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Amino Acid Conjugated Self Assembled Molecules Modified Titanium Surfaces For Investigating Osteoblast Behavior

Osteoblast Davranışının Titanyum Yüzeylere Amino Asit Konjüge Edilmiş Kendiliğinden Oluşan Tek Katman Moleküllerin Modifikasyonu ile İncelenmesi

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ABSTRACT

In this study, human fetal osteoblasts behavior was investigated on titanium surfaces that has been modified with amino acid conjugated self-assembled molecules. For this purpose, 3-aminopropyltriethoxysilane (APTES) was conjugated by histidine and leucine and these newly synthesized molecules were used in different combinations to modify titanium surfaces via creating amino acid conjugated self-assembled monolayers (SAM) on titanium surfaces. The modification of the surfaces to introduce hydrophilic and hydrophobic regions on the surface was achieved with varying concentrations (v/v,100:0 20:80, 50:50, 80:20, 0:100). X-ray photoelectron spectroscopy (XPS) analysis and water contact angle measurements were performed for characterizing all of the modified surfaces in order to verify presence of amino acid specific bonds and wettability behavior to find suitable concentrations to support initial cell adhesion. In order to confirm that the surface modification supported cell adhesion and proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Our results have shown that, amino acid SAM modification can be used to fine tune surface wettability and adherent cells were able to proliferate at different rates using different mixture concentrations. This presented approach can prove useful for expanding fine tuning surface chemistry methods for more specific applications and research.

Key Words
Titanium, surface modification, self-assembled molecules (SAMs), amino acids.

ÖZ

Bu çalışmada insan fetal osteoblast hücrelerinin amino asit konjüge edilmiş kendiliğinden oluşan tek katman molekül modifikasyonu (TKM) yapılmış titanium yüzeyler üzerindeki davranışı incelenmiştir. 3-aminopropyltriethoxysilane (APTES), histidine ve lösin ile konjüge edilmiş ve bu yeni moleküller değişken konsantrasyonlarda karıştırılarak titanium yüzeylerde TKMler oluşturmaları sağlanmıştır. Hidrofobik ve hidrofilik bölgeler bu amino asit konjüge moleküllerin değişken konsantrasyonlarının yüzeylere modifiye edilmesiyle elde edilmiştir (v/v, 100:0 20:80, 50:50, 80:20, 0:100). X-ray fotoelektron spektroskopisi (XPS) analizi ve yüzey su temas açısı analizleriyle modifiye edilmiş yüzeylerde amino asit konjüge moleküllerin varlığı gösterilmiş ve yüzey ıslanması değerlerine göre ilk hücre tutunmasını destekleyecek uygun karışım oranı tayin edilmiştir. Hücre tutunması ve çoğalmasının gösterilmesi ve tayini için MTT testi kullanılmıştır. Sonuçlar, amino asit TKMlerinin yüzey ıslanılabilirliğini ayarlamak için kullanılabileceğini ve farklı karışım oranlarında farklı hücre çoğalması davranışlarının görüldüğünü göstermektedir. Bu çalışmada kullanılan modifikasyon yaklaşımı, titanyum yüzeylerinin kimyasının isteğe göre ayarlabilirliğini ve bu yöntem ile daha spesifik araştırmaların yapılabileceğini göstermektedir.

Anahtar Kelimeler
Titanyum, Amino asit SAMs, osteoblast, yüzey modifikasyonu.

