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Yüzey Karakteristiklerinin NiTi Şekil Hafızalı Alaşımlarının *in vitro* Biyouyumluluk Davranışı Üzerindeki Etkileri

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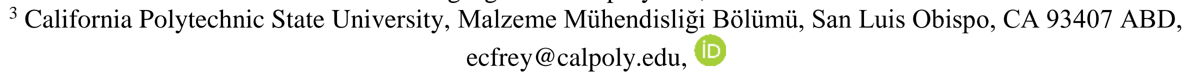

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Öz

Farklı geometrilere ve yüzey özelliklerine sahip üç set Nikel-Titanyum (NiTi) şekil hafızalı alaşımının (ŞHA) biyouyumluluğu, nitel ve nicel *in vitro* deneylerle incelenmiştir. Deneylerde kullanılan alaşımların bir seti levha, diğer iki seti ise farklı yarıçaplarda silindirik geometriye sahip örneklerdir. Hücre kültürü deneyleri öncesinde yapılan yapısal elektron mikroskobu ve profilometre incelemelerinde örneklerin geometrilerine bağlı olarak farklı yüzey özellikleri gösterdiği saptanmıştır. Yapısal karakterizasyon işlemlerinin devamında yapılan *in vitro* deneylerde ise, yüzey özelliklerinin şekil, dağılım ve derinliğinin hücre yapışması ve çoğalma davranışları üzerindeki etkileri elektron mikroskobu incelemeleri ve hücre sayımı deneyi ile araştırılmıştır. Sonuçlar örnek geometrisi ve yüzey pürüzlülüğünün ilk hücre yapışması açısından belirleyici faktörler olduğunu ortaya çıkarmıştır. Bununla birlikte, birbiriyle bağlantılı hücre ağlarının oluşumu açısından, yüzey oluklarının derinliği ve organizasyonunun daha kritik olduğu gözlemlenmiştir. Genel olarak bu çalışma, metalik biyomalzemelerin biyouyumluluğunun; yüzey özelliklerinin manipülasyonu, özellikle de yüzey karakteristiklerinin dağılım ve derinliğinin değiştirilmesi yoluyla geliştirilebileceğini göstermektedir.

Anahtar Kelimeler: NiTi, biyomedikal alaşım, biyouyumluluk, yüzey, fibroblast

Effects of Surface Characteristics on the *in Vitro* Biocompatibility Response of Niti Shape Memory Alloys

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Abstract

Biocompatibility of three sets of Nickel-Titanium (NiTi) shape memory alloys (SMAs) with varying geometries and surface characteristics were investigated through qualitative and quantitative *in vitro* experiments. One set of the alloy samples used in the experiments had a plate geometry while the other two sets had cylindrical geometries with different radii. Prior to the cell culture experiments, through the structural electron microscopy and profilometer investigations, the samples were detected to exhibit different surface properties based on their geometries. With the *in vitro* experiments which were conducted following the structural characterization procedures, the influence of surface feature shape, distribution, and depth on the cell attachment and proliferation behaviors was investigated via electron microcopy analysis and cell count experiments. Results revealed that sample

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geometry and surface roughness are determining factors for initial cell attachment. However, in terms of formation of interconnected cellular networks, depth and organization of surface grooves become more critical. Overall, this study demonstrates that the biocompatibility of metallic biomaterials can be improved through the manipulation of surface properties, especially the organization and depth of surface features.

Keywords: NiTi, biomedical alloy, biocompatibility, surface, fibroblast

1. INTRODUCTION

Nickel-Titanium shape memory alloys have been widely used as biomedical materials because of their unique shape memory and pseudoelastic properties, which enable them to return to their original shape once the applied load, heat, or combination thereof is removed—without causing any plastic deformation. These properties make them useful for various biomedical applications, such as cardiovascular stents, dental wires, and orthopedic vertebrae spacers [1,2]. However, the biocompatibility of NiTi SMAs is still subject to investigation for ensuring and improving the safe use of these materials for biomedical purposes.

