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Stability of Proteins on the Surface of Ti-6Al-4V, 316L SS and Nitinol Alloys using 2D Correlation Analysis

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Abstract

Ti-6Al-4V, 316L SS and Nitinol are often used as metallic bio-implants. However, their biocompatibility with the environment of a living organism implant surfaces needs to be enhanced by exposing them to proteins such as albumin in the human body. An analysis of the adsorption of bovine and human serum albumin on the surface of these bio-implants was investigated to confirm its biocompatibility. The adsorption of both albumins was done to obtain the time for the proteins to establish stable contact with the outermost surface of the metallic bio-implant. 2D application of correlation spectroscopy was displayed as a contour map from vibrational spectra of Amide III bands. This has allowed for the selection of an optimal time of conformational stability of protein adsorption on the surface of the biomaterials. The findings revealed that albumin was in contact with the surface of Ti-6Al- 4V after 30 minutes in BSA, while 316L SS and Nitinol took longer at 60 minutes. HSA achieved a stable configuration after 15 minutes for Ti-6Al-4V, while 316L SS and Nitinol took longer, with about 40 minutes. Dynamic changes were observed through conformation of absorption between BSA and HSA that showed a significant difference between both types of protein.

Discipline: Biomedical engineering, Biomaterials, Engineering materials, Mechanical Engineering.

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1 Introduction

In general, proteins are made from a unique sequence of amino acids. However, the composition of amino acids in each type of protein varies (Wrigglesworth, 1984). It is determined by the arrangement of the amino acids in its chain (Branko, 2015). Proteins are relatively the most complex structure. Protein adsorption takes place when any protein-containing fluid including extracellular matrix components comes into contact with the implanted area of the human body. In the field of bio-implant material, it is widely agreed that one of the main considerations that influence biocompatibility is the rapid adsorption of proteins from human body fluids onto the implant-tissue interface (Vidal et al., 2010). This paper focuses on bio-implant material and protein interaction that affect its structural conformation. Therefore, the vibrational spectroscopic technique such as Raman spectroscopy is the most suitable testing used to investigate conformations and protein interaction structures.

The ultraviolet spectrum of proteins such as aromatic amino acids and peptide bonds is significantly influenced by their confined environment due to electrical phenomena. Moreover, hydrogen bonding and ion pair interactions also influence the spectra. Although protein is a highly complicated biological sample, its environmental sensitivity of protein spectra can be used to achieve information about the structure of proteins (Hammes, 2006).

Bovine serum albumin (BSA) and human serum albumin (HSA) have been widely used and studied in terms of their structure and interactions between bio-implants. However, there is still insufficient Raman spectroscopic investigation, especially for the conformation of serum albumin on implantation. Therefore, the objective of this study is to investigate the conformation of proteins on the surface of Ti-6Al-4V, 316L SS and Nitinol alloys by using 2D correlation analysis.

2 Literature Review

2.1 Biomaterials

The use and application of biomaterials only started after World War II when scientists discovered that the use of titanium as implants showed a positive response indicating great biocompatibility (Ornberg, 2007). Biomaterials are made from natural or synthetic materials which interface with living tissues and do not produce any toxic or dangerous response in the human biological system (Abbass, 2018). Biomaterials are widely used in orthopaedics, dentistry and cardiovascular surgery. For a biomaterial to be considered suitable and good material, it should possess three main characteristics, namely great biocompatibility, high corrosion resistance and sufficient mechanical strength. Stainless steel (SS), cobalt chromium, titanium-based alloys, nitinol, composites and polymer are widely used as bio-implant materials in biomedical applications (Burnat, 2014). Each material behaves differently when interacting with tissues and bones in the human body (Jean & Laurent, 2007). When implants interact with the biological environment, some major concerns such as corrosion, biodegradation of implant surface or the instability of the implant itself should be taken into serious consideration (Magheru et al., 2013).

This is due to the interaction between the surface implant and human body fluids that contain various biomolecules and types of protein which could have further influence on the rate of ion metal release. This interaction mustn't cause any negative effects such as inflammation, toxicity, or allergy that will prolong the recovery time or at worst cause the failure of the implants (Zhang et al., 2016).