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INTRODUCTION

Bone grafting is a widely used approach for treating of bone injuries. Traditional grafting methods include autografting, allografting, and xenografting; however, in the past decades use of artificial bone substitutes like ceramic, polymeric, and metallic biomaterials have gained traction in treatment of these defects. Metallic biomaterials, especially titanium and its alloys, have an impactful role in bone treatments due to their mechanic properties closely mimicking native bone tissue such as durability, resistance to corrosion, and low elastic modulus. Additionally, its bioinert nature, good biocompatibility, and osteointegration have been important factors to make it one of the most utilized biomaterials in the history. [1, 2]

Enhancing osteointegration and bioactivity of the titanium implants have been achieved through various surface modification methods. Plasma spraying, [3, 4] acid etching, [5, 6] anodization, [7] grit-blasting, [8, 9] and self-assembled monolayer surface formation [10] are utilized to modify titanium surfaces to achieve desired characteristics. Self-assembled monolayers (SAMs) are a collection of organic molecules that is bound to a surface and spontaneously forming a one molecule thick layer across the material. A hydrophobic functional group and a hydrophilic head forms an amphiphilic structure, which allows them to form covalently bind onto material surface from head groups and organize their tail groups due to their alkyl chains. Amino acid SAMs were synthesized and utilized in the literature to measure their modification efficacy, protein adsorption behavior, and cellular responses; however, modification of different amino acids at the same time on a material surface has not been investigated. [11–13] Using different amino acids known for their cellular behavior enhancing effects, when used in tandem, can result in a synergistic effect. These exposed tail groups of SAMs alter chemical characteristics of surfaces they are bound onto, resulting in a coating that alter the materials native hydrophilicity and roughness. This difference leads to a change in cellular response and ultimately can decide on adherent cell fate. [14]

In this study, titanium surfaces were chemically modified by various amino acid conjugated SAMs. Amino acid SAMs were synthesized through a reaction of 3-aminopropyltriethoxysilane with two different amino acids, namely L-histidine and L-leucine. Imidazole group of histidine promotes the interactions with metal ions such as calcium and magnesium which play important roles in biomineralization and cell adhesion. [15] Its pKa being around 6 also helps to buffer local microenvironment at physiological pH. [16] Leucine presence adds hydrophobicity to the microenvironment, which mimics the native extracellular matrix. [17] Combination of both of these amino acids have shown to positively affect cellular attachment and proliferation. These amino acid SAM modified surfaces were investigated for osteoblast cell viability and surface wettability, as well as modification So; it is important to inspect cell differentiation, viability, and proliferation on these titanium surfaces modified with amino acid conjugated SAMs. Response of osteoblasts these SAM modified titanium surfaces were investigated after modifying the surface in different combinations of those molecules in this study.

MATERIALS and METHODS

Synthesis of Amino Acid Conjugated Self Assembled Molecules (SAMs)

Amino acid conjugated self-assembled molecules used in this study were prepared according to our previous report. [11] In summary benzotriazole (4 eq.) was dissolved in pre-dried tetrahydrofuran (THF) under N_2 atmosphere at room temperature and thionyl chloride ($SOCl_2$, 1.2 eq.) was combined with this solution dropwise and solution was stirred at room temperature for 30 minutes. Cbz-Leu-OH (1 eq.) or Cbz-His-OH (1 eq.) were added in one portion and reaction mixture left to stir for further 3 hours. Precipitate formed from this reaction was removed via filtering whereas the filtrate was concentrated using a vacuum chamber. Histidine reaction mixture, which at this point contains Cbz-His-Bt intermediate, was used without any further purification, on the other hand leucine reaction mixture was dissolved in ethylacetate and extracted for removal of excess benzotriazole using a Na_2CO_3 solution (20% by w/v). Subsequently, organic layer was dried using Na_2SO_4 to obtain Cbz-Leu-Bt. Both intermediates were then reacted further by adding 3-(trimethoxysilyl)propan-1-amine (1 eq.) dropwise to the solution of Cbz-AA-Bt in CH2Cl2 under N2 atmosphere for 30 minutes. Reaction mixtures were extracted by using Na2CO3 solution (10% by w/v). Cbz-[His-Si$(OCH_3)_3$] and Cbz-[Leu-Si$(OCH_3)_3$] were collected by drying over Na_2SO_4. Finally, Cbz-[His-Si$(OCH_3)_3$] or Cbz-[Leu-Si$(OCH_3)_3$] were dissolved in methanol and combined with Pd/C catalyst (3 eq.) to react under 50

bar H_2 gas pressure in a stainless steel autoclave for 5 h in order to remove/reduce Cbz groups. Catalyst was filtered over celite whereas filtrates were evaporated to obtain [His-Si(OCH_3)$_3$] and [Leu-Si(OCH_3)$_3$], which will be referred as His-SAM and Leu-SAM, respectively from now on.

