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Protein Adsorption on Poly-3-hydroxybutyrate and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate): Adsorption Isotherms and Conformational Analysis of the Adsorbed Protein

Poly-3-hydroxybutyrate ve Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Yüzeylerine Protein Adsorpsiyonu: Adsopsiyon İzotermleri ve Adsorbe Proteinin Konformasyonel Analizi

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ABSTRACT

The equilibrium adsorption of bovine serum albumin (BSA) on poly-3-hydroxybutyrate (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) surfaces was studied at different solution concentrations. The equilibrium adsorption data were fitted to the Langmuir and Freundlich adsorption models. Adsorption behaviour of BSA on PHB and PHBV surfaces was further investigated by analysing the conformation of the surface adsorbed protein with Fourier transform infrared (FTIR) spectroscopy with an Attenuated Total Reflectance (ATR) apparatus. The results showed that the Freundlich isotherm was a better fit for the adsorption of BSA on PHB and PHBV surfaces, which is supported by the significant conformational changes that BSA undergoes upon adsorption.

Key Words

Protein adsorption, protein conformation, adsorption isotherm, biopolymer.

ÖZ

Sığır serum albümininin (BSA) poli-3-hidroksibutirat (PHB) ve poli(3-hidroksibutirat-co-3-hidroksivalerat) (PHBV) yüzeyleri üzerinde denge adsorpsiyon davranışı farklı çözelti konsantrasyonlarında incelenmiştir. Denge adsorpsiyon verileri, Langmuir ve Freundlich adsorpsiyon modellerine uyarlanmıştır. BSA'nın PHB ve PHBV yüzeylerinde adsorpsiyon davranışı ayrıca Fourier transform kızılötesi (FTIR) spektroskopisisiyle zayıflatılmış toplam yansıma (ATR) aparatı kullanılarak yüzey adsorbe olmuş proteinin konformasyonun analiz edilmesi suretiyle araştırılmıştır. Sonuçlar Freundlich izoterminin, BSA'nın PHB ve PHBV yüzeyleri üzerinde adsorpsiyonunu açıklamak için daha uygun olduğunu göstermiş olup bu durum BSA'nın yüzey adsorpsiyonu sonucu geçirdiği konformasyonel değişikliklerle de desteklenmiştir.

Anahtar Kelimeler

Protein adsorption, protein conformation, adsorption isotherm, biopolymer.

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INTRODUCTION

Due to its strong influence on the interaction of cells with surfaces, the investigation of protein adsorption onto biomaterial surfaces is of great interest to researchers in this field. Especially, the conformational changes in the surface adsorbed proteins can be an important driving force for further adsorption [1, 2]. The type and amount of the proteins present on the surfaces affect cell adhesion, and their orientation is crucial for exposing binding sites specific to the receptors on cell membranes [3]. Accordingly, efforts have been focused on controlling and understanding the adsorption of proteins onto various surfaces. For this purpose, the adsorption kinetics, adsorption isotherms, and the conformational changes on the secondary structure of proteins that are adsorbed to the surfaces are commonly investigated [4].

Polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are natural biopolymers of bacterial origin [5]. PHB and PHBV are polyesters and thermoplastics with linear aliphatic hydrocarbon backbones. PHB has a high degree of crystallinity which is reduced for its co-polymer PHBV. Their biodegradability and biocompatibility make them attractive for use as biomaterials and scaffolds [6-8]. Several studies reported the amount of protein adsorbed onto various polyhydroxyalkanoates (PHAs) such as PHB and PHBV from different origins or PHA derivatives such as PHB-PEG [5, 8-11]. These studies usually correlated the amount of protein adsorption with the surface hydrophilicity, surface free energy or crystallinity of the polymers [5, 6, 8-10]. In this study, the adsorption behaviour of bovine serum albumin (BSA) to PHB and PHBV surfaces in terms of adsorption isotherms and the conformational changes of BSA upon adsorption is reported for the first time. BSA was chosen as the model protein because it can easily undergo conformational changes upon adsorption.