2.2 Proteins

In a healthy human body, the number of biomolecules such as protein varies between 33 g/L to 52 g/L. The human body contains blood plasma. This blood plasma is made up of three major proteins known as fibrinogen, globulin and albumin (Loh & Lim, 2016). The main component of albumin is made of 584 amino acids of known sequence. The albumin ranges between 50 % and -60 % which is the total protein content of plasma (Boldt, 2010). This type of protein is the best available known binder of biological fluids and the most abundant. Albumin is known to be synthesized in the liver. In terms of molecular size and general composition, these amino acids of human albumin are mostly found to be similar to bovine albumins. This is the main reason why albumin is often chosen to be used and added in simulated body fluid solutions (Kulkarni et al., 2015; Mudali et al., 2003). Many studies have shown that the interaction between proteins, tissues and cells has a significant role when it comes into contact with material implants (Thevenot et al., 2008; Wataha et al., 2001; Ashwin et al., 2016). However, now, there is still much debate on whether the presence of proteins leads to higher or lower corrosion rates in implants.

3 Method

3.1 Preparation of Samples

Rods of Ti-6Al-4V, 316L SS and Nitinol alloys were sequentially wet sanded from 320 up to 600 grit finish, followed by washing with distilled water, and dried with compressed air. After drying, all the samples were immersed in PBS solution with an addition of 1% of BSA (the first batch) and HSA (the second batch) in a 150 ml beaker. The duration of immersion was 15 min, 30 min, 45 min and 60 min. After immersion, the samples were taken out and dried out in the open air before being tested under Raman spectroscopy.

3.2 Raman Measurements

Raman spectroscopy measurements were performed using a Raman spectrometer working in confocal mode. The measurement configuration included a 100X high- magnification objective lens (NA = 0.90) in the excitation line of 514.5 nm. The software of this instrument was used to analyse the spectra and calculate the position, intensity and area of the characteristic peaks.

4 Result and Discussion

The 2D correlation analysis was carried out using Raman spectra to sort out complex variations in correlation maps. This method allows for an analysis of Raman scattering of the system that responded to samples under influence of external perturbation and found the relativity

between components of the system (Branko, 2015). The analysis was made up of absorbance measurements of the PBS and albumin solution remaining after the immersion of the samples. The spectrum of the alloys was assigned to the high peak of the amide band by referring to the peak of the amide band found in BSA and HSA as shown in Figure 1 and Figure 2.