As in all biomaterials, biocompatibility of NiTi SMAs is dependent on both the tissue's response to the alloy and the alloy's reaction to the body [2-4]. The alloy's response to the physiological environment is influenced by various material properties, including its chemical content, microstructure, and surface characteristics [2-4]. Among these, surface characteristics constitute a critical parameter in determining biocompatibility, as the alloy surface hosts the initial biomaterial-tissue interaction [5]. For metallic materials, including NiTi SMAs, surface properties are strongly related to processing history [3]. Therefore, constructing a relationship between a material's surface characteristics—and thus processing history—and its biocompatibility is essential for understanding the underlying mechanisms of tissue response.

Many have conducted studies focusing on larger-scale surface properties, such as roughness and surface groove frequency, depth, and organization, as well as microstructure related parameters, such as grain size, grain boundary distribution, and dislocation density [2, 6-8, 9-11]. Such studies indicate cellular response is strongly dependent on surface roughness, as well as on surface feature shape and organization [2, 6-8]. Moreover, it has been shown that alterations in microstructural features can affect the surface energy of the material, thus influencing physiological interactions [9-11]. On the other hand, some investigations emphasize the effect of surface chemistry and coating on cell response, rather than surface roughness of the metal itself [4,12-14]. The aforementioned studies carried out *in vitro* experiments with fibroblasts, osteoblasts, and malignant glioblastoma cells [2-14]. However, to the best of the authors' knowledge, studies focusing specifically on the time-dependent attachment and proliferation behavior of fibroblast cells on NiTi SMA surfaces of various surface characteristics have not been forwarded yet.

With this motivation, the current study investigated the *in vitro* biocompatibility of NiTi SMAs with three different geometries. Each exhibited different surface characteristics based on the different processing routes applied to obtain these specific geometries. The main objective of this study was to isolate and examine the influence of NiTi's surface properties—specifically, surface characteristic shape, distribution, and depth—on cell attachment and proliferation behavior. Results indicated that, in regards to favoring intercellular communication and cell proliferation following initial attachment; surface groove depth, a determining parameter for surface roughness, was found to be more critical than surface roughness itself. Therefore, the outcomes of this study indicate that cellular response can be modified by manipulating not only surface roughness, but also groove depth and organization.

2. MATERIALS AND METHODS

The NiTi samples (Memry, Germany) under investigation featured three different geometries: one plate specimen and two cylindrical specimens with different radii (referred to as thin and thick wire). Dimensions and chemical compositions of the three types of NiTi samples are summarized in Table 1.

Table 1. NiTi sample geometries and chemical contents. Chemical compositions are provided in atomic percentage of Ni, where the balance is Ti.

Specimen Type	Dimensions	Available Surface Area for Cellular Attachment	Ni content
Plate	1.3 cm × 0.65 cm	0.85 cm^2	50.7 at.%
Thin wire	r=1 mm, h=2.75 cm	1.73 cm^2	50.4 at.%
Thick wire	r=1.75 mm, h=1.5 cm	1.65 cm^2	50.7 at.%

Qualitative and quantitative structural analysis of the three types of NiTi samples was performed prior to the cell culture experiments. Scanning electron microscopy (SEM) (FEI Quanta 200, hi-vacuum) was used to examine the surfaces to be tested, after which surface roughness measurements were taken with a Mitutoyo Surftest SJ-201P stylus profilometer. For the profilometry analysis, three sets of each geometry of as-received NiTi samples were used. Average roughness (R_a) values were calculated at 40° angles from the wires' longitudinal axis to incorporate the effect of longitudinally-aligned grooves. Cut-off length was set to 0.25 mm.