Activation of the titanium surfaces for modification

Ti coated glass slides were cleaned by submerging into HNO3 solution (10% v/v) at 80°C for 30 min. Then, they were washed by deionized (DI) water and dried in an oven at 50°C. In order to activate Ti coated glass slides, they were treated with oxygen plasma (March Plasma Systems, PM-100) for 10 minutes at 200 mT and 50 sccm oxygen flow. At the end of the surface activation procedure, hydroxyl groups were introduced on Ti surfaces that were ready for further a single step modification.

Preparation of the SAM Solutions

Conditions for the self-assembled monolayer formation, the effecting factors as dipping time and concentration of the solutions were picked as 24 hours and 10 mM, respectively, according to a previous study that investigated same amino acid SAMs. [12] Solutions used in modifications were prepared in absolute ethanol by dissolving the appropriate amounts of SAMs.

Modification of Ti Surfaces by SAMs

Surfaces were modified at room temperature by submerging activated Ti coated glass samples (diameter: 14 mm) in the solutions containing 10 mM of His-SAM, Leu-SAM and three different concentrations of His-SAM and Leu-SAM mixtures (80% His-SAM+20% Leu-SAM, 50% His-SAM+50% Leu-SAM and 20% His-SAM+80% Leu-SAM (v/v)). After incubation for 24 hours, samples were heated at 120°C for an additional 5 minutes to complete the reaction between silane groups of SAMs and hydroxyl groups on Ti surfaces. Then, modified Ti surfaces were washed with absolute ethanol and deionized H_2O. Samples were sterilized with 70% ethanol, rinsed with deionized H_2O, and finally treated for 30 minutes under UV light before cell culture studies.

Characterization Studies

Ellipsometry

Thicknesses of the modified surfaces were measured through an ellipsometry equipment (Auto-Nulling Ellipsometry, Nanofilm EP3, Germany). All measurements were with a green laser beam with a wavelength of 658 nm as well as with an incidence measurement angle of 65°. During thickness measurements, one spot auto-nulling to integrate on 50 µm x50 µm representative area of the specimen in correspondence to appropriate algorithm was utilized.

For a single layer, an assumption of Ti/TiO_2/organic layer/air as four-phase surface layer model was made to carry out measurements. SAM modification presents layers with carbon bonds on surfaces after modifications. The relationship between layer thickness and its refractive index for very thin (<10 nm) films requires an estimation of refractive indexes of other three layers to measure modification thickness. In this system, refractive indexes of Ti, TiO_2, organic layer (His-SAM and Leu-SAM) and air were estimated as 2.24, 2.493, 1.460 and 1.000, respectively.

Determination of hydrophilicity through water contact angle measurements

Hydrophilicity of modified Ti surfaces, namely, bare Ti, activated Ti, His-SAM and Leu-SAM, were measured with a contact angle goniometer (Model DSA 100, Krüss, Germany) by sessile drop method. A water droplet of 1 µL was placed on the materials and measurements were taken after a minute. All measurements were taken as triplicates for each sample.

X-Ray Photoelectron Spectroscopy (XPS)

Elemental composition of modified Ti surfaces was analyzed by XPS (ThermoScientific K-Alpha X-ray Photoelectron Spectrometer) with a monochromated aluminum Kα radiation at 72 W. Spot diameter was selected to be 400 µm and measurements were taken with an angle of 90° with a 128-channel detector.