MATERIALS and METHODS

Surface wettability and surface free energy

PHB sheets (BU393201, GoodFellow, UK) and PHBV films (PHB92/PHV8, BV301010 GoodFellow, UK) were cut into 1x1 cm pieces and were cleaned for 5 min sequentially in acetone, ethanol and deionised water using an ultrasonic water bath (Isolab, Germany). The water contact angle (WCA) of the biopolymers were determined by the sessile drop method using an optical tensiometer (Attension Theta, Biolin Scientific, Sweden). The surface free energy of the samples was calculated by using the Contact Angle (CA) values of deionised water and diiodomethane with the Owens, Wendt, Rabel and Kaelble (OWRK) model. At least 3 measurements at different points on each surface were taken.

Protein adsorption

Cleaned samples were placed in BSA (Serva, Germany) solutions having different concentrations (0.25; 0.50; 0.75; 1.0; 1.5 and 2.0 mg/mL) in a pH 7.4 PBS solution. Samples were incubated with slow shaking at 60 rpm (Mikrotest, Turkey) at 37 °C for 24 h. BSA adsorbed on the surfaces was recovered by placing the samples in 1 % aqueous sodium dodecyl sulfate (SDS; Sigma Aldrich, Germany) solution for 6 h, then by sonication for 5 min. The protein concentration in the SDS solution was determined by the bicinchoninic acid (BCA) method using a micro BCA protein assay kit (Thermo, USA) following the instructions on the kit's manual. The absorbance at 562 nm was measured using BioDrop µLITE+ spectrophotometer (BioDrop, UK). Adsorption experiments were performed in 3 replicates.

FTIR-ATR and protein conformation

Fourier transform infrared (FTIR) spectroscopy was used to determine the functional groups on PHB and PHBV surfaces and analyse the surface adsorbed BSA's conformation. Thermo Fisher Nicolet is50 FTIR spectrophotometer equipped with a diamond crystal and an Attenuated Total Reflectance (ATR) apparatus was used to collect the spectra of pristine and BSA adsorbed sample surfaces. For each sample, 512 scans were performed with a resolution of 4 cm^{-1}. The spectra of BSA adsorbed surfaces were further processed by subtracting the pristine sample's spectrum from that of the BSA adsorbed samples. Second derivative and curve fitting procedures were performed as described elsewhere [2]. For the fitting procedure the baseline of the amide I band was first treated to give a linear baseline between 1700 and 1500 cm^{-1}. Fitting was performed assuming Gaussian band profiles and the peak positions were let to be varied during the fitting procedure [2].

RESULTS and DISCUSSION

Contact angle and SFE

With a WCA of 80.68° ± 5.24° the PHB surface was slightly more hydrophobic than the PHBV surface which

had a WCA of 70.55° ± 7.89°. The SFE of PHBV was 37.12 mN/m with a dispersive component of 34.62 mN/m and a polar component of 2.50 mN/m. The SFE of PHBV was calculated as 44.40 mN/m with a dispersive component of 38.14 mN/m and a polar component of 6.26 mN/m. Bonartsev et al. reported a WCA of 70.5° ± 3.5° and 75.3° ± 6.1° for the smooth and rough PHB surface, and a WCA of 70.2° ± 2.1° and 77.7° ± 2.6° for the smooth and rough PHBV surface respectively [3]. The total SFE of PHB was 41.1 mN/m, and the polar and dispersive components of the SFE were 11.3 mN/m and 29.8 mN/m, respectively. The total SFE of PHBV was close to the SFE of PHB (45.2 mN/m) but had a lower value of the dispersive (17.7 mN/m) component and a higher polar component (27.5 mN/m). Domínguez-Díaz reported a WCA of 72° for the PHB film [12]. Although the WCAs and the SFEs of the biopolymers were close to the ones reported in the literature the polar and dispersive components were significantly different. This might be due to the different sources of the polymers as well as the different polymer processing and the additives present in the PHB and PHBV used in this study.

