Verifying the effect of silanized nitinol surface: An in-vitro study

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Abstract. Although Nitinol is a popular orthopedic implant material, there is still need of surface modification of nitinol to control nickel leaching. Main challenge with such surface modification of orthopedic implant is that along with cost effective modification the surface, there should be good integration with the surrounding bone tissue. In this study surface of the shape memory Nitinol was modified by simple silanization process using (3-aminopropyl)triethoxysilane(APTES). Here we report the interaction between the Nitinol surface (both bare and silanized) and the human osteoblastic cells (MG63cell) over a period of 48 hours. From detailed investigations, involving MTT assay and immunocytometry it was found that silanized Nitinol performed marginally better than bare Nitinol. The effect of silanization on surface composition and roughness of the specimen is also reported here to explain the superiority of silanised samples in case of cell-material interaction.

1. Introduction

Nitinol is a very promising biomaterial to be used in medical treatment like surgical implants [1]. In particular, its use in orthopedic implants or as stents, highlights many valuable yet subtle behaviors, including shape memory, pseudo-elasticity, enhanced fatigue resistance, low magnetic susceptibility, and good biocompatibility. The specifications of Ni-Ti alloys used for medical devices are controlled under ASTM F2063-05[2], which prescribes chemical ingredients, mechanical characteristics, superelastic characteristics, metamorphic temperature, inclusions and grain size[3,4].

It may be noted that Nitinol, be it shape memory or superelastic, is often provided with a biocompatible coating or subjected to some other form of surface modification through mechanical and electrochemical treatments, chemical etching, heat treatments, [6] conventional and plasma ion immersion implantation [5] laser and electron-beam irradiation, design of bioactive surfaces [7], coating [8,9] etc. Such surface modifications are done to reduce nickel release without compromising its otherwise advantageous properties. Researchers have also attempted bio-functionalisation of Nitinol surface [10-13]. In most of the cases the step preceding such biofunctionalisation was silanization in which the surface is covered with organo-functional alkoxysilane molecules. In-vitro performance of bio-functionalised Nitinol is assessed in recent past to study their effect on Ni release and cytotoxicity [11-13]. In most of the cases, bio-functionalisation yielded better results. But none of these studies focused on the effect of silanization alone on in-vitro performance although it is a well-known process of primary surface modification of nitinol. Usually, in silanization, silane (tri-alkoxy-silanes) is used as coupling agent which forms covalent bond between the underlying metal and the polymer coating to be formed on top. Silane also forms a strong chemical bond with
the metal oxide layer which is durable [8] but how far it can inhibit nickel release and promote cell material interaction is yet to be reported.

In this study, we performed surface modification of nitinol surface using silanization technique and evaluated the behavior of silanized nitinol in terms of nickel release, hemo-compatibility, roughness, contact angle and in-vitro cell –material interaction.

2. Materials and Methods

2.1. Materials

The initial material for the experiment was a commercially available Nitinol shape memory wire and plate consisting of 54.5 wt%Ni, 45.5 wt%Ti (Manufacturer: Nitinol Devices & Components, NDC, USA; Supplier: VRAS traders, India). The supplier confirmed the composition which conforms well to standard prescribed for orthopaedic use (ASTM F-2063).

Cell lines: Human osteoblast-like cells (MG 63) were bought from National Centre for Cell Science (NCCS), Pune, India and following the standard protocol the cells were preserved.

2.2. Methods

2.2.1. Nitinol substrate preparation: 1.5 mm diameter NiTi wire was chopped into number of 1 cm long pieces and the plates were cut into 1cm x 1cm pieces. The pieces were then washed thoroughly for 15 min in acetone (70% by volume), ethanol, and deionized water, respectively, and ultrasonically cleaned to remove residual surface impurities and finally dried to use them for various studies.

2.2.2. Silanization: Four groups of nitinol samples (both wires and discs/plates) were silanized by immersing them into 2% v/v solution of APTES ((3-aminopropyl)triethoxy saline)(Sigma Aldrich, St. Louis, USA); in toluene(Merck) for 10 hrs to generate an amino-silane surface. After that, the silanized Nitinol surface was washed three times with pure toluene to remove the excess APTES. Then the samples were washed two times with ethanol to remove toluene from the silanized Nitinol samples and then the samples were rinsed two times using deionised water to remove ethanol( Merck). X-ray photo-electron spectroscopy study (PHI5000 Versa Probe II, Ulvac-Phi Inc. Japan.) was done on silanized samples so as to confirm the presence of Si.