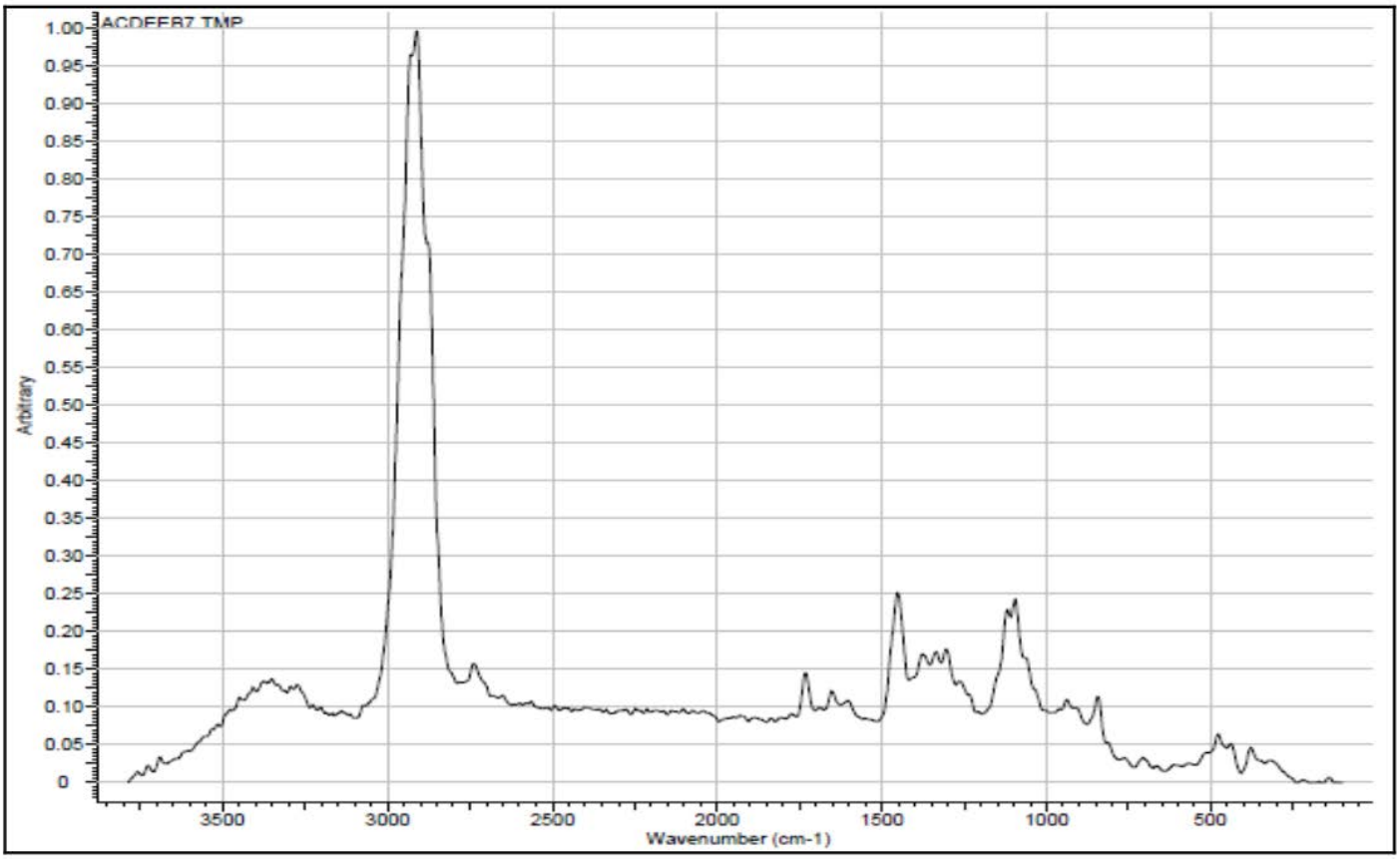

Figure 1: Peak of Raman Spectrum of Amide Band found in BSA

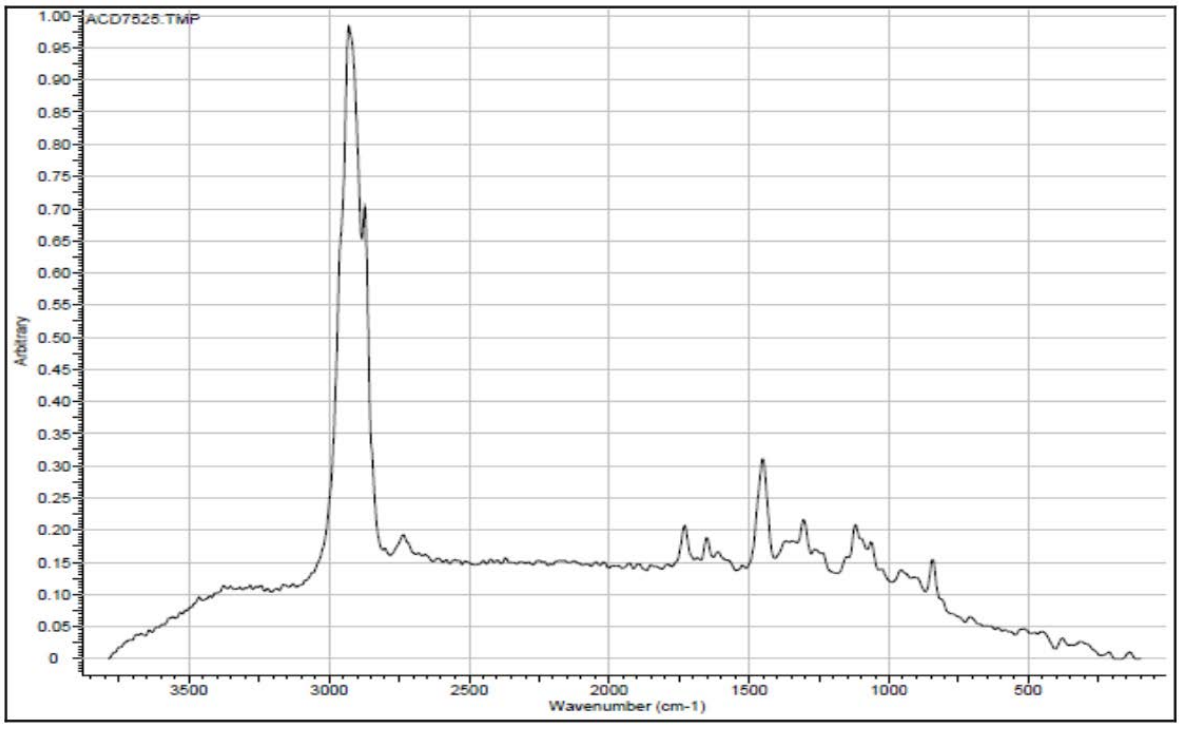

Figure 2: Peak of Raman Spectrum of Amide Band found in HSA

Table 1 shows the Raman measurements which clearly indicate the similar peak position of Amide III bands for BSA and HSA, that is 2929.95 cm^{-1}. The bands characteristic of BSA and HSA, respectively are indicated for characterization of the intensity level: vw: very weak, w: weak, m: medium, s: strong, and vs: very strong.

Table 1: Observed significant Raman bands (cm^{-1}) and their assignments.

Peak position (cm-1)	Arbitrary	Assignment	Intensity
BSA			
2929.95	0.965	Amide III	vs
HSA			
2929.95	0.984	Amide III	vs

Raman measurements were taken at four different areas on the outermost surface of the Ti-6Al-4V, 316L SS and Nitinol alloys obtained in different immersion times as shown in Figures 3 to 5 and tabulated in Tables 2, 3 and 4. In the spectrum of the alloys, it is assigned to the high peak of the amide band by referring to the peak of the amide band found in BSA and HSA (Table 1). From the immersion time periods, it was identified that the C–H stretching (νC–H) vibration bands of side chains were observed in Amide III regions around 2910 cm^{-1} to 2964 cm^{-1}.