Adsorption isotherms

The Langmuir and Freundlich isotherm equations have been employed to investigate the adsorption behaviour of BSA on PHB and PHBV surfaces.

Langmuir isotherm

According to the Langmuir isotherm theory, the adsorbate covers the surface of a homogeneous substrate in a monolayer. Therefore, saturation is reached at equilibrium where no more adsorption/desorption occurs [13]. The equation for the Langmuir isotherm can be expressed as;

$$q_e = \frac{q_m K C_e}{1 + K C_e}$$

and,

$$\frac{C_e}{q_e} = \frac{1}{q_m K_m} + \frac{1}{q_m} C_e$$

Where, C_e is the equilibrium concentration of the adsorbate in the solution (mg/mL), q_e is the equilibrium amount of the adsorbate on the adsorbent (mg/cm^2), K is Langmuir's equilibrium constant which indicates to the strength of interaction between the adsorbate and adsorbent, and q_m is the maximum amount of the adsorbate on the surface which reflects the adsorption capacity. When C_e is plotted vs q_e, the values of K and q_m can be calculated from the intercept and slope, respectively.

Freundlich isotherm

Freundlich isotherm theory is used to calculate the equilibrium adsorption constant assuming that the concentration of adsorbate on the adsorbent surface increases with the increasing concentration of the adsorbent [14]. This method can be applied to heterogeneous surfaces but over a limited range of concentration [15]. Freundlich isotherm equation can be expressed as;

$$q_e = K_F C_e^{1/n}$$

or,

$$\ln q_e = \ln K_F + \frac{1}{n} \ln C_e$$

Where, K is a constant reflecting the measure of adsorption capacity related to the bonding energy and the n is a constant indicating the measure of adsorption intensity or surface heterogeneity. The value of 1/n can range between 0-1 and as its value approaches 0 it indicates a more pronounced heterogeneity [1, 16]. From the graph of $\ln q_e$ vs $\ln C_e$ the values of K_F and n can be calculated.

The adsorption isotherm of BSA on the PHB and PHBV surfaces are given in Figure 1. Based on correlation coefficient values (R^2), for BSA adsorbed on the PHB surface, a higher correlation fit for Freundlich isotherm (R^2=0.99) than for Langmuir isotherm was calculated (Figure 2). Still, the R^2 of 0.93 for the Langmuir isotherm can be considered significant. The R^2 values of both Langmuir and Freundlich isotherm fits were calculated as 0.99 for BSA adsorbed on the PHBV surface. These results are further discussed in the light of the findings form the conformational analysis in the following section.

According to the Langmuir isotherm fit, the maximum amount of BSA adsorbed on the PHB and PHBV surfaces at full saturation were 0.022 mg/cm^2 and 0.0012 mg/cm^2, respectively (Table 1). The n parameter of Freundlich's isotherms is a representative of the ave-

