Bioreactivity of titanium implant alloys

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A study was conducted regarding the adsorption of peptides on commercially pure (cp) Ti and Ti-6Al-4V. The peptides used were arginine-glycine-aspartic acid-alanine (RGDA), arginine-glycine-aspartic acid-serine (RGDS), and arginine-phenylalanine-aspartic acid-serine (RFDS). The tripeptide RGD is known to be important for biologically specific adhesion reactions. This research was conducted to investigate the reason for a tendency toward thrombus formation with Ti-6Al-4V that is not observed with cp Ti. After argon plasma cleaning, coupons of the titanium alloys were inserted into solutions with variable concentrations (0.0625–2 mg/ml) of an individual peptide group under constant temperature and time conditions. The samples were rinsed, dried, and analyzed with x-ray photoelectron spectroscopy (XPS). Adsorption isotherms were obtained by plotting the relative amount of peptide adhesion as a function of solution concentration. It was postulated through the XPS and adsorption isotherm data that the major adhesion mechanism for the peptides to the titanium alloys was hydrogen bonding. CP titanium and Ti-6Al-4V are hypothesized to react differently as implants because Ti-6Al-4V has a more electropositive surface, which allows fewer hydrogen bonds to form. Hydrophilic reactions were proposed to be of secondary importance during bioadhesion, influencing the structure of the second layer adsorbed. There was no correlation found between the net charge of the peptide groups and their adhesion to the alloys.

I. INTRODUCTION

When a material is implanted into living tissue, proteins immediately adsorb onto the surface of the foreign object. Any subsequent reaction between the material and the host is a function of these adsorbed proteins and surrounding tissue. An understanding of the interaction between materials used for implants and proteins may help to increase the success of implants. The most common titanium alloy is Ti-6Al-4V (titanium–6 wt % aluminum–4 wt % vanadium), and at this time it is the only titanium alloy used in load-bearing implants such as hips and knees because it offers the best combination of strength, ductility, and freedom from environmental effects such as stress corrosion cracking. Heart valves, however, are usually made of unalloyed titanium (also called cp titanium, for ''commercially pure'') because of a reported tendency toward thrombus formation[1] with Ti-6Al-4V. A study by Johansson *et al.*[2] determined a more natural-like tissue reaction occurred with cp titanium than with Ti-6Al-4V. It is theorized that the clotting in the region of the Ti-6Al-4V structures is associated with the aluminum in the alloy. The differences in which cp titanium and Ti-6Al-4V interact with known, well characterized peptide systems is investigated.

Both fibronectin and fibrinogen are proteins that exist in high concentration in animals. Fibronectin is a large protein that can mediate adhesion and spreading of cells on an extracellular matrix. Fibrinogen is essential to blood clotting. To perform these adhesive functions, both proteins are required to interact with other proteins, primarily receptors. A chain of amino acids with a specific electronic configuration acts as a receptor area to accomplish this interaction. Both fibronectin and fibrinogen have the same specific sequence of amino acids (arginine-glycine-aspartic acid, abbreviated RGD) that act as this specific cell receptor and mediate cell adhesion. Although it will not have specific binding to implant surfaces in the same fashion as it does to other biomolecules, this peptide series is well characterized and is known to be important at implant sites. Adsorption isotherms of RGD-related peptides on titanium alloys were obtained.

The mechanism of bioadhesion to titanium and its alloys may be due to hydrogen bonding, hydrophilic interactions, and/or charge transfer. To study these parameters, the substituted RGD-based peptides had different amino acid side chains and different total charges. The four amino acids of arginine-glycine-aspartic acid-serine (RGDS) have charges of +, 0, +, − for a net charge of +1. In RFDS, the second position amino acid glycine (with a –H side group) is replaced by phenylalanine, an aromatic-containing amino acid. The charges on the RFDS amino acids are +, −, +, −. In the tetrapeptide RGDA, the final serine (with an –OH side group) is replaced by alanine (with a $-CH_3$ group). The charges on the RGDA amino acids are +,0,+,+. These three relatively differently charged peptide groups were adsorbed onto the two titanium alloy surfaces. Additionally, because of the difference in the side chains, RFDS is more hydrophobic than RGDA and RGDS, and RGDS and RFDS should form more hydrogen bonds than RGDA. The relative amount of peptide adsorbed will be determined with x-ray photoelectron spectroscopy (XPS). A review of the use of XPS to study protein adhesion was completed by Paynter and Ratner.[3]