Biocompatibility tests followed this initial structural analysis. For this purpose, mouse primary embryonic fibroblast cells (American Type Culture Collection (ATCC), NIH 3T3-L1) were used. The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) with Fetal Bovine Serum (FBS), Fungizone anti-fungal agent, and Penicillin Streptomycin (Pen Strep) antibiotic reagent. Cell media was replaced accordingly every other day. The cell culture environment remained constant using a humidified incubator at 37 °C and 5% CO_2. Using a 1X Trypsin-EDTA solution (Sigma-Aldrich, T3924), each cell type was passed a total of two times in 1:3 to 1:6 ratios to obtain approximately 8.375×10^6 3T3 cells per sample. Briefly, 3T3s were harvested using 1X Trypsin-EDTA solution (Sigma-Aldrich, T3924), centrifuged to a pellet, and re-suspended in 1 ml per sample of cell media. A gravity sodding technique was employed to gradually drip-deposit cells on the sample surfaces and ultimately achieve an approximate cell density of 326,625,000 cells/cm^2. 4 mL of cell media was added to each well after one hour to ensure cell survival while allowing for maximum adherence. The samples were cultured in a humidified incubator at 37 °C and 5% CO_2 for varying different time periods: 1, 3 and 7 days. Following each incubation period, a glutaraldehyde agent was used to fix the cells onto the sample surfaces. Once fixed, cell attachment and proliferation behaviors on the various NiTi specimens were analyzed via SEM.

Scanning electron microscopy of the as-received samples was conducted at 30kV to generate maximum topographical information. Conversely, analysis of the cell-seeded samples was done with a 10kV acceleration voltage to minimize charging and cell damage.

After collecting SEM images displaying cellular response to the different sample surfaces at specific incubation periods, an additional cell counting experiment was conducted. Cellometer Auto T4 Bright Field Cell Counter (Nexcelcom Biosciences) was used to quantify adhesion at each time point. Samples were removed from cell culture media and rinsed in 2 mL Dulbecco's cation-free phosphate-buffered saline (DCF-PBS). After rinsing, 1 mL of 1X Trypsin-EDTA solution (Sigma-Aldrich, T3924) was applied at the given time point per sample and analyzed for the number of attached cells per square millimeter surface.

3. RESULTS

Surface characterization of the NiTi samples of varying geometries via SEM and profilometer revealed notable differences between the three surfaces. Specifically, SEM images collected from representative areas of the as-is metal surfaces (Figure 1) show that groove shape and frequency vary significantly with sample geometry. Both of the cylindrical samples (thin and thick wire) exhibit linear grooves, whereas circular grooves are prominent on the plate specimen surface. Moreover, groove depth and thickness appear to vary between the thin and thick wire samples.

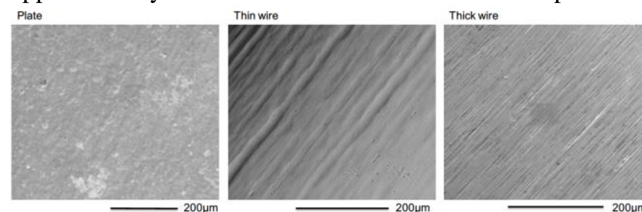

Figure 1. SEM images of the three NiTi sample surfaces in their as-received conditions.

These observations were supported through profilometry measurements by correlating these differences in groove characteristics with differences in surface roughness values. The thin wire had the highest average surface roughness value (R_a), and the plate sample exhibited a relatively low surface roughness as compared to both cylindrical samples (Table 2).

Table 2. Average surface roughness (R_a) values of the three NiTi surfaces.

Sample	Plate	Thin wire	Thick wire
R_a (µm)	0.19	0.70	0.45
stdev	0.02	0.11	0.06

SEM images demonstrating the fibroblast attachment and spreading behaviors on the representative areas of the different metal surfaces at the incubation periods of 1, 3 and 7 days are provided respectively in Figures 2, 3, and 4.

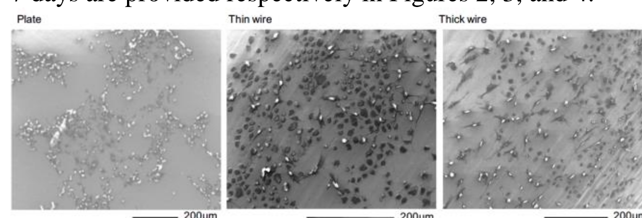

Figure 2. SEM images of the three NiTi sample surfaces after 1 day of incubation with 3T3 fibroblast cells.