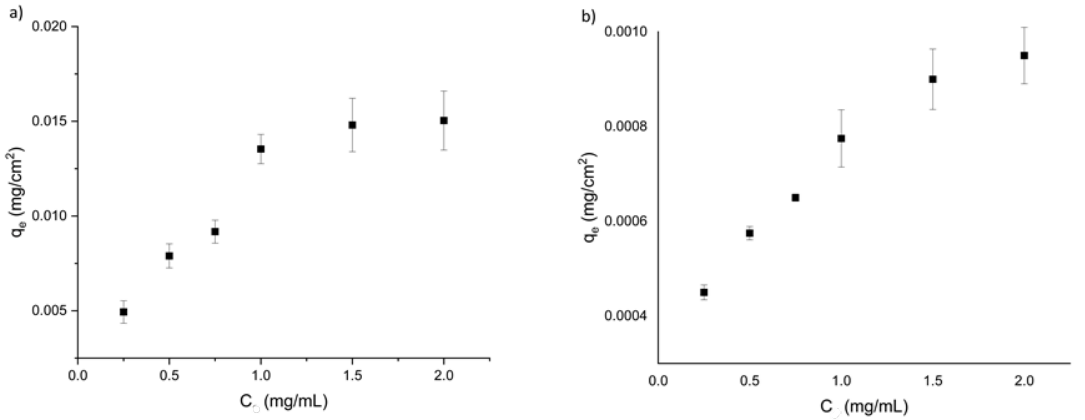

Figure 1. The adsorption isotherms of BSA on a) PHB and b) PHBV.

rage energy of adsorption where a lower value indicates a higher the affinity between the surface and adsorbate [16] and was calculated as 1.8 and 2.7 for the BSA adsorbed on PHB and PHBV surfaces, respectively. The n parameter is markedly lower for the PHB surface, indicating a higher affinity of BSA to the PHB surface. This result is consistent with the maximum amount of surface adsorbed BSA being higher for PHB than PHBV. The amount of protein adsorption on PHB and PHBV surfaces are previously reported in the literature. For example, Bonartsev *et al.* reported BSA adsorption on 3D porous PHB scaffold surface as 2.9 ± 0.1 µg BSA/ mg polymer [4]. In another study, Bonartsev *et al.* calculated the amount of protein adsorbed from Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) to be around 80 µg/cm^2 for both PHB and PHBV and didn't seem to be affected by the components of SFE [5]. Lee *et al.* reported that the BSA adsorption was almost identical onto poly(3-[R]-hydroxybutyrate) ([R]-PHB), poly(3-[R]-hydroxybutyrate-co-3-[R]-hydroxyvalerate) ([R]-PHB/HV), (racemic) poly(3-[RS]-hydroxybutyrate) ([RS]-PHB). The amount of adsorbed BSA was close to 1 µg/cm^2 for [R]-PHB and [RS]-PHB while it was lower than 1 µg/cm^2 for [R]-PHB/HV [8]. The WCA of all PHB derivatives were around 70°. Rouxhet *et al.* reported the HSA adsorption on PHBV surface incubated in 1 mg/mL HSA solution to be less than 1 µg/cm^2 [10]. The PHB and PHBV used in this study were produced by the authors using *Azotobacter chroococcum* and shaped into films using the solvent casting method. Zheng *et al.* reported the protein adsorption form DMEM containing 10% (v/v) FBS to be around 100 µg/cm^2 for both the PHB with a receding WCA of 53.4° ± 1.3° [11]. However, none of these studies investigated the adsorption isotherm of BSA on PHB and PHBV surfaces.

BSA conformation

The FTIR-ATR spectra of PHB and PHBV are given in Figure 3. For both biopolymers, the strong band at around 1720 cm^{-1} is characteristic of the carbonyl groups. The bands between 2800-3000 cm^{-1} are attributed to the asymmetric and symmetric stretching of CH_3 and antisymmetric stretching of CH_2 while the band at around 1380 cm^{-1} is attributed to the symmetric wagging of CH_3 groups. The bands in the region between 600-1500 cm^{-1} are associated with the ether groups and the CH_3 and CH groups on polyhydroxyalkanoates [17].

The conformational changes of BSA adsorbed on the PHB and PHBV surfaces were evaluated using the difference spectra of BSA adsorbed and pristine sample surfaces. For this purpose, the amide I/II intensity ratios and the components of the amide I band were considered (Figure 4). The amide I/II intensities for BSA adsorbed on PHB and PHBV surfaces were 1.95 and 2.45, respectively. The amide I/II ratio of BSA measured using KBr disks, and ATR were previously reported as 1.1 and 1.24 by Ishida *et al.* [18] and 1.6 and 1.76 by Lenk *et al.* [19]. The experimental methods used by these authors differed in temperature and incubation time, which strongly affected the protein's secondary structure, resulting in different amide I/II ratios. The amide I/II ratio of BSA adsorbed on both PHB and PHBV was higher than the amide I/II ratios reported for BSA measured using KBr disks indicating that the conformational change in the surface adsorbed BSA was significant for both surfaces. In addition, the amide I/II ratio of BSA adsorbed on the