II. EXPERIMENT

Samples of commercially pure titanium (cp Ti, grade 2) were obtained from Titanium Industries (Fairfield, NJ) and of Ti-6Al-4V from Intermedics Orthopedics, Inc. (Austin, TX). The surfaces were mechanically polished to a bright finish by Efco Finishing Corporation (Butler, WI), utilizing No. SS-35

stainless steel compound (alumina in an organic binder) from Kocour Co. (Chicago, IL). The samples were cleaned with a solvent method consisting of 5 minutes each in ultrasonic isopropanol and acetone. The test coupons were subsequently argon plasma etched. Cleaning of implants by this method has been shown to yield clean surfaces that have a beneficial reaction with neighboring tissue.[4–7] The sputtering system used for plasma etching was a Materials Research Corporation model 822 Sputtersphere with an argon radio frequency (rf) plasma operated at 13.56 MHz. The 8 in. diameter pallet voltage was 1350 V. The etching process was done for 15 minutes. The samples were allowed to cool in argon before exposure to the atmosphere. The cleanliness of the samples was verified with XPS. The analysis system used was a VG ESCALAB system operating with an Al $K\alpha$ anode. Survey spectra were collected with a pass energy of 50 eV; high resolution spectra with a pass energy of 20 eV.

The cp titanium and Ti-6Al-4V samples were cut into 15 mm$\times$7 mm coupons. One coupon of each alloy was inserted vertically (back, unpolished sides touching) into new, cleaned 9 mm test tubes. Solutions of RGDA, RGDS, and RFDS obtained from Bachem, Inc. (Torrance, CA) were made with double distilled, deionized water (resistance >10 MΩ) by successive dilution. The nominal peptide concentrations were 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.00 mg/ml (distilled water control). These nominal weights were corrected for the percentage of the peptide in the vial as given by Bachem: RGDA 76.3%, RGDS 90.9%, and RFDS 93.7%. All of these weights are $\pm 3.0\%$. The titanium alloys were exposed to the solutions for 26 hours at 25.0 ± 0.5 °C using a temperature controlled water bath. Each concentration experiment for each peptide was replicated fifteen times. After the exposure was completed, the solution was decanted and the samples were rinsed twice with double distilled, deionized water and allowed to air dry. The samples were analyzed with XPS. The XPS spectra were quantified by the ESCALAB system using the sensitivity factors of Wagner *et al.*[8] The surface concentration of carbon, nitrogen, oxygen, and titanium was tabulated. The nitrogen signal from XPS is used by Ratner[3] as a quantitative measure of the amount of protein present on a surface. Adsorption isotherms were obtained by plotting the sensitivity factor-corrected nitrogen (400.9 eV)/titanium (458.5 eV) ratio as a function of solution concentration. The ratios were additionally corrected to account for the different theoretical amounts of nitrogen in the peptide. The amount of peptide on the surface cannot be precisely quantified due to uncertainties in sensitivity factors and attenuation lengths in these systems. Throughout this study, the data are used on a comparison basis. Additionally, a powder sample of RGDS peptide was analyzed by embedding the material in indium foil and immediately inserting the sample into the ESCALAB system.

III. RESULTS

XPS analysis of the cleaned samples indicated the two alloys have a relatively similar surface composition. A small amount of nitrogen detected on the surface of the control samples occurred at 397.4 eV. This correlated to nitride at the surface. Nitrogen on both the RGDS powder and on the peptide-exposed titanium samples occurred at 400.9 eV.