2.2.3. Contact angle measurement: In the static mode at room temperature (25 °C) sessile drop technique (DSA4, Kruss Easy drop) was used to measure the contact angle of bare Nitinol and silanized Nitinol surface. Probe liquid was water. For each surface, the contact angle was measured at number of spots. All the data are expressed as mean ± S.D.

2.2.3. Surface Profilometry: The roughness of the Nitinol specimens were measured by profilometry using a TalySurf Plus Surtonic 3D Profilometer. Number of surface roughness parameters are measured. Five individual measurements were made for each sample.

2.2.4. MTT assay: The viability of the MG-63 cells (human osteoblast cell) on the NiTi samples were tested for 48 hours. Nitinol samples were cast into the wells of a 24-well plate (Tarsons, India). MG-63 cells were maintained incomplete Dulbecco's modified eagle media ((DMEM), Gibco by life technologies), (10% Fetal Bovine Serum (FBS, Himedia) and 1%antibiotic solution( peniciline, streptommmicine, antimicocene)) at 37°C, 5% CO₂, 95% humidity. Cells were harvested by trypsinization(Himedia) and 1X10⁴ cells were seeded to each well of a 24-well plate and incubated for 12hrs for adherence (37°C, 5%CO₂ and 95%humidity). NiTi samples at a concentration of 100μg/ml were added to the adhered cells in respective wells for a period of 48hours. The complete
media was taken as control. Using MTT assay kit (Himedia, Mumbai, India) following manufacturer's instruction, MTT assay was carried out at definite time interval. Dissolving the formed formazan crystals in DMSO (100μl) the absorbance was measured at 595nm. Cell proliferation was expressed in terms of the cell proliferation index (CPI). The data was reported in terms of CPI.

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\text{CPI} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{control}}}
\]

For cell count, the cells were trypsinised first and then we added complete media to the cells. Then it was centrifuged for 3minutes and then the supernatant was discarded. After that complete media was added and 10μl cell suspension was taken from that. It was put into hemocytometer and counting of cells under the phase microscope (Axioam ERc5s, Zeiss) was done.

2.2.4. Immunocytochemistry: In a 24well plate MG-63 cells (1X10^4cells/well) were seeded. The seeded cells were kept in the incubator for a period of 12 hrs for adherence. Thereafter, in the adhered cells Nitinol samples were added. After 24hrs, cells were first fixed with 4% paraformaldehyde, permeabilized, and then subjected to 15minutes incubation with gentle shaking. After fixing, the samples were washed with PBS thrice. Then 0.25% Triton X was added and subjected to 15minutes incubation with gentle shaking. Finally, the samples were washed thrice with PBS and counter-stained with DAPI (4’, 6-diamidino-2-phenylindole, 0.2-2μg/ml, 50µg/ml) and TRITC (tetramethylrhodamineisothiocyanate) Phalloidin. The expressions were visualized using a fluorescence microscope (IX 71,Olympus). Analysis of the expressions from fluorescence micrographs was done using standard Image-J software.

3. Results and Discussion

X-ray photo-electron spectroscopy study confirmed the presence of Si. Detailed data on XPS is reported elsewhere [14]. Si is a known element to promote bone cell growth, the following parts focus on the effect of silanization on cell-material interaction and verify the reasons behind that.

![Figure 1: Contact angle measurement of (a) Bare Nitinol (b) Silanized Nitinol](image)

**Table 1: Average data of contact angle measurement**

|          | CA(M) [deg.] | CA(L) [deg.] | CA(R) [deg.] | t [sec] | Vol [ul] | MD [mm] |
|----------|--------------|--------------|--------------|---------|----------|---------|
| Bare Nitinol | 72.87        | 72.87        | 72.87        | 268.55  | 8.71     | 3.81    |
| Silanized Nitinol | 81.13        | 81.13        | 81.13        | 809.63  | 9.78     | 3.76    |
CA(M) - Contact Angle Mean (in degree); CA(L) - Contact Angle (Left) (in degree); CA(R) - Contact Angle (Right) (in degree); t - Drop Age (in second); Vol - Drop Volume (in microliters); MD - Maximum Drop Diameter (in millimeter).