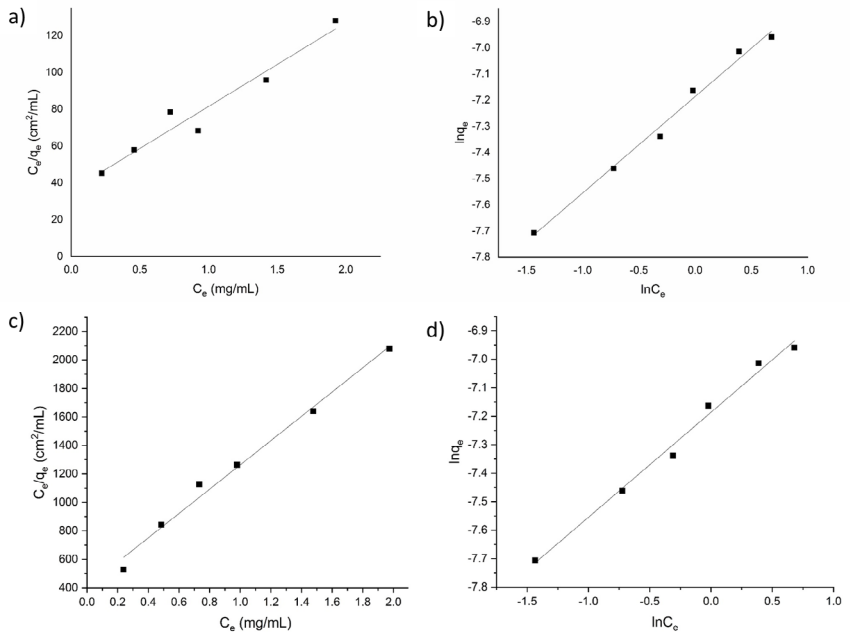

Figure 2. a) Langmuir and b) Freundlich fitting curves of BSA adsorption on PHB and c) Langmuir and d) Freundlich fitting curves of BSA adsorption on PHBV.