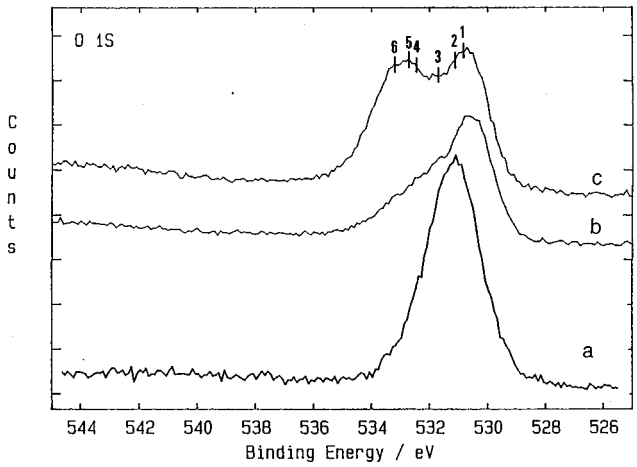


FIG. 1. XPS high resolution O 1*s* spectra from (a) RGDS powder, (b) Ti-6Al-4V exposed to distilled water, and (c) 2 mg/ml RGDS adsorbed from aqueous solution on Ti-6Al-4V. The centroids of deconvoluted peaks are noted with vertical dashes. Peaks are assigned as No. 1 TiO_2 (530.7), No. 2 C═O (531.2), No. 3 $Ti(OH)_4$ (532.0), No. 4 $TiO_2 \cdot nH_2O$ and C–OH (532.5), No. 5 speculated to be oxygen* in Ti–O*–H–peptide, and No. 6 speculated to be oxygen* in C–O*–H–O–Ti.

The C 1*s* peak of the RGDS powder (not shown) readily demonstrated the expected carbonyl and COH groups as well as the C_xH_y and C_mN_n backbone. On the peptide-exposed titanium samples, the primary carbon peak coincided with that of adventitious carbon, except for minor high energy broadening. At higher peptide concentrations, there was increasing carbon with an increase in solution concentration, but the carbon background present on the control samples was too large to allow the detection of peptide carbon at lower concentrations.

As with carbon, oxygen was not a good quantitative indicator of peptide at the surface because it is also present not only in the peptide, but also to a large degree on the surface of distilled-water exposed titanium. The oxygen spectrum from the RGDS powder is shown in Figure 1(a). The primary peak occurs at 531.2 eV, coinciding with C═O; high energy tailing is also found, due apparently to C–OH oxygen at 532.5 eV. High resolution oxygen spectra of the as-cleaned titanium and distilled water-exposed titanium were very similar and are illustrated in Figure 1(b); the results are consistent with hydrated TiO_2. The major O 1*s* peak occurs at 530.7 eV and rutile titanium dioxide occurs at 530.6 eV.[9] There was high energy broadening [Figure 1(b)]. High resolution XPS of the oxygen peaks for the samples exposed to peptide solutions yielded additional information about the adsorption processes. A series of peaks [Figure 1(c)] ranging from 531.5 to 533.5 eV increased (relative to the main oxygen peak at 530.7 eV) with increasing peptide solution concentration. The spectra depicted in Figure 1 are typical of both alloys and all three peptides analyzed.

As expected, no aluminum was detected on the surface of cp titanium; only a small Al 2*p* peak at 75.2 eV was visible on the Ti-6Al-4V. This peak is due to the native oxide from aluminum.[8] No vanadium was detected. The low aluminum

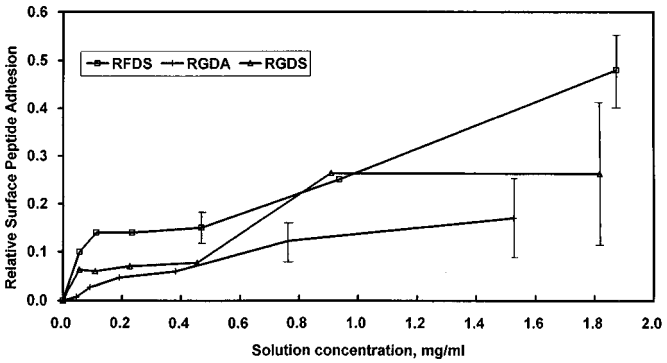


FIG. 2. Adsorption isotherms of RFDS, RGDS, and RGDA on cp titanium.

surface concentration and lack of detectable vanadium is similar to the data of Maeusli.[10] Low levels of aluminum were only sporadically detected on the survey scans of the peptide-exposed Ti-6Al-4V. Because of this fact, the surface aluminum was not included in the peptide:alloy ratio. The qualitative ramifications of this omission are discussed subsequently.