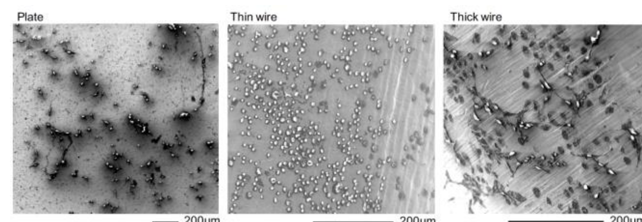

Figure 3. SEM images of the three NiTi sample surfaces after 3 days of incubation with 3T3 fibroblast cells.

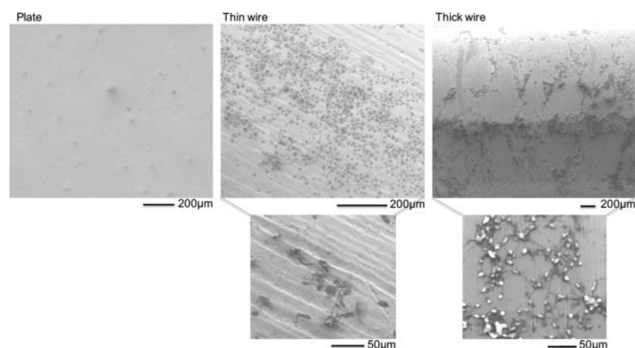

Figure 4. SEM images of the three NiTi sample surfaces after 7 days of incubation with 3T3 fibroblast cells under two different levels of magnification.

After one day of incubation, the 3T3 cells appeared to be well-attached to all three sample surfaces and even began spreading and forming networks. This was especially so for the plate sample surface (Figure 2). However, following 3 days of initial cell seeding, the spreading behavior of the cells on the plate sample had significantly diminished as compared to day 1. Similarly, the cells on the thin wire surface lacked extensions and showed almost no sign of interconnection with each other, whereas cells on the thick wire sample were observed to spread better and even started to form small networks (Figure 3).

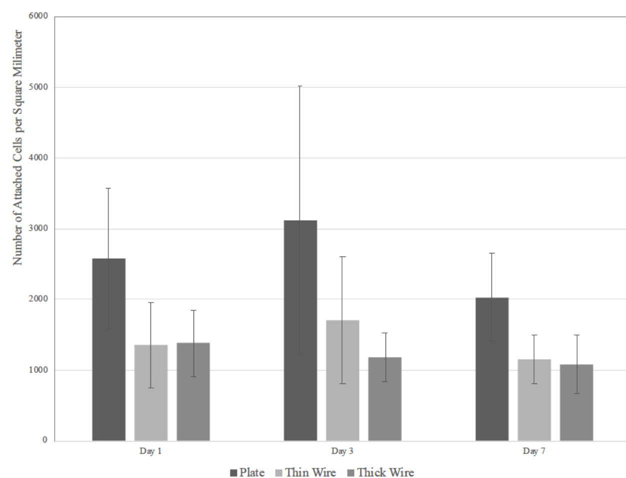

Figure 5. Number of attached cells per square millimeter on the three NiTi surfaces over three different incubation periods.

SEM observations of the cell cultured samples following 7 days of incubation revealed that the fibroblast cells on the plate sample exhibited minimal cytosolic extensions and therefore no indication of spreading. A higher density of viable cells was observed on the thin wire surface; however, these cells also mostly lacked extensions. As made evident in higher magnification images, cell spreading and network formation was very limited. On the other hand, the thick wire sample surface was mostly occupied by viable cells, whose interconnection and relatively advanced networking are evident at both lower and higher magnifications (Figure 4).

