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幹細胞於表面生物活性改質鈦金屬之生物性反應

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一、中文摘要

良好生物相容性是植體的首要要求，而植體材料的本質及其表面特質決定其生物相容性，尤其是與宿主組織直接接觸的材料表面。雖然目前對於植體表面與組織之間的反應、其長期穩定性及在臨床上的顯著性仍無定論，但已知植入後最早的生物反應為組織液蛋白質在植體表面的吸附，這層緊密吸附的蛋白質直接決定接下來宿主對植體表面的細胞反應，所以為了提昇植體與組織間整合，可藉由控制界面反應，吸附特定蛋白質，進而導引有利之組織癒合。本研究利用低溫電漿來活化鈦金屬表面以進一步連接白蛋白，企圖改變鈦金屬表面化學性質，發展一種能鍵結已知生物活性分子到鈦金屬表面的技術。首先鈦金屬圓片以氬氣電漿去除表面污染物，來產生可重複取得的清潔表面，接著用丙烯胺電漿處理，使丙烯胺聚合在鈦金屬表面，再以交鏈劑戊二醛將白蛋白與丙烯胺的胺基($-NH_2$) 鍵結。研究顯示使用電漿處理技術可使鈦金屬表面胺基化，加上交鏈劑戊二醛的處理後，可使鈦金屬表面接上白蛋白產生表面改質，確可提供一個已知幹細胞分子到鈦金屬表面的方式，以期植體植入後能引導及促進組織復原。

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Abstract

Biocompatibility is the prime requisite for implant material and is determined by the bulk properties and especially the surfaces of implant which directly contacting the host tissue. Even though the relationships and reactions between the surface of implant and tissue and their long-term integrity and clinical efficacy are still not well understood, the first biological reaction known to occur after implantation of a biomaterial is the adsorption of tissue fluid proteins onto its surface and these tightly bound proteins strongly influence the subsequent interactions of cells with the surface. In order to optimize the integration of implants, it is desirable to control interfacial reactions such that nonspecific adsorption of proteins is minimized and beneficial molecules are selectively adsorbed onto biomaterials prior to their implantation. In this regard, our goal was to develop a glow-discharge method to functionalize titanium surfaces by the covalent immobilization of bioactive organic molecules. Based on the above investigation, the surface characteristics was successful modified on the titanium plates by glow

discharge technology and this method could offer the possibility of covalently linking stem cell on titanium surface in order to guide and promote the tissue healing that occurs during implant integration in bone and soft tissue.

Keywords: Titanium, glow discharge, cross-linking agent, and stem cell

II. Introduction and Purpose

The use of osseointegrated implants for retention of fixed or removable prostheses is well established. The concept of osseointegration was documented in fundamental experimental studies by Brånemark et al. [1-2]. The most used materials for implant anchorage with direct bone-implant contact is commercially pure titanium [3]. Titanium is the material of choice for bone-anchored implants because of its biocompatibility. The specific water and biomolecule interactions and high corrosion resistance of the titanium dioxide covering titanium implants are the factors supposed to be responsible for its biologic acceptance. At present, however, the relationships between the surface of an implant, its reactivity with tissue constituents, and its long-term integrity and clinical efficacy are still expected to improve. Hence, the surface physical and chemical properties of biomaterials are today considered to play an essential role in the interaction between the biologic tissue and the implant [4]. Surface modification techniques could be used to tailor the surface chemistry of implant parts to optimize their interfacial properties in critical, bone-contacting areas [5-7]. Modifications of both surface morphology and surface composition have been shown to affect the cell response on Ti surfaces [1-3]. With regard to surface chemistry, surface cleaning and sterilization, the nature of the surface oxide layer, and nitridation treatments are the chemical variables most frequently discussed [1,8-13]. Surface modification of organic materials to direct interfacial interactions with cells and bacteria undoubtedly presents many more possibilities for manipulation of the details of the surface chemistry than does modification of inorganic surfaces. In principle, a polymer coating on an inorganic substrate could provide an organic surface on which further chemistry could be performed [9]. The aim of the present work is to demonstrate that the methods of surface modification of polymers can be used to manipulate the interaction between cells and Ti surfaces. The effect of the surface modification treatments on Ti surface is evaluated by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), electron spectroscopy for chemical analysis (ESCA), and atomic force microscopy (AFM) and growth of a continuous osteoblast-like cell (MC3T3-E1) is performed to assess the effect of the surface modification on the material and cell interaction.

III. Experimental procedure

The grade II titanium substrates used in these experiments were 2-mm-thick plates with a diameter of 1 cm and 99.7% purity. The titanium plates were polished using 600-grit SiC metallographic paper. After being polished, specimens were