Typical titanium spectra were obtained from both alloy surfaces, with $2p$ peaks occurring at 458.5 eV (due to Ti^{4+} titanium oxide) and at 453.8 eV (metallic titanium). There were no substantial changes in the titanium metal or titanium oxide peak energies as a function of peptide concentration. Because the TiO_2 signal was so strong, peaks from titanium hydroxides were obscured. $Ti(OH)_4$ has been reported to occur as a leading edge of the Ti $2p_{3/2}$ peak at 457.9 eV. One may presume that titanium hydroxides are present because of the oxygen upfield tailings that are consistent with a hydrated surface [Figure 1(b)]. The titanium peak ratio of metal to oxide was also essentially constant. It can be argued that the peptide was adhering in discrete islands with a thickness of greater than 50 Å. The size of the peptides are on the order of the analysis depth for XPS—30–50 Å.

Adsorption isotherms are illustrated in Figures 2 and 3; error bars represent one standard deviation of 15 analyses. With the exception of one data point (0.91 mg/ml RGDS on cp titanium) out of 42 points, a surface concentration progression of RFDS>RGDS>RGDA was found on both cp titanium and Ti-6Al-4V. There was more RGDA adsorbed on cp titanium than on Ti-6Al-4V.

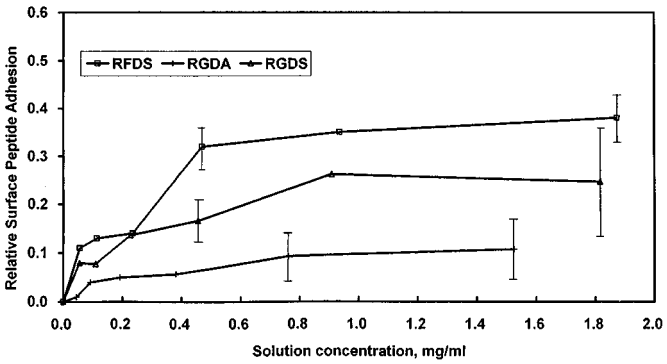


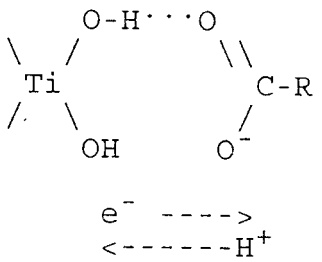
FIG. 3. Adsorption isotherms of RFDS, RGDS, and RGDA on titanium-6 aluminum-4 vanadium.

IV. DISCUSSION

A. Interpretation of the O 1*s* binding energy

The complexity of these spectra precludes any absolute judgements, but the many repetitive detailed features that are detected and their obvious relationship with the results of previous studies strongly support the following scenario. (1) The O 1*s* spectrum of the RGDS powder [Figure 1(c)] confirms the substantial presence of carbon–oxygen groups with the major peak at about 531.2 eV, typical of the presence of C═O units with obvious tailing upfield, indicative of C–OH units. (2) The O 1*s* spectra for the water-exposed titanium [Figure 1(b)] is typical of the oxide portion of TiO_2.[11] The high energy tailing in the spectrum reflects the terminal hydroxide and aquation ($TiO_2 \cdot nH_2O$) experienced by all air exposed oxides. TiO_2 is well known to exhibit several n values that should result in a manifold of peaks, the most prominent of which should occur about 1.3 eV upfield from that for the oxides, i.e., at about 532 V. (3) For the peptide-exposed samples, there is a series of peaks suggested by the numerical designations in Figure 1(c). First, there is evidence in peak 1 for the retention of substantial amounts of largely undisturbed TiO_2. It should be apparent from the comparison with Figure 1(b) that most of the balance of the upfield O 1*s* structure found in Figure 1(c) is due to the presence of and interaction with the peptide. The broadened low binding energy structure suggests an additional peak near point 2 that is indicative of the C═O part of the metal adsorbed carboxyl unit. Peak 4 is due to $TiO_2 \cdot nH_2O$ and C–OH (532.5).[12] The significant peak structures in the vicinity of positions 5 and 6 (about 533 eV) are indicative of adsorptive bonding through the $C–O^-$ part of the

carboxyl unit, thus confirming that the peptide is interacting with the titania with its oxygen down. On the other hand, peaks 2–4 are indicative of unattached units of the peptide plus very important reflections of the retention of titanium metal hydroxide peak structures that still exist on the outer surface of the metal oxide. Some of the latter reflect undisturbed surfaces of $TiO_2 \cdot nH_2O$, but some of these metal hydroxide peaks are additionally shifted to new positions (peak 5), suggesting possible involvement with the adsorbed peptide. These shifts may reflect the creation of a hydrogen bond. Surface reactions with organic acids can occur by electron donation:[13]