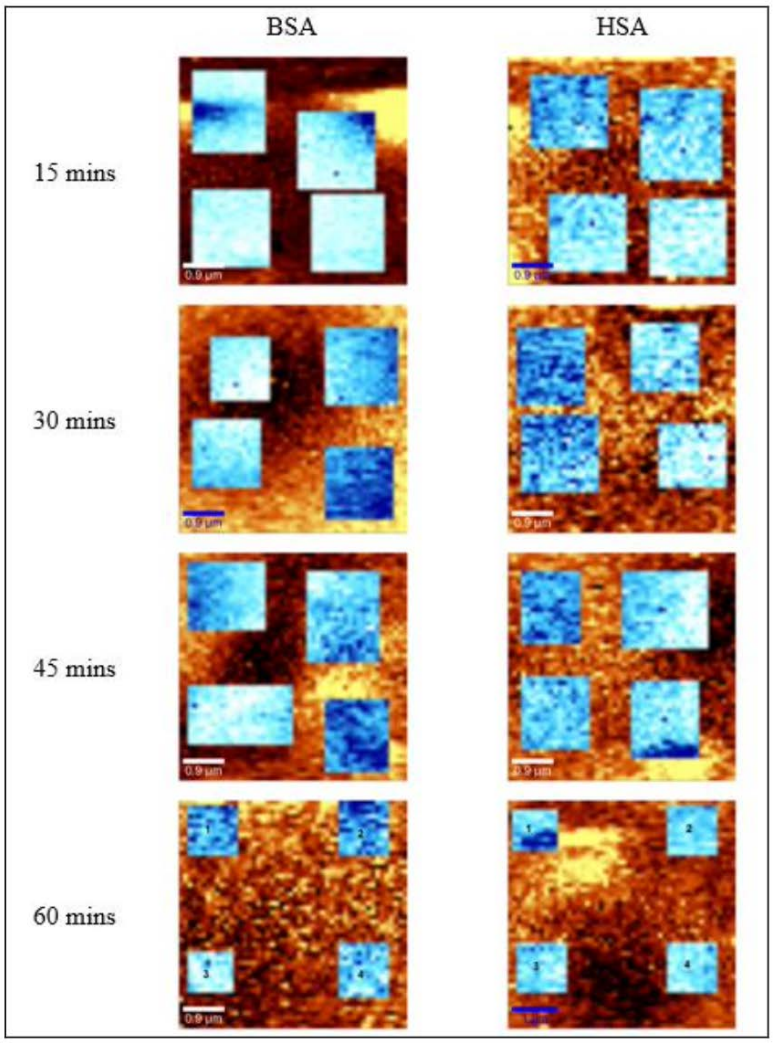

Figure 3: The 2D Raman spectra of Ti-6Al-4V obtained at different times in four different areas.

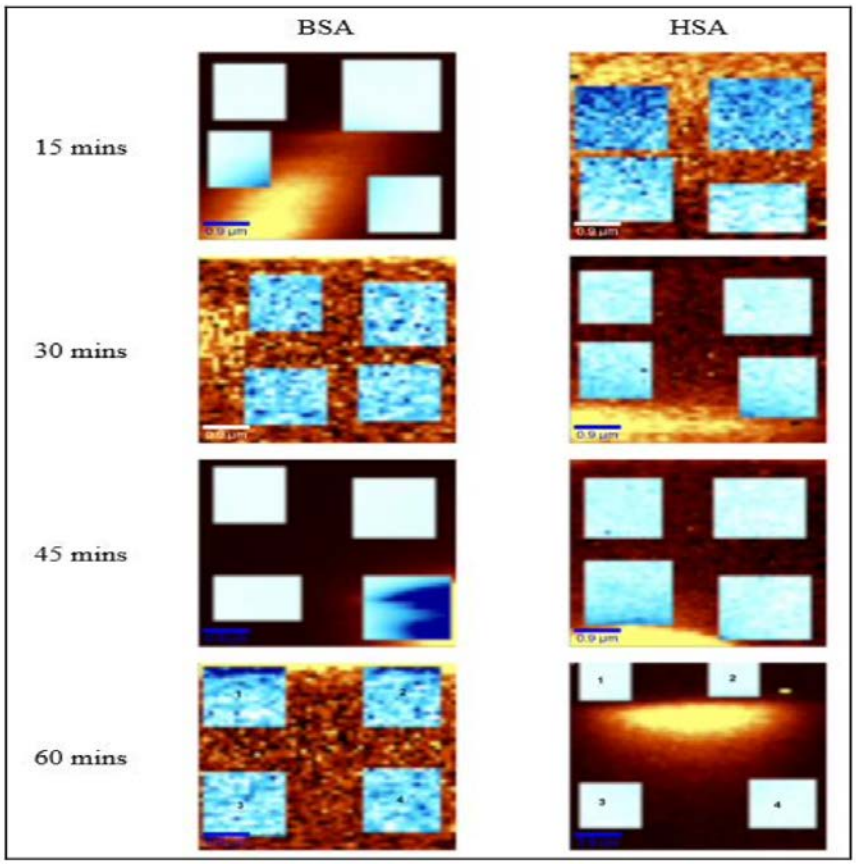

Figure 4: The 2D Raman spectra of 316L SS obtained at different times in four different areas.