solvent-cleaned in methylethylketone for 5 min, washed in distilled water for 20 min, acid pacified in 30% nitric acid for 30 min according to the ASTM procedure and rinsed again in ultrapure water for 20 min. Before samples were coated by deposition of allylamine plasma, they were subjected to argon-plasma treatment in the same reactor. Plasma cleaning was performed at a discharge power of 85 W, a working pressure of 250 mTorr, and an argon flow rate of 20 sccm, after the base pressure was reduced to below 50 mTorr. The plasma treatment time remained at 600 s. In situ allylamine was deposited as the base pressure was reduced to below 50 mTorr again. Treated Ti plates were immersed in a 1% glutaraldehyde solution in 0.1 M phosphate buffer (pH adjusted to 7.0 with NaOH) for 4 h at 25 °C and rinsed with phosphate buffer. The plates were then placed overnight in solutions containing albumin dissolved in phosphate buffer. In order to analyze the properties of plasma-treated and albumin-grafted titanium plates, surface morphologies of the treated titanium plates were analyzed using a Nanoscope III D5000 atomic force microscope (AFM) with a Si probe. The AFM probe was scanned over an area of 5 × 5 μm with 512 scans at a scanning rate of 1 Hz in the tapping mode. The compositions of the films were analyzed by x-ray photoemission spectroscopy (XPS) with a monochromatic Ag Kα source. The x-ray power was 250 W (15 kV at 16.7 mA). The XPS energy scale was calibrated by setting the binding energy of the Ag $_{3d_{5/2}}$ line of clean silver to exactly 368.3 eV as referenced to the Femi level. The angle of incidence of the x-ray beam to the specimen normal was 45°. High-resolution scans were run for Ti, C, O, and N using an x-ray beam with about a 15-nm diameter. Furthermore, cell proliferation on the titanium and surface-modified Ti samples was determined after 48 h. At that time, cells on the samples were gently rinsed with PBS and removed from the growth surface by incubation in 1 mL of a sterile trypsin-EDTA solution in PBS for 2 min. An aliquot of the cell suspension was then counted with a hemocytometer. An osteoblast cell line (MC3T3-E1) was directly seeded onto the surface of the specimens at a density of 5 x 10^4 cells/ml. The culture medium was alpha-modified minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA). Cells were cultured for 96 h in α-MEM, supplemented with 10% inactivated fetal calf serum. Cells were incubated at 37 °C in an atmosphere containing 5% CO_2, and the medium was changed every 72 h. Cell were seeded into 24-well plates at a density of 5 x 10^4 cells per well in 500 μl of medium. After 48 h, when the cells were in the log phase of growth, the medium was replaced with 500 μl of extracts or control medium. Cells were exposed to the extracts for 24 h, the liquid was aspirated, and 50 μl of 10 mM MTT in PBS, pH 6.75, was added to each well. After an incubation period of 4 h, the liquid was aspirated, and the formazan reaction product was dissolved in 200 μl dimethyl sulfoxide. The optical densities were measured using an Anthos 2020 Labtec instrument at 595 nm. For immunolabeling of albumin-linked specimens, treated titanium plates were

incubated for 10 min with 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 1% ovalumine to block nonspecific sticking and then for 90 min with a rabbit anti-rat albumin antibody (Cappel) diluted 1: 250. Specimens were rinsed in PBS and again briefly exposed to PBS-ovalbumin as above. The sites of antibody-antigen binding were then revealed by incubation with the protein A-gold complex for 30 min. The complex was prepared as previously described using 8-nm gold particles. Controls consisted of incubating the samples with a non-immune antibody followed by protein A-gold. The samples were rinsed first with PBS and then with distilled water, dried, and examined by AFM using the tapping mode to visualize good particles at the surface of the specimens. In addition, to test the significance of the observed differences between the percentages of attached cells on different surfaces, *t*-test was applied.

IV. Results and Discussion

Figure 1 shows titanium surfaces with and without plasma cleaning, as well as plasma polymerization by SEM. Figure 1(a) shows contaminants are obvious on the non-plasma cleaning surfaces. In Fig. 1(b), the level of absorbed substance on an argon plasma cleaned surface has decreased substantially. As shown in Fig. 2, similar results are obtained from images captured using atomic force microscope (AFM). In Fig. 2(a), there are small particles on the non-plasma cleaning Ti surfaces. Figure 2(b) is a Ti surface that has undergone ten minutes of Ar plasma cleaning. Since there are no contaminants left on the surface, the s conclude that they were effectively removed by Ar plasma cleaning. Furthermore, as seen under AFM, from examining the Ti surface after Ar plasma cleaning form 1 min. to 10 min., it is found that the surface roughness decreases as treated time increases. In Fig. 2(a), a non-plasma treated Ti surface has roughness of 22 nm and after ten minutes of plasma treatment, it has decreased to 2.5 nm, as shown in Fig. 2(b). The variation also reveals that surface roughness decreases dramatically after plasma-treatment, thus indicating that the length of Ar plasma treatment is directly proportional to the surface flatness of the Ti plate. Ar plasma cleaning is set to be ten minutes long because there are no contaminants found on the Ti surface after this period. After the Ar plasma treatment, the Ti surface is treated with allylamine plasma. AFM is used to measure the allylamine deposition and the results are displayed in Fig. 3. It is found that after the 30 min. and 60 min. allylamine plasma treatments, the depositions are approximately 371 nm and 558 nm, respectively. The results indicate the deposition layer becomes thicker as the time of allylamine plasma treatment increases. Chemical compositions of titanium with plasma-polymerized allylamine for various time is conducted with XPS and the results are shown in Table I. The results suggest that the surface elements of allylamine plasma that have been polymerized for 30 and 60 min are very similar. One-sample t-test suggests that the atomic percentages of carbon and nitrogen in the two groups are not statistically different (p=0.172 and

0.065). In addition, the atomic ratio of carbon to nitrogen in the 30-minute group (3.247) is not statistically different than that of the chemical composition of allylamine in Table I (one-sample t-test). This shows that after plasma polymerization for 30 minutes, the allylamine has already decomposed allylamine into amines (amino groups) and reached its steady state. As a result, the allylamine plasma treatment time is set to be 30 minutes.

Figure 4 shows energy dispersive spectrometer (EDS) is performed to detect the titanium surface that has undergone various chemical treatments. The results indicate that titanium is the primary elemental composition of non-treated and Ar plasma treated Ti surfaces. On the other hand, as shown in Fig. 4(a), nitrogen is found on allylamine plasma treated Ti surfaces. This finding suggests that amines may have remained on the Ti surface after the allylamine plasma treatment. As for the gluteraldehyde treated Ti surfaces, oxygen is detected (in gluteraldehyde, the atomic ratio of C : O : H is 5:2:8). Figure 4(b) shows that carbon and oxygen elements are found on ALB treated Ti surfaces (in ALB, the atomic ratio of C : O : N : S is 63:20.1:16:0.95) [14].