Cell Culturing

All cell culture studies were conducted using a human fetal osteoblast cell line (ATCC-CRL-11372, Rockville, MD, USA) in 24-well plates. Seeding density for cells was $2x10^4$ cells per well. Complete DMEM-F12 (Biosera, France) containing 10% FBS and 1% penicillin/streptomycin solution used as growth medium. Cellular viabilities were analyzed with am MTT assay and measurements were taken on days 1 and 4. Number of replicated for each group was six.

MTT Assay

10% MTT solution (5mg/mL) in complete growth medium were added into the wells and incubated for 3 hours. MTT solution was aspirated and formazan crystals were dissolved with 150 µL of DMSO. Solutions were transferred to 96-well plates and absorbance values were measured at wavelengths of 570 nm and 750 nm. Titanium surfaces without any surface modification were treated as controls.

Statistical Analysis

One-way ANOVA and a post-hoc Tukey's honest significant difference test was performed for all applicable analyses and $p<0.05$ was considered to show a meaningful statistical difference.

RESULTS and DISCUSSION

The NMR spectra recorded for both His-SAM and Leu-SAM, and intermediates obtained during synthesis routes fully confirmed that all molecules were successfully synthesized [12, 18].

Aforementioned, ellipsometry, water contact angle measurement, and XPS analyses were utilized to confirm SAMs formation on Ti surfaces while characterizing thickness, wettability, and surface chemistry variation during surface modification steps. In this context, thicknesses of His-SAM and Leu-SAM modified surfaces were measured via ellipsometry and determined as 1.8 ± 0.3 nm and 1.8 ± 0.2 nm, respectively. The results pointed out that a single layer coverage was achieved in the light of the geometrical descriptors calculation performed by using the software, MarvinSketch via default parameters. As given the respective figure, both SAMs have quite close values in respect to dreiding energy, van der Waals volume, and length perpendicular to area. As a result, the algorithmic model chosen for ellipsometry measurements was proper to measure thickness that confirmed aforementioned monolayer coverage.

Thickness of His-SAM was measured 1.8 ± 0.3 nm in ellipsometry analysis, which is higher than the theoretical value of 1.63. Theoretical value for thickness is calculated when the surface binding is assumed at an angle of 90° with the surface. Even though the values are relatively close to each other, this discrepancy may differ due to the binding angle of SAMs onto surface being different from 90° Some regions of SAM functionalized Ti surfaces may have resulted in more than a monolayer and created multilayers built upon the angled SAMs found on the surface. A similar trend is also observed with Leu-SAM modified surfaces, which had a measured thickness of 1.8 ± 0.2 nm. This measurement is also greater than the theoretical value for Leu-SAM and it might also point out a similar multilayer formation. Furthermore, hydrophilicity of bare Ti and modified Ti surfaces were measured with a water contact angle measurement device. Water contact angle values of bare Ti, His-SAM, Leu-SAM, His-SAM/Leu-SAM (80:20 v/v), His-SAM/Leu-SAM (50:50 v/v), and His-SAM/Leu-SAM (20:80 v/v) are determined as 60.2° ± 3.8°, 32.28° ± 2.34°, 68.05° ± 2.84°, 65.99° ± 4.81°, 55.85° ± 2.82°, and 39.1° ± 6.92° respectively (Figure 1). Ti surfaces modified with His-SAM, exhibited a hydrophilic water contact angle characteristic (32.28° ± 2.34°). Water contact angle of Leu-SAM modified surfaces was measured as 68.05° ±2.84. His-SAM coverage has decreased water contact angle due to its relatively hydrophilic heteroaromatic charged ring, whereas; Leu-SAM coverage has increased the value because of its hydrophobic alkyl chain and it is reported previously that leucine is more hydrophobic than histidine and their wettability properties in comparison to each other have not changed after being modified onto titanium surfaces. [19] Between all surfaces modified with SAMs, His-SAM and Leu-SAM resulted in most hydrophilic and most hydrophobic, respectively. Mixture of different amounts of His-SAMs and Leu-SAMs resulted in a ranging wettability behavior on titanium surfaces. As expected, most hydrophilic group of SAM mixtures is 80% His-SAM+20% Leu-SAM and most hydrophobic group is 20% His-SAM+80% Leu-SAM. Combination of His-SAM and Leu-SAM in different concentration ratios allowed to easily tune surface contact angle value, in other words surface wettability properties can be adjusted using amino acid SAMs