Ouantitative analysis of fibroblast cell attachment on the different metal surfaces at varying time periods is summarized in Figure 5. According to the results of this analysis, the number of attached cells per square millimeter was highest on the plate sample for all incubation times. Moreover, the plate sample achieved maximum cell attachment on day 3 and minimum cell attachment by day 7. The thin wire exhibited a similar temporal trend, but at a much lower cell count. Conversely, the thick wire exhibited a small, steady decrease in cell count with time, such that the obtained cell densities remained closer to each other as compared to the other two samples.

4. DISCUSSION

According to the initial characterization, the three NiTi sample types exhibit significantly different surface characteristics. This is qualitatively apparent from SEM inspection in terms of surface groove shape and depth, as well as quantitatively evident from the surface roughness values obtained by profilometry measurements (Figure 1 and Table 2). These differences in surface roughness and topography are expected to influence the biocompatibility of the metal surface at various levels, namely; at *ex-situ* and *in-vitro* as well as *in-vivo* levels.

A recent study investigated the *ex-situ* biocompatibility of the same NiTi sample geometries used in the current work. After exposure to varying simulated body fluids, the different NiTi surfaces displayed differences in new structure (i.e., corrosion product) formation [3]. This study demonstrated the significant effect of surface properties on the alloy response to physiological fluid media. The current study complements this past work by revealing that these differences in surface characteristics also significantly influence the cell response to the sample surface.

SEM observations on the cell cultured samples demonstrated that 3T3 cells were able to attach to all three sample surfaces on day 1 (Figure 2). However, as the incubation period was prolonged, significant differences were observed in terms of the cellular interconnection on the different metal surfaces (Figures 3 and 4). Specifically, networking of the cells on the plate sample diminished on day 3 through day 7, by which point the cells were fairly isolated from each other. On the thin wire surface, although the cells weren't observed to become more isolated from each other, no improvements were prominent in terms of the interconnected cellular network formation. On the other hand, a significant improvement was observed in the formation of interconnected cellular networks on the thick wire sample over time.

The difference in cell response to the various specimen surfaces can be attributed to their significant surface roughness differences (Table 2). The plate sample exhibits a rather smooth surface with the lowest R_a values among the three samples. As increased surface roughness also indicates higher surface energy—and therefore a more suitable

environment for cell attachment [5,10,11], it is reasonable that the plate specimen provides a less favorable surface for cell attachment and spreading. Although initial cell attachment is possible due to the biocompatible nature of NiTi, the rather smooth, low surface-energy plate specimen does not favor interconnection of cells over longer incubation periods.

When comparing the two cylindrical samples, namely thin and thick wire, notable differences are evident—especially in terms of cell spreading behavior at extended incubation durations. Similar to the plate sample, both surfaces provide an initially favorable environment for cell attachment. Specifically, after one day of incubation, both surfaces exhibited similar cell densities and spreading behaviors (Figure 2). However, on the third day of incubation, the 3T3 cells on the thin wire surface appeared isolated from each other where the cell geometry is rather circular, lacking extensions necessary for cellular interconnection (Figure 3). A similar behavior was observed on the seventh day of cell culture on the thin wire sample, where still a significant number of cells are prominent, but without extensions or signs of forming networks. On the contrary, the cells on the thick wire surface demonstrated signs of spreading, starting from the first day and progressing through the third day of incubation (Figures 2 and 3). Especially on the third day of incubation, extensions of the cells were more apparent and intercellular networking was prevalent in contrast to thin wire (Figure 3). Moreover, further cell spreading and improved cellular interconnection was observed on the thick wire as the incubation period is extended, as evident from the dense interconnected cellular networks on many areas of the thick wire surface on the 7th day of incubation (Figure 4). The thin wire exhibits a rougher surface than the thick wire, but provides a less favorable environment for cellular networking: cell morphology observations demonstrated the presence of much healthier cellular networks on the thick wire sample (Figures 3 and 4). This is contrary to the plate sample, where lower surface roughness appears to have resulted in diminished cell attachment, proliferation, and networking. This would seem evidence against the positive correlation between surface roughness and cellular proliferation. However, it is also important to consider the effect of groove depth on intercellular interaction [6]. Specifically, high surface roughness of the thin wire was the result of the presence of deeper grooves, which acted as obstacles against the bridging and interaction of cells. Thus, while cells were able to successfully attach to the thin wire surface, they were incapable of communicating with cells in neighboring grooves because of the significant groove depth in which they were positioned. This impeded cell-to-cell communication and therefore diminished cellular interconnection on the thin wire surface. The thick wire, on other hand, exhibited shallower grooves than those on the thin wire, as supported by its lower R_a values. Therefore, cells positioned within grooves on the thick wire surface were able to extend their cytoplasmic "arms" far enough to overcome these grooves and consequently interact with each other. Moreover, the distance between the grooves on the thick wire surface was much shorter as compared to those on the thin wire surface (Figure 1). For the cells, this translated to increased frequency in sites for cellular adhesion and better chances for inter-groove connection. Cumulatively, the increased frequency and decreased depth of the thick wire grooves resulted in enhanced cellular networking (Figures 3 and 4).