X-ray photoelectron spectroscope (XPS) is used to analyze surface deposition changes of Ti surfaces that have undergone various chemical treatments. Local spectra show that after the different treatments, the binding energy and the ratio of Ti : N : C : O has changed significantly, as shown in Fig. 5. As shown in Fig. 5(a), after the Ar plasma treatment, the titanium binding energy, Ti $2p_{3/2,}$ shifts from 459 eV to ~ 457 eV. This suggests that the Ti surface has been bio-activated and other kinds of bindings have been formed. Nitrogen-local spectrum (Figure 5(b)) analyses indicate that chemical shifts did not occur after plasma cleaning. However, the binding energy of allylamine plasma, N 1s, shifted from 398.1eV to ~ 402eV due to the fact that the formation of amino groups ($-NH_2$) causes changes to the nitrogen bindings. After the gluteraldehyde (cross-linking agent) and ALB treatments, XPS shows that the nitrogen-binding energy, N 1s, shifted from 402 eV to ~400.5 to 401 eV, which suggests that the amines (amino groups) ($-NH_2$) have been binding with gluteraldehyde and some dangling bindings remain on the Ti surface. Thus, the nitrogen binding energy shifts from high to low binding energy. Carbon-local spectrum (Fig. 5(c)) analyses indicate that chemical shifts did not occur after plasma cleaning. XPS analysis of allylamine plasma treatment shows that the carbon binding energy, C1s, shifts from 285.5eV to ~ 287.5eV, suggesting that carbon and nitrogen are forming bindings. In other words, the formation of allylamine deposition has occurred. After gluteraldehyde and ALB treatments, the carbon binding energy, C 1s, shifts from 287.5 eV to ~286.5 eV, indicating that carbon and oxygen are forming bindings. Furthermore, oxygen-local spectrum (Fig. 5(d)) analyses indicate that chemical shifts did not occur after plasma cleaning. XPS analysis suggests that the oxygen binding energy, O1s, of the allylamine plasma and gluteraldehyde treated Ti surface, shifted from 531 eV to

~534 eV. This demonstrates that they are no longer the binding energy of metal-oxides.

To analyze the chemical composition of Ti surfaces that have undergone various treatments, the specimens are performed by XPS. As shown in Table II, the main elemental compositions of non-treated and Ar plasma treated surfaces are titanium, oxygen, and carbon. Whereas the main elemental composition of allylamine plasma treated surfaces are nitrogen and carbon. As for the gluteraldehyde and albumin groups, the main elemental compositions are oxygen and carbon. Based on the ANOVA statistical analysis in Table II, it can be determined that the various treatments change the ratio of titanium, nitrogen, carbon, and oxygen on the Ti surfaces. The Scheffe Post Hoc Test has found that the non-treated and Ar plasma treated surfaces have a higher concentration of titanium than the allylamine, gluteraldehyde, and albumin treated groups. We also determined that the non-treated surfaces have a higher concentration of oxygen than the allylamine plasma treated group, and the Ar plasma treated surfaces have a higher concentration of oxygen than the allylamine, gluteraldehyde, and albumin groups. The amount of carbon is notably lower in the non-treated surfaces are than the allylamine and gluteraldehyde treated surfaces. On the other hand, the amount of carbon is higher in the allylamine, gluteraldehyde, and albumin groups than the Ar plasma cleaned group. As for the allylamine plasma treated group, the amount of nitrogen is significantly higher than the other four groups.

In order to determine whether the various treatments have achieved the anticipated effects, the Independent T-Test analysis was used to compare the amount of titanium, nitrogen, carbon, and oxygen on the Ti plate surfaces before and after the treatments. The results have found that after Ar plasma treatment, only carbon has decreased significantly ($p=0.000$). The carbon came primarily from organic contaminants and deterministic hydrocarbon contaminants in the x-ray photoelectron spectroscopy reaction chamber. The decrease in carbon indicates that the amount of organic contaminants has lessened. Furthermore, this value (17.07%) can be perceived as the deterministic hydrocarbon contaminants in the x-ray photoelectron spectroscopy reaction chamber [15]. While the allylamine plasma treated surface has a significant decrease in titanium and oxygen ($p=0.004$ and 0.000), there is a considerable increase in carbon and nitrogen ($p=0.000$). This indicates that the allylamine has successfully polymerized on the Ti surface and is covering the titanium and oxygen. As a means of proving this hypothesis, the main surface elements of the allylamine plasma group (carbon to nitrogen ratio=3.247, standard error=0.303), were compared to carbon to nitrogen ratio (=3) in the acrylamide chemical composition. The one-sample t test has shown that there are no major differences between the two groups, indicating that the allylamine has successfully polymerized on the Ti surface. After the gluteraldehyde treatment, there is a considerable increase in oxygen ($p=0.037$) and decrease in nitrogen ($p=0.000$). The carbon to oxygen ratio in the primary