with the transfer of electrons and hydrogen ions in the directions shown. *R* represents the balance of the peptides. Using the above diagram, it is speculated that peak 5 could be due to the oxygen* in Ti–O*–H-peptide and peak 6 could be due to C–O*–H–O–Ti. Neither of these oxygen states was present in the initial systems, consistent with the XPS data obtained. It should be noted that several characteristics of the materials involved support this supposition. For example, the surface titanium involved is completely oxidized before adsorption of the peptide units, thus no direct positions exist on the metal itself to accommodate the O^- unit of the peptide. Also, the latter (O^-) is not compatible with direct attachment to the oxygens of any terminal TiO_2. Therefore, it seems consistent to reason that the attachment of at least some of the peptide to the titania surface is through surface hydroxyls in the form of a hydrogen bond. As one finds in the case of DNA and related systems, there is also a driving force for organic units such as the one illustrated above to form a stabilizing ring structure.

The terminal ends of the tetrapeptide groups each have an organic acid of the form shown above for reaction with the hydrated titanium oxide surface. In addition, each of them has an aspartic acid (abbreviated D) group that can allow bonding in this fashion and RFDS and RGDS also contain serine (S), with an OH-containing side chain. Thus, additional bonding seems to occur between the OH and O^- forming six-member ring structures, very similar to the manner that paired carboxylic acids form two hydrogen bonds.

B. Relative peptide surface concentration

Trends in the data are visible. There is a pattern in the surface concentration of RFDS>RGDS>RGDA on both alloys; this is more apparent above concentrations of 0.4 mg/ml. The only difference in the surface composition of the two alloys studied is a small amount of aluminum on the surface of Ti-6Al-4V. The native surface of cp titanium is negatively charged while the native surface of Ti-6Al-4V is relatively more positively charged because of the aluminum.[1] The net charge of RFDS is 0, of RGDS it is +1, and of RGDA it is +3. If net peptide charge was the strongest motivation for the adsorption to occur, the peptide with the highest surface concentration on cp titanium should be RGDA because it has the highest net positive charge. This was not the case; indeed, RGDA had the lowest concentration on cp titanium. This lack of effect is due to the high dielectric constant of water, i.e., only when unlike charges are very close and there are no water molecules in between would this type of electrostatic force be dominant.

RGDA, with an end group acid and aspartic acid available, is hypothesized to form a hydrogen bonded structure on the surface. Because of the electropositive nature of the hydrogen atom in covalent bonds, fewer hydrogen bonds would form on the relatively electropositive surface of Ti-6Al-4V than on the more electronegative surface of cp titanium. This seems to be the case. Inspecting Figures 2 and 3, there appears to be slightly more RGDA on cp titanium than on Ti-6Al-4V. This difference would be accentuated if a correction could have been done for the amount of aluminum at the surface.

In RGDS, the final amino acid of RGDA (alanine, $-CH_3$ side chain) is replaced with serine ($-CH_2^-$–OH side chain). Serine hydrogen bonds through the removal of its hydrogen atom. Theoretically, it should be easier for RGDS than RGDA to form a hydrogen bond to the surfaces because there is a higher likelihood that the molecule is in the correct orientation for hydrogen bonding to occur in at least one of the three molecular sites. Therefore, it is expected that more RGDS would be on the surface of the materials if hydrogen bonding was the dominant force; this was indeed found. More RGDS than RGDA was detected on the surface of both titanium alloys.

RGDS can be altered by changing the second position glycine amino acid (–H side chain) to phenylalanine ($-CH_2$–benzyl group side chain) to become RFDS. The phenylalanine side chain is very hydrophobic.[14] RFDS could be expected to bond to the surface of hydrated titanium oxide in the same fashion as RGDS; the end group acid, aspartic acid, and serine are all present in the same configuration as in RGDS and all near the terminal side. As a result, the hydrophobic phenylalanine is probably pointed outward, away from the surface. Water tends to order at this type of hydrophobic interface. Because this ordering is entropically undesirable, there is a driving force to minimize the water/phenylalanine interface in solution. This could cause the adsorption of a second layer, with the phenylalanine side chains grouping together, in much the same fashion as hydrophobic forces are responsible for protein folding. In agreement with this theory, the concentration of RFDS was generally higher than RGDS on each alloy.