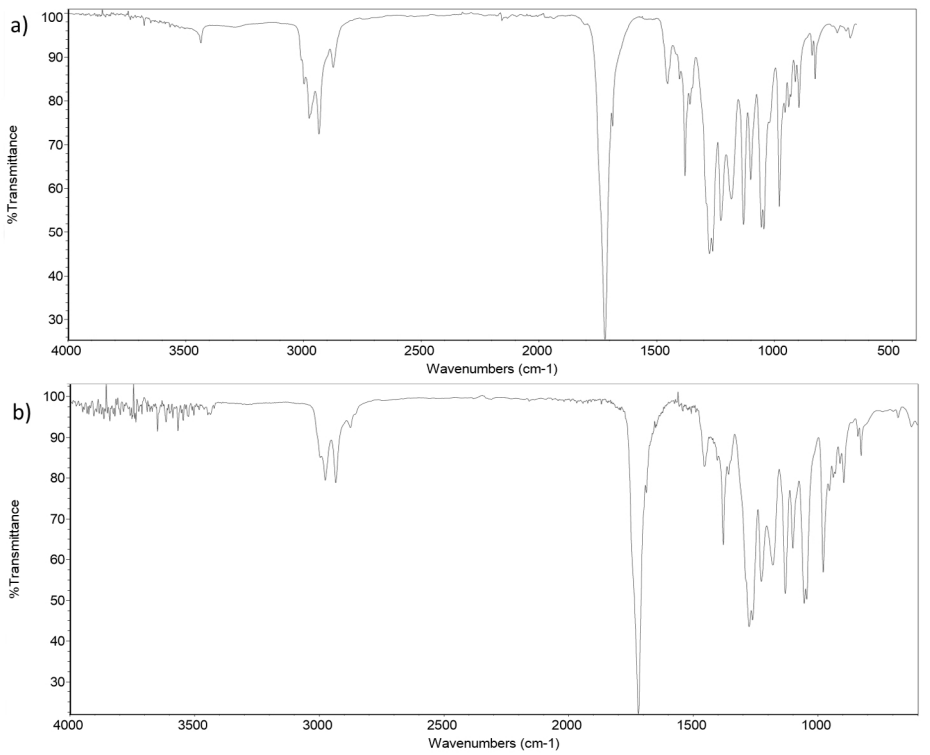

Figure 3. FTIR-ATR spectra of a) PHB and b) PHBV. BSA adsorption on PHBV.

Table 1. Langmuir and Freundlich parameters for BSA on PHB and BHPV.

Material	Langmuir parameters			Freundlich parameters			
0	R^2	qm (mg/cm^2)	K (L/mg)	R2	K_F	n	1/n
PHB	0.93	0.022	0.0013	0.99	0.012	1.8	0.56
PHBV	0.99	0.0012	0.0021	0.99	0.00076	2.7	0.37

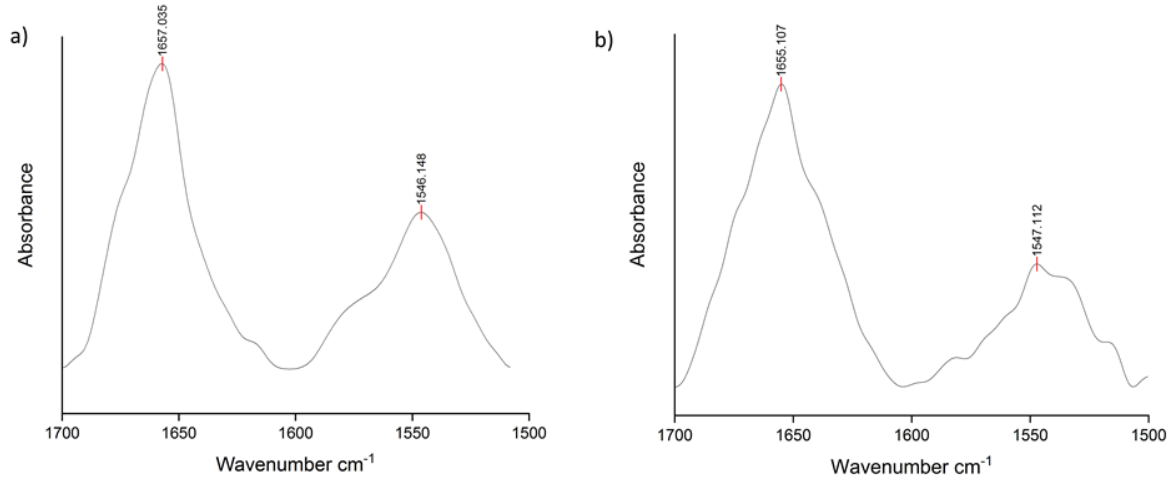

Figure 4. Amide-I and II bands of BSA adsorbed on a) PHB and b) PHBV.