surface composition is 2.404 (standard error=1.333). In comparison to the carbon to oxygen ratio in the gluteraldehyde chemical composition (=2.5), the one-sample t test has found that there are no major differences between the two groups (p=0.879), proving that the amination reaction has successfully taken place between gluteraldehyde and the Ti surface. Albumin was detected by immunogold labeling, where the distribution and density of the labeling were visualized by SEM-EDS. Both freshly prepared titanium plates and similar samples stored in buffer for 1 week provided comparable results and demonstrated a generally uniform labeling of the titanium surface, although some areas of the plates illustrated a lesser density of gold particles. These data demonstrate that the albumin is stably bound and retains its antigenicity following the various steps of the linking procedure. Furthermore, the density of labeling obtained suggests that the albumin linkage is efficient. Figure 6(a) illustrates a titanium plate without plasma treatment after labeling. It displays the average number of gold particles on a non-treated Ti surface is $17/\mu m^2$. The control sample was incubated only with protein A-gold and displays a background labeling of fewer gold particles. Figure 6(b) shows a representative area of a plasma treated titanium sample with a labeling density of 175 gold particles/μm^2, which is below the experimental value, reflecting the expected nonspecific adsorption of albumin. The T-Test has found that there are major differences between the two groups (p=0.000), proving that after plasma treatment, the albumin can successfully cover the Ti surface. In addition, the number of gold nano-particles is much higher on the treated than the non-treated Ti surfaces. In general, cells spread well on all titanium plates, indicating good attachment to titanium surfaces, as shown in Fig. 7. The morphology of the cells was heterogeneous. The cells were intimately adapted to the plasma-treated titanium surfaces. After cell culture for 1 h, SEM examination revealed that the cells were round with central, protruding nuclei surrounded by a thin rim of cytoplasm (Fig. 7(a)). The cell surface morphology after 8 h of culture is shown in Fig. 7(b). No clear orientation of cells can be noted on the surfaces. The cells were flat, well spread, and polygonal. After 24 h of culture, as shown in Fig. 7(c), cells were more elongated than after 8 h of culture. In addition, most of the cells had begun to align along the fine irregularities. After 48 h of culture, as shown in Fig. 7(d), cultured cells were intimately attached, elongated, and flat, and were aligned in the direction of the fine irregularities, revealing that the plasma-treated plate had the better biocompatibility. On the plasma-treated titanium surface, cells were mainly polygonal, flat, and well spread with no specific orientation.

As investigated through scanning electron microscopy (SEM), the non-plasma treated Ti surfaces, like surfaces that were treated with normal cleaning, are covered with contaminants. Further analysis was conducted with X-ray photoelectron spectroscopy (XPS) and the main surface elements are found to be titanium, oxygen, and carbon. Carbon primarily came from the

organic contaminants and deterministic hydrocarbon contaminants in the XPS reaction chamber. The spectrum of titanium indicates that the non-plasma treated Ti surface is chiefly comprised of titanium dioxide (TiO_2); however, the atomic ratio of titanium and oxygen (1:3.7) is not the expected 1:2 ratio. The excessive amount of oxygen might have come from the oxygen and water in the atmosphere [16]. A small amount of nitrogen also came from the air, and trace amounts of sodium, chlorine, and phosphorus came from contaminates such as cleaning products and impurities during the preparation process.

After Ar plasma treatment, there is a notable decrease in absorbed substance as shown in SEM. The investigation by atomic force microscopy indicates that as the time of the Ar plasma cleaning increases, the surface roughness decreases obviously. This finding reveals that Ar plasma not only cleans, but also refines the surface. Moreover, it is much faster and cleaner than the wet cleaning process. The Aronsson's group showed that the ideal Ti surface roughness, 3 nm, can be achieved in ten minutes by using plasma treatment [17]. Aronsson's result is similar to that of our current research, which is 2.5nm. The result has once again proved that the Ar plasma treatment is an efficient method to modify the surface properties. XPS has found that after plasma treatment, there is an oxidized titanium layer on the Ti plates. Similar researches suggest that this thin oxidized titanium layer does not have a crystalline structure. It is deemed that this finding will be valuable to the future investigation of albumin corsslinking [18]. After local spectra of nitrogen, carbon and oxygen, it has been found that chemical shifts did not occur after plasma cleaning. The binding energy of titanium, $Ti2p_{3/2,}$ shifted from 459 eV to ~ 457 eV, suggesting that in addition to titanium dioxide (TiO_2) there are other oxidized titanium substances. The main reason for this shifting is that the thickness and composition of the 5~10 nm oxidized layer is unevenly distributed. After Ar plasma treatment, the amount of carbon dropped from 30.43% to 17.07%, signifying that the amount of organic pollutants was decreased. In other words, Ar plasma can remove organic pollutants obviously. The remaining 17.07% of carbon is the determined contaminants in the XPS reaction chamber. Since there is no obvious variation in the other elemental compositions, it can be safely inferred that Ar plasma will not change the Ti surface's oxidized titanium composition. The atomic ratio of titanium and oxygen is similar to the non-treated group, which is 1: 3.4.

Based on AFM observation, there are little variation to the allylamine plasma treated Ti surface. This finding confirms the AFM analysis of an even distribution of a 371nm allylamine deposition over the Ti surface. The presence of nitrogen in SEM-EDS suggests that there may be deposition of amines. X-ray photoelectron spectroscopy shows that after allylamine plasma treatment, the bindinging energy of nitrogen, N1s, shifts from 398.1 eV to ~ 402 eV because the formation of amino groups ($-NH_2$) causes the Ti to undergo significant variation. Binding energy of carbon shifts

from 285.5 eV to ~ 287.5 eV, suggesting that carbon and nitrogen are binding. The oxygen binding energy, O1s, shifts from 531 eV to ~534 eV, indicating the presence of non-metal oxide binding energy. In addition, the allylamine plasma treated group shows a greater amount of nitrogen than the other four groups. The ratio of carbon to nitrogen is also greater than the non-treated and the Ar plasma treated groups. However, the amount of titanium and oxygen is much lower than the non-treated and the Ar plasma treated groups. This suggests that the allylamine plasma has successfully polymerized and covered the titanium oxides. The One-sample t test has found that when comparing the ratio of carbon to nitrogen in the allylamine plasma group to the ratio of carbon to nitrogen in the allylamine chemical composition group, there are no significant differences ($p=0.143$), proving that the allylamine has successfully polymerized on the Ti surface. Moreover, since the average standard error of the allylamine plasma treatment group is low and other than the 5.4% of oxygen and water that may have come from the atmosphere, only carbon and nitrogen (94.5%) remain on the surface. Thus, this finding is different from that of normal wet wafer cleaning, which has a large amount of residue remaining on the Ti surface [19]. The allylamine plasma treatment of the Ti surface creates nitrogen and hydrogen ions but does not induce nitridation. Instead, the plasma ionized allylamine forms dangling bindings ($-NH_2$) on the surface and this facilitates the linking of the albumin to the surface. Similar sedimentations have been found on gluteraldehyde and albumin. Therefore the plasma treatment is crucial for the subsequent processes of attaching amines to the Ti surface and using gluteraldehyde as the cross-linking agent to connect the albumin to the treated surface.