This type of double layer adsorption behavior has been noted in the literature for protein structures. For example, an extensive study of protein adsorption isotherms on polymeric biomaterials was published by Young *et al.*;[15] the isotherms showed the possibility of two layers of adsorbed protein. Studies by Arnebrant *et al.*[16] of hydrophilic substrates showed that the plateau values of the adsorption isotherms correspond to a bilayer. In that case, the protein adsorbed into a bilayer, with the bottom layer unfolded and attached by strong polar bonds to the surface. On top of that layer, additional protein molecules are attached by hydrophobic interaction and/or ionic forces. The upper layer results in large electrical charges. Similarly, Johnston *et al.* reported[17] that, as the bulk fibrinogen concentration was increased, the protein fractional coverage detected on polytetrafluoroethylene reached a constant value but the adsorption isotherm continued to increase, indicating multilayer growth in patches on the surface. This was consistent with the incomplete coverage indicated by their XPS results. Incomplete substrate coverage was also detected in our titanium study.

C. Proposed mechanism for the shape of the RFDS isotherm

The isotherms for RFDS are illustrated in Figures 2 and 3. The plots for cp titanium and Ti-6Al-4V are different in form, with substantially smaller standard deviations in the data than the other peptides. Although not fully illustrated in the plots, there were no overlaps in the surface concentration error bars above 0.25 mg/ml. The plot for cp titanium is a

classic Brunauer–Emmett–Teller (BET) isotherm with a monolayer concentration corresponding to a solution concentration of 0.1 mg/ml. The BET theory implies that multilayer adsorption is present. Within the concentration range used, the amount of adsorbed peptide does not plateau. In contrast, RFDS on Ti-6Al-4V shows a distinct bilayer adsorption, with each layer behaving in accordance with Langmuir theory.

A possible mechanism for RFDS adsorption can be proposed. At low solution concentrations, the major adhesion mechanism is the formation of a hydrogen bond through the terminal acid group, the aspartic acid, and the serine end group. The nitrogen/titanium ratio is the same for both alloys and the distribution of RFDS is random on the surface throughout the solution concentration range of 0–0.25 mg/ml. As solution concentrations continue to increase, the RFDS continues to be adsorbed onto the initial surface of cp titanium; additionally, hydrophobic interactions with the exposed phenylalanine can begin and a second layer is adsorbed in spots. It is conceivable that adjacent, bonded RFDS molecules could rearrange themselves on the surface due to hydrophobic forces of adjacent phenylalanine groups. Because the ability to form hydrogen bonds is high, the RFDS density is relatively high on cp titanium and this rearrangement would be sterically feasible.

In contrast, the mechanism for Ti-6Al-4V could be different because it is more electropositive than cp titanium. At low solution concentrations, the major adhesion mechanism is once again bonding through the terminal acid group, the aspartic acid, and the serine. As solution concentrations increase, the sites for the hydrogen bonding on the alloy begin to be used up, sooner than on cp titanium. The surface concentration of RFDS increases and becomes saturated on Ti-6Al-4V at a lower solution concentration than for cp titanium. The RFDS groups on Ti-6Al-4V may be too far apart to minimize the hydrophobic surface energy by rearrangement and as a result they could then adsorb additional hydrophobic RFDS groups from solution. This is done relatively quickly until saturation of the initial RFDS layer is complete. After this second layer of adsorption has occurred, there is no hydrophobic outer layer to allow additional adsorption, and equilibrium has been attained. Consistent with the data observed, this proposed mechanism could allow for a gradual increase of RFDS on cp titanium and bilayer adsorption on Ti-6Al-4V.

V. CONCLUSION

XPS nitrogen/titanium ratios were used to obtain adsorption isotherms of RGD-based peptides on titanium alloys. Differences in the isotherms were noted and possible explanations discussed. In correlation with the high resolution oxygen XPS data, it has been postulated that biomolecules may adsorb differently on cp titanium and Ti-6Al-4V due to the difference in the abilities of the alloys to form hydrogen bonds and the ultimate effect that this has on hydrophobic interactions. There was no correlation found between the net charge of the peptide groups and their adhesion to the alloys.

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