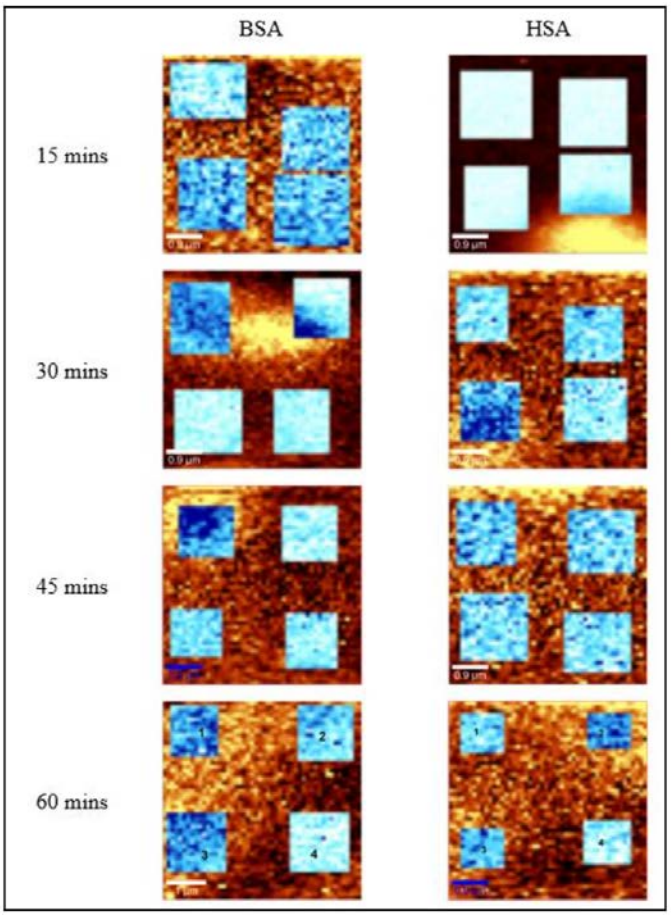

Figure 5: The 2D Raman spectra of Nitinol obtained at different times in four different areas.

From Tables 2, 3 and 4, the most likely similar peak positions of BSA and HSA were found at immersion time of 30 minutes (BSA) and at 15 minutes (HSA) for Ti-6Al-4V, 60 minutes (BSA) and 45 minutes (HSA) for 316L SS and Nitinol. The time conformation of both albumins on the metal surface of Ti-6Al-4V, 316L SS and Nitinol was different, where BSA took 15 minutes longer than HSA.

Table 2: Interpretation of the Reference Raman Bands (cm^{-1}) and Their Assignment with the Highest Peak Among Four Peaks of Each Immersion Time for Ti-6Al-4V Excited with 514.5 nm Laser Line.

Immersion time	Peak position (cm^{-1})	Arbitrary	Assignment	Intensity
BSA				
15 mins	2929.96	0.969	Amide III	vs
30 mins	2910.11	0.282	Amide III	s
45 mins	2935.91	0.270	Amide III	vs
60 mins	2925.98	0.532	Amide III	s
HSA				
15 mins	2927.97	0.727	Amide III	vs
30 mins	2925.99	0.740	Amide III	vs
45 mins	2923.01	0.672	Amide III	vs
60 mins	2917.06	0.931	Amide III	vs

Table 3: Interpretation of the Reference Raman Bands (cm^{-1}) and Their Assignment with the Highest Peak Among Four Peaks of Each Immersion Time for 316L SS Excited with 514.5 nm Laser Line.

Immersion time	Peak position (cm^{-1})	Arbitrary	Assignment	Intensity
BSA				
15 mins	2935.91	0.773	Amide III	vs
30 mins	2926.98	0.943	Amide III	vs
45 mins	2930.95	0.969	Amide III	vs
60 mins	2925.99	0.923	Amide III	vs
HSA				
15 mins	2927.97	0.727	Amide III	vs
30 mins	2964.68	0.486	Amide III	vs
45 mins	2929.95	0.734	Amide III	vs
60 mins	2930.95	0.919	Amide III	vs

Table 4: Interpretation of the Reference Raman Bands (cm^{-1}) and Their Assignment with the Highest Peak Among Four Peaks of Each Immersion Time for Nitinol Excited with a 514.5 nm Laser Line.

Immersion time	Peak position (cm^{-1})	Arbitrary	Assignment	Intensity
BSA				
15 mins	2929.96	0.710	Amide III	vs
30 mins	2930.95	0.948	Amide III	vs
45 mins	2931.94	0.935	Amide III	vs
60 mins	2929.95	0.932	Amide III	vs
HSA				
15 mins	2927.97	0.727	Amide III	vs
30 mins	2964.68	0.486	Amide III	vs
45 mins	2929.95	0.734	Amide III	vs
60 mins	2930.95	0.919	Amide III	vs

Raman spectroscopy is particularly sensitive to subtle spectral changes, therefore when the bond strength of protein changes, it scatters a laser photon during protein conformation and interaction structures. The amide band increases due to bond vibrations in the peptide chains. The Amide I band consists of C=O stretching vibrations from CN stretching vibration, CCN deformation,

as well as N-H, bending. Meanwhile, NH in-plane bending is combined with CN stretching vibration received from CO bending to form the Amide II band (Premasiri et al., 2005). The Amide III band is a complex band made of C-N stretching and C-N-H in-plane bending modes. This band is commonly used in defining the conformation of proteins (Parker, 1983). In Tables 2, 3 and 4, the region from 2910 cm^{-1} to 2964 cm^{-1} in the Raman spectrums showed a readily distinguishable and very strong Amide III for both albumins.