SEM observation has shown that after gluteraldehyde treatment, there are obvious changes on the Ti surface and the presence of oxygen suggests that gluteraldehyde has been successfully grafted. XPS analysis reveals that the carbon binding energy, C1s, shifts from 287.5eV to ~286.5eV and proves that carbon and oxygen are binding. The oxygen binding energy, O1s, is ~534eV, suggesting that there is non-metallic oxide binding energy. The amount of oxygen is much higher than the allylamine group, but the amount of nitrogen is much lower. This finding indirectly shows that there is the formation of gluteraldehyde cross-linking, which covers the allylamine. The one-sample t test has found that when comparing the ratio of carbon to oxygen in the gluteraldehyde group to the ratio of carbon to nitrogen in the gluteraldehyde composition group, there are no significant differences ($p=0.879$), indicating that the amination reaction has successfully taken place between gluteraldehyde and the Ti surface. However, due to the solution treatment and the PBS wash during the gluteraldehyde cross-linking, there are sodium, chlorine, and phosphorus residues deposited on the surface. Since the standard error of the gluteraldehyde group is high, it proves that it is difficult to reach consistent results when using wet wafer cleaning, which is a similar

problem found in the albumin group.

The observation of sphere-shaped covering on the albumin treated Ti surface through SEM, along with the appearance of SEM-EDS oxygen and carbon indirectly suggests the successful albumin coverage of the Ti surface. After the cross-linking agent, gluteraldehyde, is attached to the amines on the albumin, the x-ray photoelectron spectrum indicated that the carbon's binding energy N1s is shifted from 402eV to approximately between 400.5eV and 401eV. The presence of dangling bindings indicates that some of the amino groups ($-NH_2$) on the Ti surface are bindinging with the gluteraldehyde; therefore, nitrogen shifts from high binding energy to a lower one. After gluteraldehyde and albumin treatments, the carbon binding energy, C1s, shifts from 287.5 eV to ~286.5eV, suggesting the carbon and oxygen binding. The observation of colloidal gold immunolabeling with SEM-EDS indicates that the plasma treatment can successfully graft albumin on the Ti surface. As for the non-plasma treated Ti surfaces, the average number is 17 gold grains/μm^2, which is much less than the albumin experimental group. It only has an average of 175 gold grains/μm^2. In comparison, the untreated group also had a minute amount of gold particle attachment, implying it also contains some unavoidable non-specific protein (A-gold) adhesion. The XPS result shows that there are some differences in the surface atomic ration and the known albumin atomic ratio in the albumin group, implying that the albumin was unable to completely cover the Ti surface; however, since the theoretical contact surface area between a single cell and the Ti surface is 20 μm × 20 μm (400 μm^2), one gold particle represents one albumin molecule. The albumin coverage based on the methods used in this experiment will provide 48533 albumin molecules to come in contact with a single cell. The number is much higher than one cell per ten thousand albumin crosslinking [20]. Thus, this experiment can be viewed as a successful chemical modification method. It can link the binding specific albumin to the Ti surface and expedite the recovery and osseointegration between the implants and tissues.

V. Plan Conclusion

This study confirms that titanium surface with plasma cleaning and plasma polymerization will cause it to become aminated. After the gluteraldehyde treatment, albumin can be bindinged to the surface. This provides a bioactive surface to link the existing bioactive molecules onto the Ti surface, which may stimulate and promote tissue healing in implants. Since plasma can perform continuous ion bombardment, Ar plasma can be used to remove organic contaminants that regular cleaning procedures cannot prevent other forms of contamination. Furthermore, since chemical modification by glow discharge does not require the making of solutions, the amount of residues in solution can be reduced. The chemical modification by can effectively attach albumin to the Ti surface. This model can be applied on various types of proteins and attach them to the Ti surface. Further in

vivo experiments will test the effects that specific proteins have on specific cells, which may increase the success rate of osseointegration.

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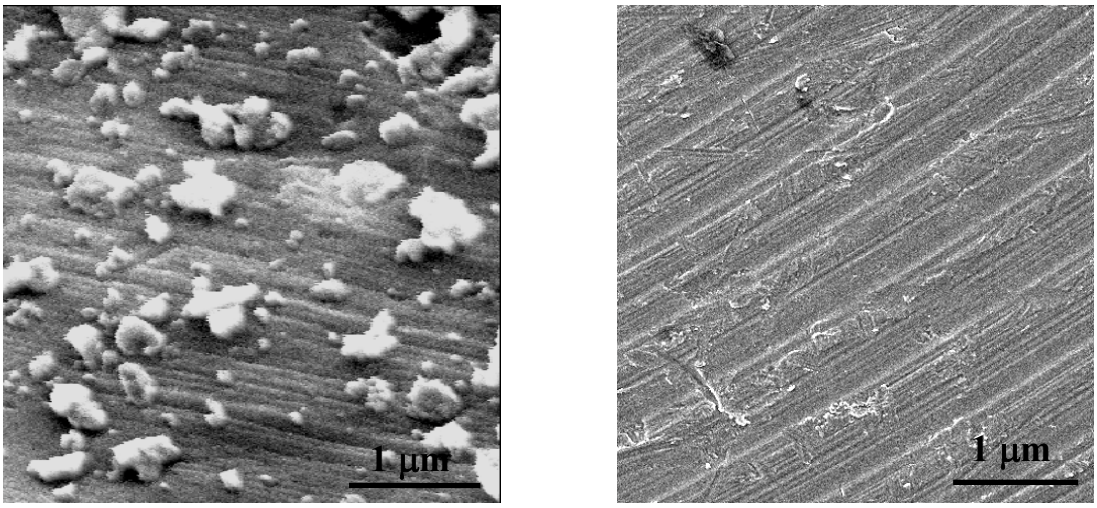
Table I. Chemical compositions of titanium surface after allylamin deposition