XPS analysis were performed to confirm the success of Ti surface modification with His-SAM and Leu-SAM. In Figure 2a, general survey of bare Ti, and SAMs modified Ti were given and three specific binding energy peaks at 284.9, 457.3 and 530.3 eV were found for C1s, Ti2p and O1s, respectively. [20] Amino acid conjugated SAMs – specific N1s peaks were showed in both His-SAM and Leu-SAM modified Ti to prove surface modification, as bare Ti is lack of nitrogen atoms. [11] After deconvolution analysis for C1s spectra (Figure 2b), bonds having binding energies of 284.2, 285.9 and 287.4 eV were found in both modified Ti, which were assigned to C-C/C-H, C-N and C=O, respectively. [21] These peaks at

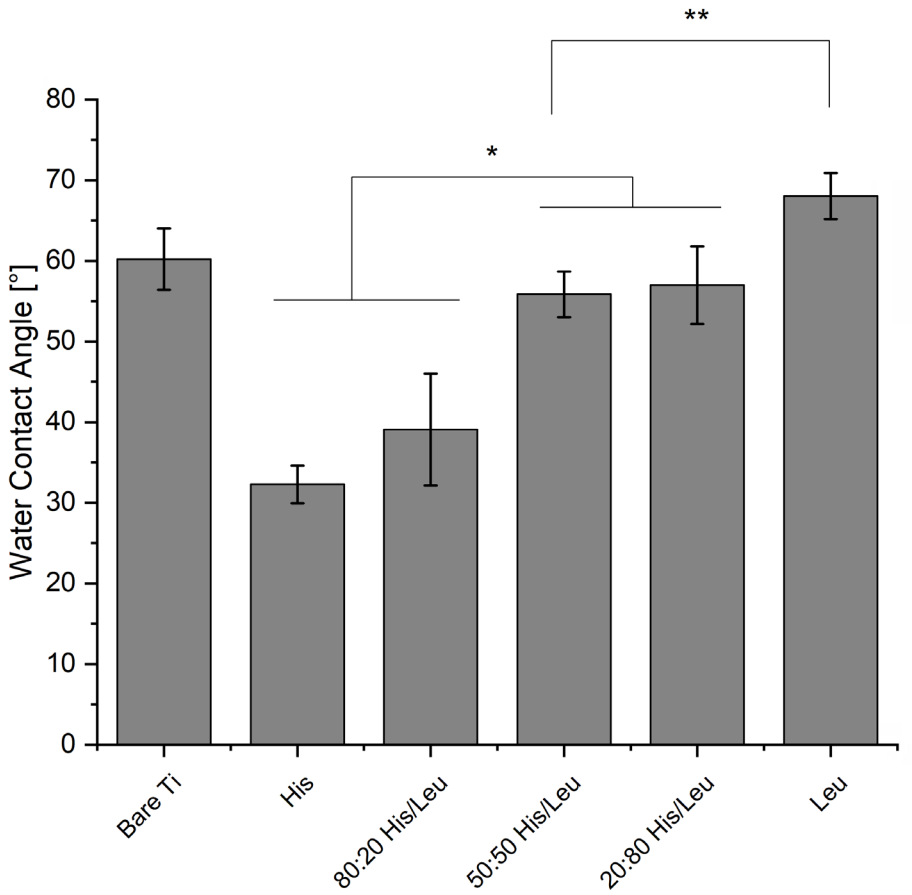

Figure 1. Water contact angle values for Ti coated glass samples modified with His-SAM and Leu-SAM in different combination after 24 hours of modification treatment. (*: p<0.01, **:p<0.05).