After silanization of the bare nitinol, it showed an increase in the contact angle( 72.87º ±6.7º to 81.3º ± 1.9º ) which means after silanization a decrease in hydrophilicity happened, that may affect the cell adhesion to some extent. The following parts verify whether such effect was evident or not.

Figure 2 shows the 2D surface roughness of Bare Nitinol and silanized Nitinol surface. From table 2 it was observed that in case of silanized Nitinol the centre line average (Ra) value was slightly high. Ra values denote that the surface was rough and it helped us to identify the effect of silanization on cell response. Rz value was calculated from the summation of height of the highest peak (Rp) and the lowest valley (Rv) contained by a single measuring length [15]. It was a little higher in Silanized nitinol sample.

It is expected that higher roughness will provide better support for the cells for proper anchorage. From the literature [16] it was also observed that the surface irregularities can be advantageous for anchorage for cells.

Table 2: Surface roughness of (a)Bare Nitinol and (b) Silanized Nitinol

|               | Bare Nitinol | Silanized Nitinol |
|---------------|--------------|-------------------|
| Ra (µm)       | 0.09         | 0.142             |
| Rp (µm)       | 0.945        | 5.12              |
| Rq (µm)       | 0.13         | 0.21              |
| Rt (µm)       | 3.16         | 8.028             |
| Rv (µm)       | -2.215       | -2.908            |

Though Ra values did not change much with silanization , it only provides an average data. In case of other parameters, it was evident that not only big peak value was there , the values corresponding to Rt and Rv are also higher in silanized samples. So it is likely that silanized samples provide more anchorage sites for the cells in case of cell-material interaction.
Figure 3: Phase contrast micrographs A) Control, B) Bare NiTi, C) Silanized NiTi

The micrographs of cells harvested with bare and silanized nitinol samples Fig (3 B, 3C) showed the live cells under the phase-contrast microscope. From these micrographs it is clearly noted that the cells were more or less in same condition in case of bare and silanized nitinol samples. No major difference could be observed.

Figure 4: Cell count data of bare nitinol and silanized nitinol

Figure 4a and 4b showed the cell count data of control, bare and silanized nitinol samples for 48hours. Silanized nitinol samples showed slightly better result than bare nitinol samples.
Figure 5: Cell proliferation (48 hours)

In case of bone repair and regeneration, proliferation and differentiation of osteoblast are important. Using MTT assay the cell proliferation was checked in this study. From standard literature it is known that in case of bare nitinol there is a high chance of toxicity as there is nickel present. Figure 5 showed that on day 2, silanized nitinol samples showed higher cell proliferation result than bare nitinol samples. As the tests were done for a short span of time and nickel release is reported to be a time dependent process, no straight forward conclusion could be made however the initial results suggests that silanized samples primarily offer better surface for the cells to attach and grow on.

Figure 6: Fluorescence micrographs: Here blue corresponds to (DAPI) and red (TRITC Phalloidin) corresponds to the nucleus and F-actin (a)Control  (b)Bare NiTi (c)Silanized NiTi

The morphology of the adhered cells was examined under a fluorescence microscope. Fluorescence micrographs (figure 6) showed that after 24 hrs of adhesion, slightly more number of nucleus vis-a-vis cells were observed in case silanized samples. In none of the cases the cells seemed to be stressed, rather they were extending their filopods which denotes that the surfaces did not have any adverse effect on the osteoblast cells.

Cell count data, Cell proliferation data and fluorescence micrographs clearly showed that silanized nitinol samples are slightly better than bare nitinol samples. This marginal betterment may be attributed to higher roughness of silanized samples, its better resistance to leaching [14], presence of Si, a known element to promote osseous bone growth. All these might have played a crucial yet combined role in this regard. So silanization proves to be a simple yet effective option of surface modification as far as cell material interaction is concerned and its scope can be further explored in case of bone implants like nails, screws etc.
4. Conclusion

The results clearly revealed that silanized samples offer slightly better cell count and cell proliferation than bare nitinol samples. In both the cases, cells did not show any sign of stress, rather in both the cases, they were found to extend number of filopods. Silanization increased surface roughness and provided more anchorage sites in the form of peaks and valleys and as a result the cell material interaction was found to be comparatively better. It may be noted that the experiments were done for a short duration of time but still showed that silanization offers advantage over its bare counterpart.

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