	O	C	N	Na	Cl	P	S
Allylamin 30 min.	5.352	76.214	18.328	0.002	0.056	0.004	0.018
Allylamin 60 min.	2.6	77.3	20.1	0	0	0	0

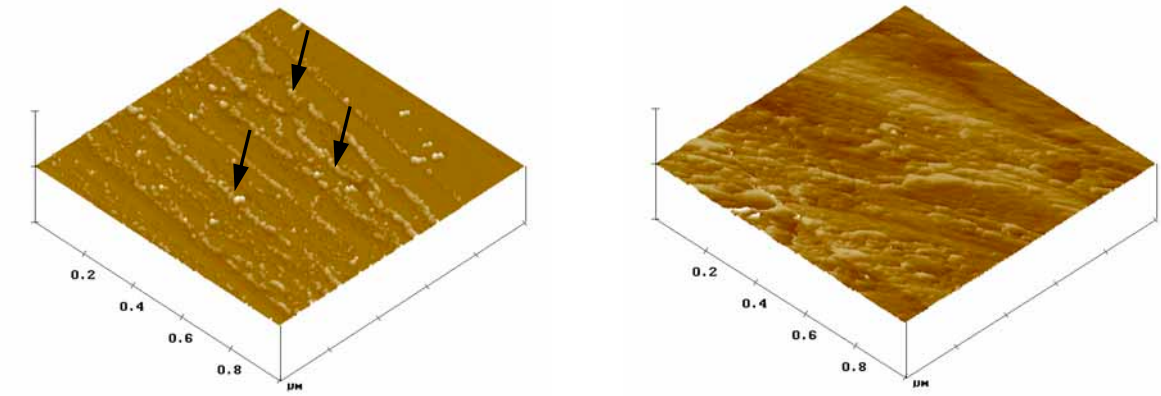
Table II. Chemical compositions of titanium surface before and after post treatment (ANOVA)

	Ti	O	C	N	Na	Cl	P	S
Group1 Non-treatment n=5	13.898 (*4.822*)	51.314 (*6.038*)	30.432 (*3.777*)	1.746 (*0.793*)	2.522 (*1.444*)	0.520 (*0.049*)	0.04 (*0.057*)	0.018 (*0.04*)
Group 2 Ar plasma treatment n=5	17.276 (*6.652*)	58.52 (*7.051*)	17.072 (*2.485*)	3.138 (*1.384*)	3.872 (*2.184*)	0.032 (*0.044*)	0.036 (*0.07*)	0.05 (*0.071*)
Group3 Allylamine (30 mins.) n=5	0.026 (*0.058*)	5.352 (*1.857*)	76.214 (*1.461*)	18.328 (*1.569*)	0.002 (*0.004*)	0.056 (*0.082*)	0.004 (*0.009*)	0.018 (*0.025*)
Group4 Glutaradehyde n=5	2.01 (*4.495*)	25.862 (*18.296*)	59.808 (*21.514*)	4.782 (*3.094*)	2.27 (*3.281*)	5.128 (*11.333*)	0.112 (*0.218*)	0.01 (*0.017*)
Group5 Albumin n=5	3.598 (*5.23*)	27.786 (*20.866*)	48.684 (*19.708*)	7.474 (*5.951*)	3.854 (*5.385*)	8.334 (*18.596*)	0.08 (*0.117*)	0.192 (*0.396*)
F	12.909	13.276	15.656	21.997	1.342	0.774	0.635	0.893
P	0.000	0.000	0.000	0.000	0.289	0.555	0.643	0.486
Scheffe Post Hoc Test	1>3,4,5 2>3,4,5	1>3 2>3,4,5	1<3,4 2<3,4,5	3>1,2,4,5	N.S.	N.S.	N.S.	N.S.



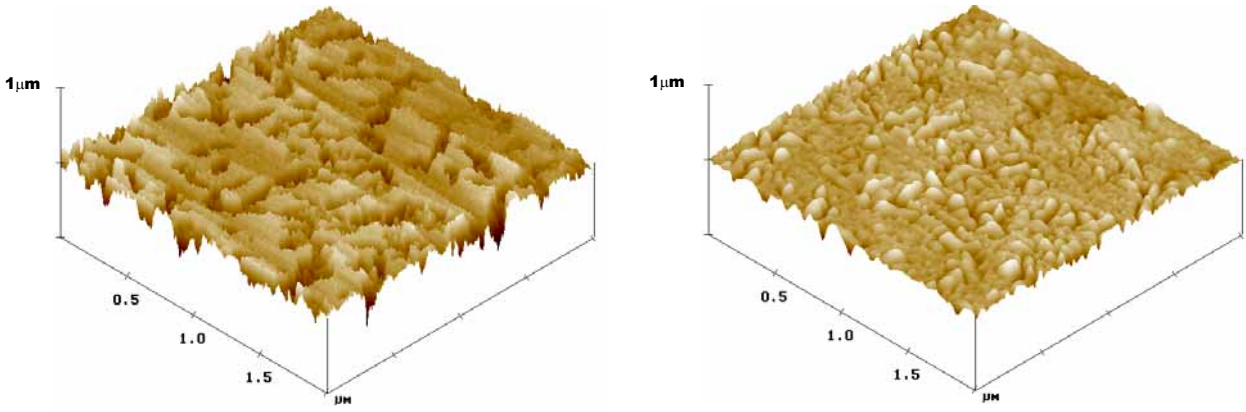
(a) (b)

Figure 1 shows titanium surfaces with and without plasma cleaning, as well as plasma polymerization by SEM.