BSA is crystallized in a monoclinic crystal form. In contrast, serum albumin is crystallized in a hexagonal crystal form of the C2 space group (Anna et al., 2017). This finding is supported by a previous study done by Majorek et al. (2012) where it was found that rabbit serum albumin (RSA) is crystallized in an orthorhombic crystal form. Both horse serum albumin (ESA) and RSA are crystallized with one molecule in asymmetric units. The albumins are composed of three structurally identical helical domains arranged in a heart-shaped molecule. The helical contents of the three albumins are 74% (BSA), 75% (ESA), or 72% (RSA), and each has 17 conserved disulfide bonds and a free thiol group related to Cys34. The structures of all three albumins show a strong resemblance to the HSA structure. The disulfide bond is a covalent bond that results in the formation of sulphur units on cysteine residues and is critical for maintaining the natural protein structure (Siddhanta & Narayana, 2012). The crystalline structure of HSA has been determined and the interaction with proteins has been studied for HSA and BSA (He & Carter, 1992; Sugio et al., 1999; Jean & Laurent, 2011). The conformation of BSA is thought to be like HSA due to 76% of amino acid sequence homology (Bunaciu et al., 2015).

Tables 2, 3 and 4, show that the conformation of proteins, which is BSA and HSA at the surface of Ti-6Al-4V, 316L SS and Nitinol, happens at different times. These findings underline the conformation of proteins affected by their environment in the solutions which include factors such as pH, ionic strength and the presence of ions (Irene et al., 2012). The finding of time-dependent protein conformational could be attributed to the corrosion behaviour of metal implants due to its protein-surface interactions. Protein adsorption is complex and involves electrostatic interactions such as van der Waals, hydrophobic and hydrogen bonding (Hȍhn et al., 2019). This phenomenon indicates the adsorption of proteins onto the surface of biomaterials that can cause limitation of oxygen to diffuse to certain regions of the surface which are resulted from the van der Waals bonding, hydrophobic-corrosion of oxygen-deficient regions and breakdown of the passive layer (Thevenot, 2008).

Tissues in the human body contain water, dissolved oxygen, proteins and ions such as chloride ions (Cl-) and hydroxide (OH-), and they present an aggressive environment to metallic materials used for implantation (Wataha et al., 2001). Protein adsorption reaction onto the surface of implanted material takes place after the materials have been implanted into the body (Ashwin et al., 2016). This phenomenon can cause corrosion and lead to the deterioration and dissolution of metals through chemical reactions. Moreover, corrosion can occur due to various conditions around the tissues and body fluids with implant surfaces. Chenglong et al. (2015) concluded that these

conditions may contribute to the formation of electrochemical cells at the implant-body fluid interface.

5 Conclusion

A 2D correlation analysis displayed as a contour map by using Raman spectroscopy confirmed that samples that are in contact with body fluids with the presence of proteins have different times in terms of protein conformation. The adsorption of albumin to the Ti-6Al- 4V, 316L SS and Nitinol surfaces was applied to verify the time consumption for proteins to completely be in contact with the surface of the implants. BSA achieved a stable conformation on the Ti-6Al-4V surface perfectly after 30 minutes, while 316L SS and Nitinol took a long time of 60 minutes to confirm. Meanwhile, HSA performed a stable configuration only after 15 minutes for Ti-6Al-4V and took much longer which was 40 minutes for 316L SS and Nitinol. In a conclusion, the adsorption of HSA is considerably much faster as compared to BSA.

6 Availability of Data and Material

Data can be made available by contacting the corresponding author.

7 Acknowledgement

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