mentioned binding energies were certain indications of amino acid structures. In addition to these bonds, presence of C=N and C=C double bonds of His-SAM at 285.0 and 284.5 eV was attributed the imidazole group of histidine on Ti surface. [22, 23] Surface modification of Ti was also proved with the deconvoluted N1s spectrum (Figure 2c). Two N1s sub-peaks were fitted for His-SAM and Leu-SAM modified Ti. Peaks at binding energies of 398.5 and 399.7 were associated to C-NH2 and C-N bonds of SAMs, respectively. [24, 25] The intensity of C-N bond is higher in Leu-SAM, as it corresponds almost all of the nitrogen species in the group. In the His-SAM modified Ti surfaces, an extra peak is observed at 399.4 eV. The detection of this relatively narrow C=N peak in the N1s spectra was an indicate of histidine's specific imidazole group. [24].

Viabilities of adherent cells on all surfaces were measured with an MTT assay on day 1 and day 4, shown on Figure 3. All experimental groups resulted in significantly different viability values (100% His, 80% His+20% Leu, 20% His+80% Leu, 100% Leu) in comparison with bare Ti on the day 4. Viable cell amounts on all six different surfaces were comparable to each other and didn't result in a statistical difference. Starting on day 4, effect of surface properties on cell viability became apparent. On day 4, greatest cell viability was observed on bare Ti surface, and followed by 50% His+50% Leu modified surfaces. Herein, His-SAM might have decreased the cell viability because of its charged heteroaromatic ring which enhanced surface wettability and inhibited cell attachment/growth on the surface when being used it as a single modifier (100%). Moreover, Leu-SAM also decreased the cell viability even though it contains relatively hydrophobic alkyl chain which inhibited surface wettability when being used it as a single modifier (100%). Only significant difference observed was measurement taken from 100% His group, observed on day 1. All cells on different surfaces have increased their cell population on day 4. Bare Ti resulted in the highest number of viable cells on day 4, followed by 50% His-SAM + 50% Leu-SAM group, where they resulted in similar wettability properties. Viable cell concentrations were at their minimum on the most hydrophilic (100%