(a) (b)

As shown in Fig. 2, similar results are obtained from images captured using atomic force microscope (AFM).



(a) (b)

AFM is used to measure the allylamine deposition and the results are displayed in Fig. 3

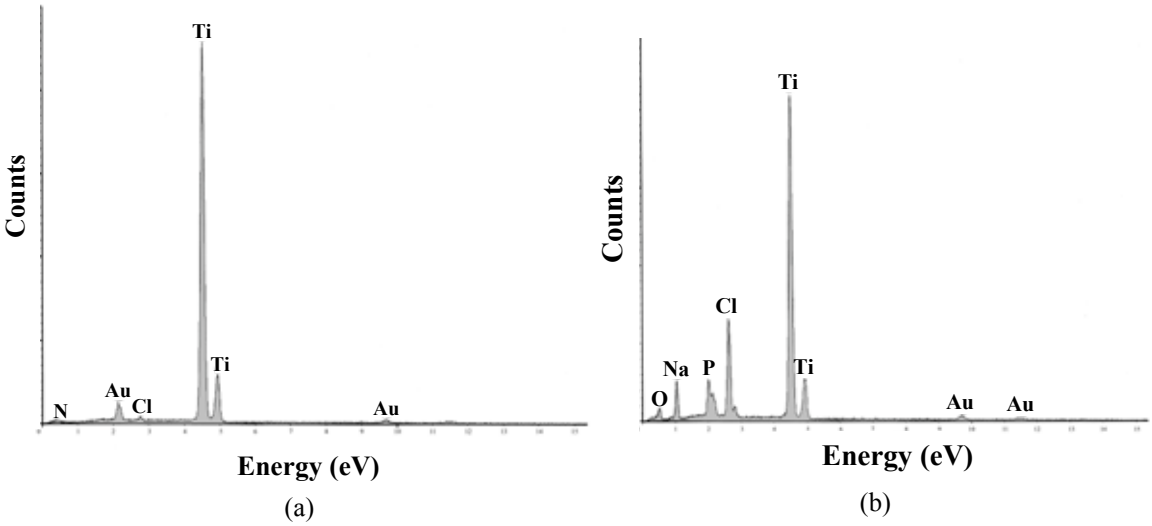


Figure 4 shows energy dispersive spectrometer (EDS) is performed to detect the titanium surface that has undergone various chemical treatments: (a) Ti without surface treatment, (b) gluteraldehyde treated Ti surfaces.

