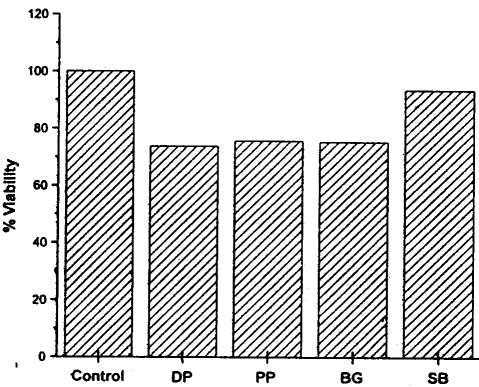


Fig 6: Cell viability of 316L SS with different surface modification at 48 hours

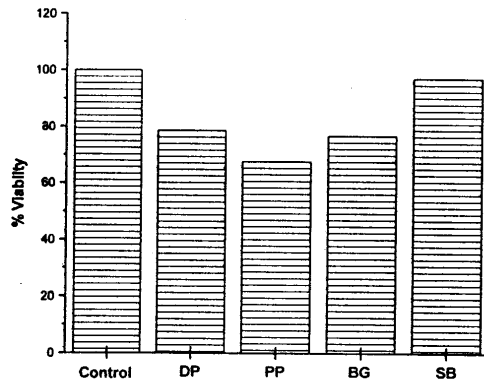


Fig 9: Cell viability of Ti-13Zr-13Nb with different surface modification at 48 hours

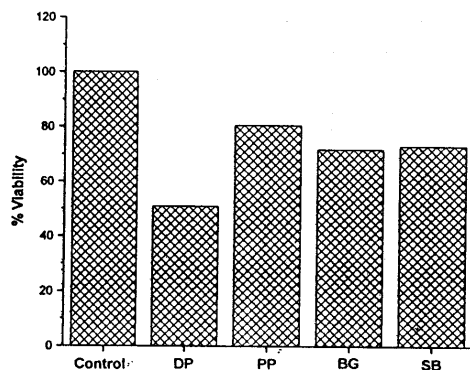


Fig 10: Cell viability of Ti-35.11 Nb-5.7Ta-7.18Zr with different surface modification at 48 hours

TNTZ alloys showed higher viability than other alloy-substrates and control in the first 24 hours and thus a higher adhesion (Fig. 4 and 5).

Cells on all the surface modifications for 316L stainless steel, which is used most commonly for any type of arthroplasty in orthopedics, showed lesser viability than any of corresponding surface modifications of Ti-alloy substrates and polystyrene (control) used in the present study. Cells grown on Ti-Al-V, Ti-Nb-Zr and Ti-Nb-Ta-Zr alloys with belt ground and sand blasted surfaces showed higher viability than that grown on polystyrene surface at 24 hours. At 48 hours, the cell viability on all the substrates showed a relatively decreasing trend in comparison to that on polystyrene surface except Ti-13Zr-13Nb sand blasted surface, which is comparable to the control (Fig. 9). In Summary therefore, sand blasted and belt ground Ti-13Zr-13Nb showed the best support for the fibroblast attachment and their viability.

Discussion

It has been found that the surface roughness is directly influencing the fibroblast viability and thus the fibroblast adhesion. Thus it can be one of the promising approaches by which cementless prosthesis in orthopedic practice can be developed and implemented. However, the effects of the increase in the surface roughness of the material on the desired physical properties of an implant have to be considered. The effect of the ions of

titanium and its alloying elements on the viability is evident with increasing the incubation period of the cells on the samples to 48 hours. The control surface, being polystyrene and thus free from ions, showed better viability trend compare with metallic substrate when cultured for longer periods. However, in the case of sand blasted TZN alloy the cell viability was still comparable with that on the control surface even after 48 hours of incubation.

The Young's modulus of Ti-13Zr-13Nb (68 GPa) is lower than SS (210 GPa), Co-Cr alloys (220 GPa) and other conventional Ti-alloys [9, 10]. In the present study it has been found that it is this substrate, which best supports the cell growth. This indicates the suitability of this alloy from cementless prosthesis. Making surfaces rough by sand blasting decreases the fatigue strength by 20 to 25%, this is not desired, in the actual practice. Thus evaluation of the efficacy of this type of substrates with roughened surface need to be performed in bio-simulating models or in vivo studies in the animals.

Fibroblasts and osteoblasts, both belong to the same group of the cells depending upon the origin (mesenchymal or mesodermal). Fibroblasts are the most fundamental cell type of mesenchymal origin so it can be taken as model to study the behavior of any of the cells of the same origin. Of course, exact behavior of the osteoblast-like cells cannot be predicted by the behavior of fibroblasts but it can be taken as a basis for the future research and development because of the common initial fundamental properties for growth and differentiation. In vitro conditions cannot replicate in vivo situations due to the large number of factors that would have to be taken into consideration. It is however, possible to determine the basic cell reaction for a single cell type, which could be representative of the cells of common origin.

Conclusion

Effect or surface condition of 316L SS, cp-titanium and some titanium alloys on cell viability and growth has been studied in vitro. It has been found that salw blasted Ti-13Zr-13Nb alloy is most suitable in terms of supporting the cell growth and viability.

References:

1. R.G. Nelissen, T.W. Bauer, L.R. Weidenhielm, D.P. LeGolvan and W.E. Mikhail, Revision hip arthroplasty with the use of cement and impaction grafting. Histological analysis of four cases, *J. Bone Joint Surg. Am.* 77,1995,412.
 2. P .B. Chang, K.A. Mann and D.L. Bartel, Cemented femoral stem performance. Effects of proximal bonding, geometry, and neck length, *Clin. Orthop.* 355, 1998, 57.
 3. Roland Strietzel, Andreas Hosch, Horst Kalbfleisch, and Dieter Buch, In vitro corrosion of titanium, *Biomaterials*, 19, 1998, 1495.
 4. S.J. Simske and R. Sachdeva, Cranial bone apposition and ingrowth in a porous nickel-titanium implant, *J Biomed Mater Res*, 29, 1995,527.
 5. L. C. Baxterl, V. Frauchiger, M. Textor, I. ap Gwynn and R. G. Richards, Fibroblast and Osteoblast adhesion and morphology on calcium phosphate surfaces, *European Cells and Materials*, 4, 2002, 1.
 6. Anselme K, Bigenelle M, Noel B, Dufresne E, Judas D, Lost A, Hardouin P, Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughnesses, *J Biomed Mater Res* 49, 2000, 155.
 7. Anselme K, Osteoblasts adhesion on biomaterials, *Biomaterials*, 21, 2000, 667.
 8. L. Ponsonnet, K. Reybier, N. Jaffrezic, V. Comte, C. Lagneau, M. Lissac, C. Martelet, Relationship between surface properties (roughness, wettability) of titanium and titanium alloys and cell behaviour, *Materials Science and Engineering C* 23 (2003) 551.
 9. M. Windler and R. Klabunde, Titanium for Hip and Knee Prostheses, in *Titanium in Medicine*, edited by D.M. Brunette, P. Tengvall, M. Textor and P. Thomsen, Springer, 2001, 703.
 10. P. Majumdar, S.B. Singh and M. Chakraborty, Effect of Heat Treatment on Microstructure and Mechanical Properties of Ti-13Zr-13Nb Alloy, *Souvenir of Indo-Australian Conference on Biomaterials, Implantable Devices and Tissue Engineering, BITE 2005, Jan 19-21, 2005, SCTIMST, Trivandrum*, p98.
-