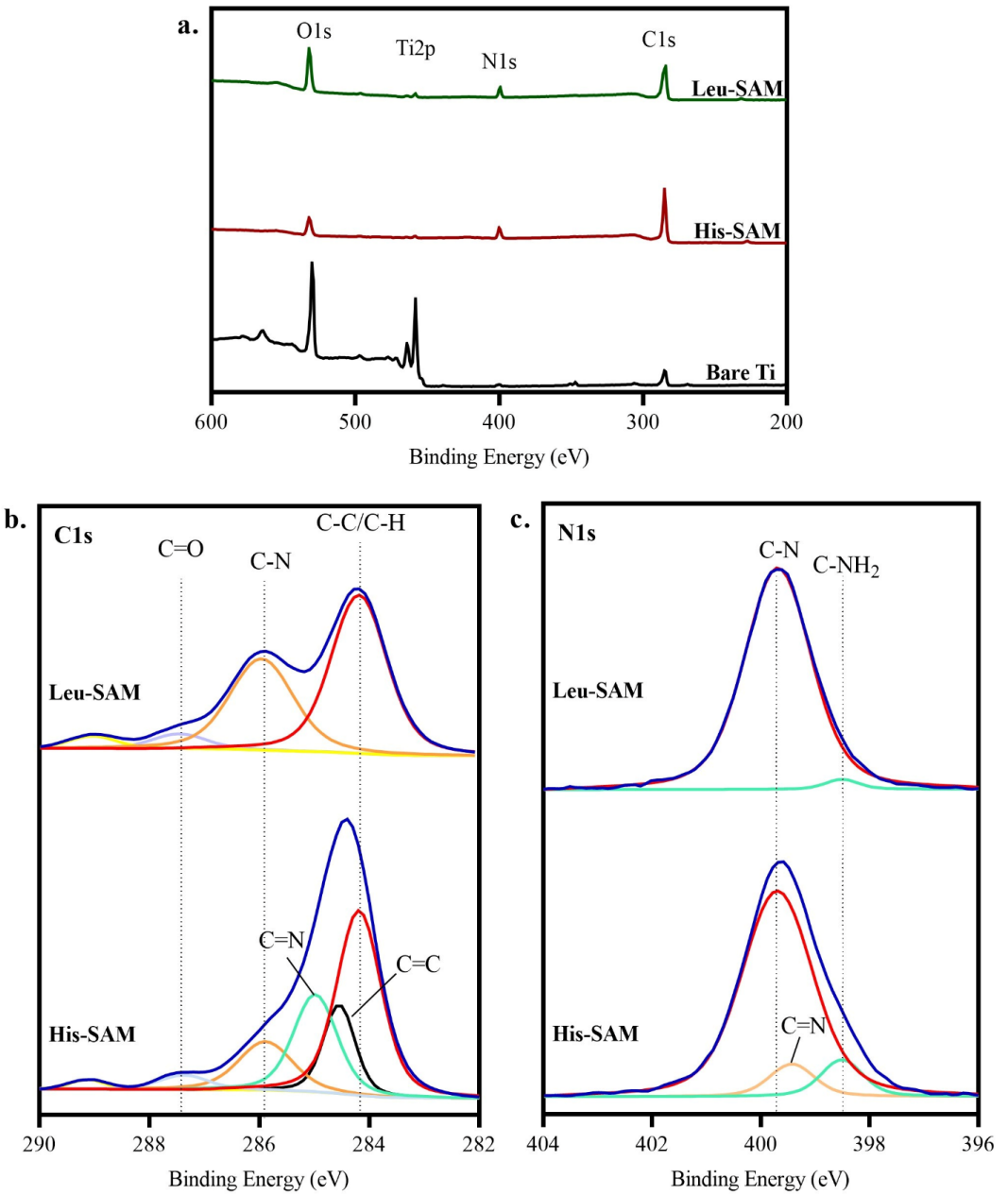

Figure 2. XPS analyses of bare and SAMs modified Ti surfaces. (a) Full-range survey spectra for bare, His-SAM and Leu-SAM modified surfaces, (b) high resolution C1s and (c) high resolution N1s spectra of His-SAM and Leu-SAM modified Ti surfaces.

His-SAM) and the most hydrophobic (100% Leu-SAM) surfaces on day 4. Combination of both SAMs interestingly enhanced the cell viability while the increasing rates of His-SAMs firstly enhanced cell up to 1:1 rate, then decreased this contribution. As a conclusion, the results emphasized that it is possible to tune the cellular attachment and cell integration properties of Ti surface by varying the combination of amino acid SAMs. Highest cell viability on modified surfaces was from the surfaces functionalized with 50% His-SAM+50% Leu-SAM, which can be attributed to 50% His-SAM+50% Leu-SAM surface resulted in a water contact angle value of 60°. It is known that moderately hydrophilic Ti surfaces are suitable for osteoblastic cell behavior and promote osteoblast adhesion, and optimal water contact angle for osteoblast cells were reported to be 64°. [26, 27] Results are consistent with the literature where moderately hydrophilic surfaces have been shown to optimally

promote cell proliferation. [26] Additionally, a similar study modifying the surfaces with small peptides rather than amino acids themselves have seen similar synergistic results when modifying surfaces with different peptides at the same time and saw similar enhancing proliferative effects. They have hypothesized that the mixtures created specificity for initial cell attachment to the surfaces, which might also be the reason of our observation as well. [28] On the other hand, with surfaces around similar water contact angle values, namely Bare Ti and 100% Leu-SAM, had shown significantly higher and lower levels of viability, respectively. Even though surface wettability is an indicator for enhanced cell adhesion and proliferation, it is not the only factor affecting cellular adhesion on the biomaterial surfaces. [29]