Quantitative analysis of cellular attachment appears to contradict the behavior observed by SEM examinations (Figure 5). Specifically, the highest cellular attachment levels were achieved on the plate sample at all incubation times, despite the poor cellular networking observed via SEM. This behavior can be attributed to the flat surface of the plate sample, which enabled easier initial cell adhesion. In contrast, the curvy surfaces of the wire samples were not ideal for cell adhesion, as reflected by the two wires' similarly low number of attached cells per square millimeter. However, the thick wire demonstrated a more stable trend in terms of the change in numbers of attached cells, as compared to the plate and thin wire samples. Specifically, on the plate and thin wire samples, the peak values of cell attachment were attained on the third day of incubation, which was followed by a decrease in the numbers of attached cells through day 7. In other words, after reaching a maximum cell attachment level, further proliferation was not favored on the plate and thin wire samples. This behavior was due to the limited cellular interconnection resulting from surface roughness and groove properties of these two surfaces, as also observed in the qualitative examinations. On the other hand, the stable trend in the cell attachment levels on the thick wire sample over time demonstrates that the surface roughness and groove depth of the thick wire were suitable for both initial cell attachment and subsequent cellular network formation. This is another indication that among the three surfaces analyzed, the thick sample provides the optimum surface for cellular attachment and interconnection.

Finally, the general decrease in cell concentration on all samples by the 7th day of incubation can be attributed to the limited surface area provided by the samples, which became insufficient for cell attachment and proliferation over prolonged incubation periods. This also constitutes the reason for limiting the duration of the experiments with 7 days.

Overall, results of the qualitative and quantitative analysis demonstrate that while surface roughness plays a significant role in terms of determining cell attachment, it is the groove characteristics that enable cells to form large networks over prolonged incubation periods.

It should also be noted that since implants are intended for long-term use, extended duration studies into the influence of metal surface properties on biocompatibility are essential prior to implementing biomaterials with deliberately altered surface properties into clinical applications.

5. CONCLUSION

Results reiterate the importance of metallic biomaterial surface characteristics when considering cellular adhesion, proliferation, and networking. It is known that rougher surfaces provide enhanced surface area and surface energy, which together promote cellular adhesion. However, this does not necessarily mean rougher surfaces are conducive to intercellular networking. The current study demonstrated that frequency and distribution of surface features (such as surface grooves) become more critical when evaluating cellular response in terms of intercellular networking. Specifically, if surface grooves are deep and far apart, connective tissue cells such as fibroblasts will have limited success in forming intercellular networks. In this way, a high "surface roughness"—as measured by apparent variations in surface height—can be detrimental to the physiological integration of biomaterials. This study indicates that an ideal biometal surface, defined as one conducive to the successful cell attachment, networking, and proliferation of cells, should exhibit a balance of surface roughness and surface feature characteristics (i.e. groove depth). To this aim, deliberately modifying the surface topography of prospective implant materials is recommended in order to optimize the cellular response of the affected region.

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