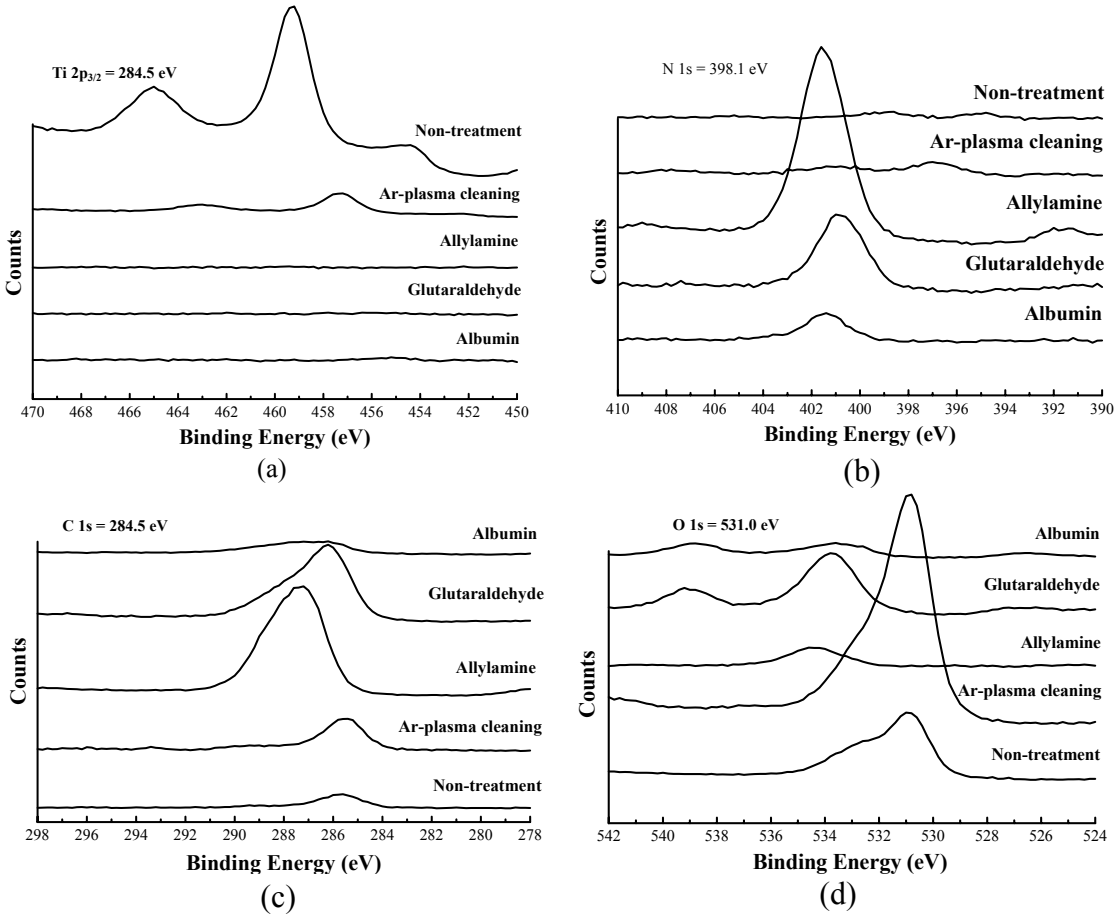
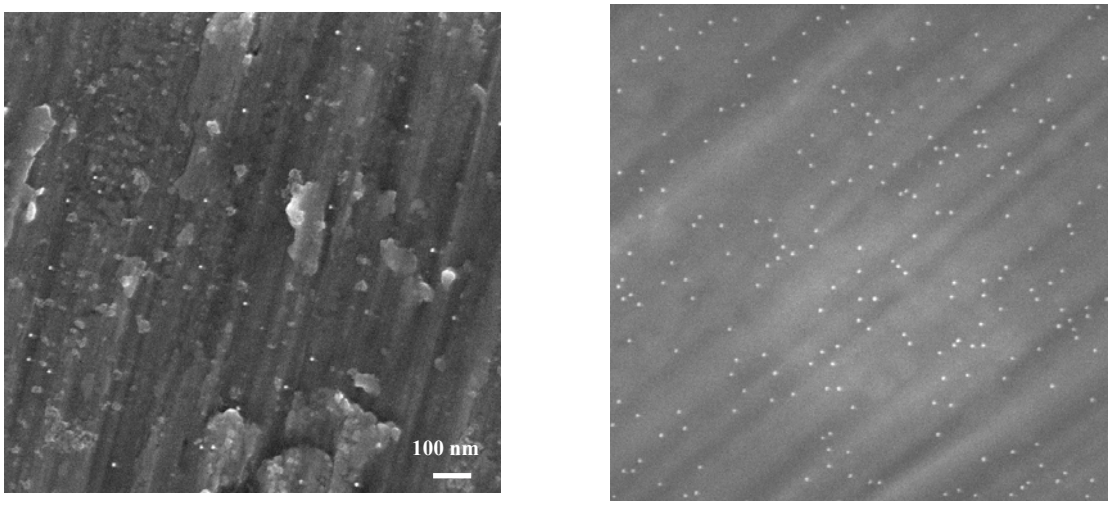
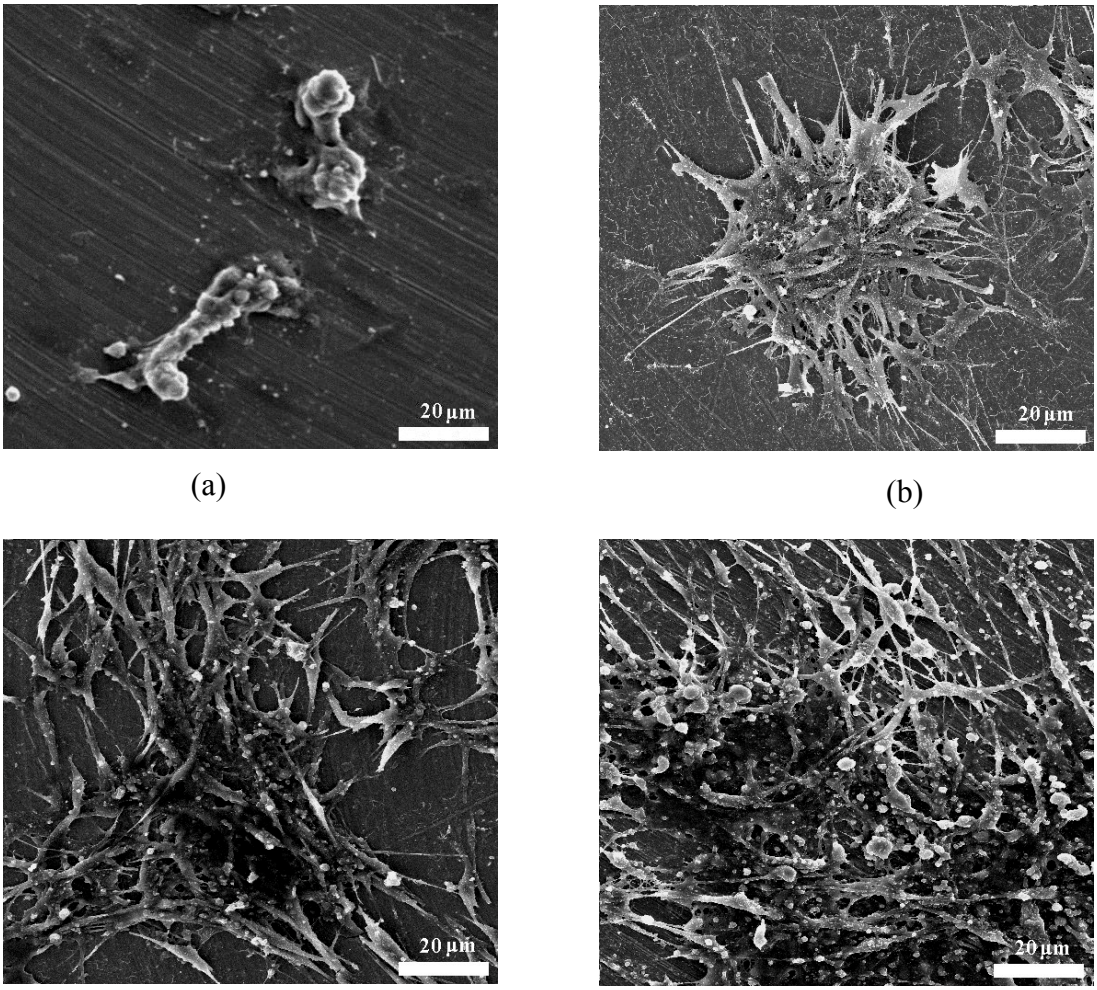


Fig. 5. X-ray photoelectron spectroscope (XPS) is used to analyze surface deposition changes of Ti surfaces that have undergone various chemical treatments.



(a) (b)

Figure 6. Albumin was detected by immunogold labeling, where the distribution and density of the labeling were visualized by SEM-EDS.



(a) (b)

(c) (d)

Figure 7. In general, cells spread well on all titanium plates, indicating good attachment to titanium surfaces.