CONCLUSION

This study investigated the feasibility and biocompatibility of titanium surfaces modified with various different concentrations of His-SAMs and Leu-SAMs. XPS and ellipsometry measurements have shown that SAMs were successfully modified onto the surfaces using a single step functionalization approach. Surface wettability was shown to be easily tunable with changing mixture ratios of SAMs. In this study, different concentrations of His-SAMs and Leu-SAMs on titanium surfaces were shown to change cell viability. We have observed that the 1:1 combination of His-SAM and Leu-SAM resulted in a greater proliferation behavior, comparatively. Presence of individually inferior surface modifications in terms of adherent cell behavior might get significantly potent when used together. This might help elevate the understanding of surface modification approaches by encouraging to combine different surface modification molecules together. However, further investigation is required to see other cell behavior such as effects on cell morphology, differences in gene expression, and possible differentiation markers to better understand and tailor amino acid SAM modified surfaces towards specific applications.

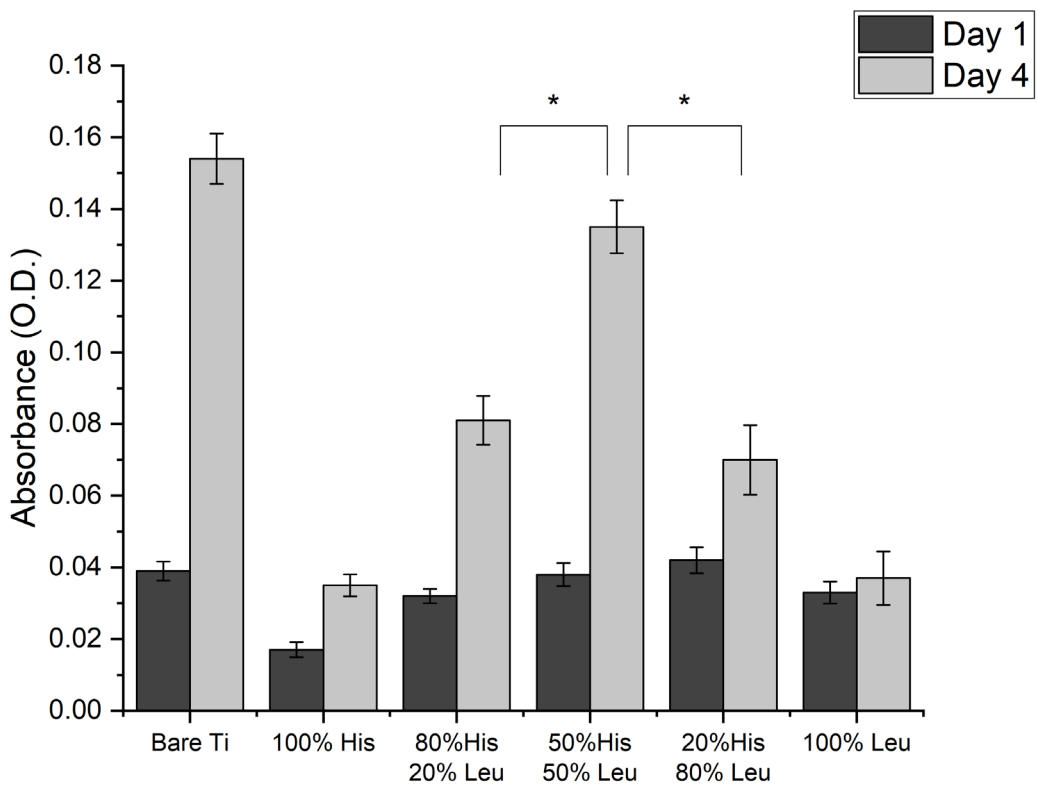

Figure 3. MTT assay results for measuring cellular viability of osteoblasts on Ti surfaces with and without SAM modification after the day 1 and 4 of incubation. (*: $p<0.05$).

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