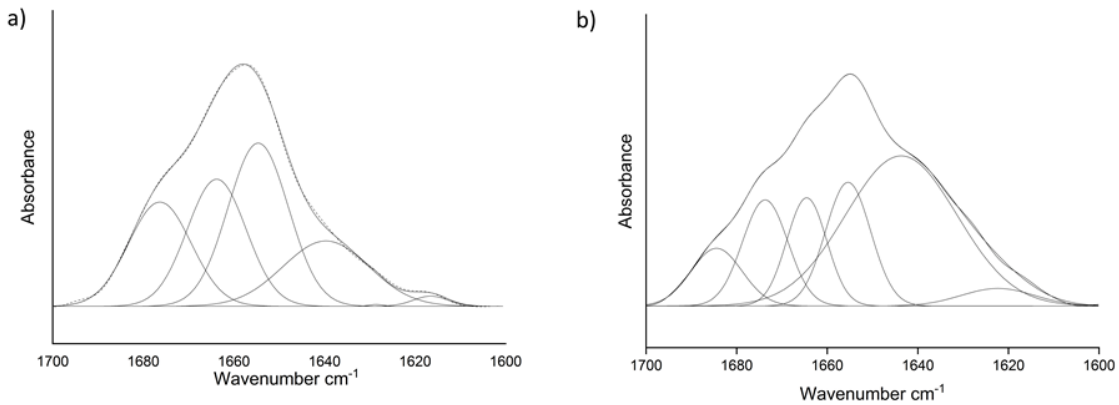

Figure 5. Deconvolution of the amide-I band of BSA on a) PHB and b) PHBV.

PHBV surface indicates a more severe change in the secondary structure of the protein. The amide I/II ratio tends to decrease with denaturation and increase with the ordered structures, helices and β-sheets [20].

Amide I/II ratio was further investigated in light of the amide I band (Figure 5). The amide I band consists of several overlapping component bands, such as α-helix, '3-turn' helix, β-structures and random structures. When the effective absorptivity of each component is assumed to be equal, their relative amounts can be determined using curve fitting [21, 22]. In this report, the bands observed at 1695–1670 cm^{-1} and 1643–1610 cm^{-1} were assigned to the β-structures (intermolecular and intramolecular), 1666–1659 cm^{-1} to '3-turn' helix, 1657–1648 cm^{-1} to α-helix and 1644–1640 cm^{-1} to random coil [11]. The α-helix content of BSA in its native form is reported to be between 50%-68%, and the amount of β-structures comprises a relatively small portion of its secondary structure (16%-22%) [18, 19, 22].

BSA, a globular protein with an average molecular of 66 kDa, have a secondary structure consisting of~ 54% α-helix and ~ 40% β-structure [23]. Since BSA is a soft protein it can easily change its structure and conformation upon surface adsorption. Surface adsorbed BSA can undergo structural changes and denaturation to optimise its interactions with the surface and the so-

Table 2. Langmuir and Freundlich parameters for BSA on PHB and BHPV.

BSA on PHB			BSA on PHBV			
Gaussian centre (cm^{-1})	Area (%)	Secondary structure	Gaussian centre (cm^{-1})	Area (%)	Secondary structure	1/n
1611	1.2	β-structure	1622	3.6	β-structure	
1629	0.1	β-structure	1643	46.9	β-structure	
1640	18.9	β-structure	1655	15.6	α-helix	
1655	33.0	α-helix	1665	12.1	3-turn helix	
1664	25.0	3-turn helix	1674	13.5	β-structure	
1676	21.8	β-structure	1684	8.3	β-structure	

lution. Upon adsorption, BSA usually loses its α-helical content while its random coil and β-structure content increases. For hydrophilic surfaces, the loss of α-helical content can reach up to 50%. Usually, the amount of surface BSA adsorbed is higher for hydrophobic surfaces than for hydrophilic surfaces, and BSA molecules interact trough $–CH_3$ groups with hydrophobic surfaces and trough –COOH groups with hydrophilic surfaces [23]. The components of the amide I band of BSA that is adsorbed on PHB and PHBV surfaces are given in Table 2. BSA adsorbed onto PHB surface had an α-helix content of 33.0% and β-structure content of 42.0%, and BSA adsorbed onto PHVB surface had an α-helix content of 15.6% and β-structure content of 72.3%. These findings are consistent with the amide I/II ratios showing that BSA adsorbed onto the PHBV surface had undergone a more extensive conformational change. The drastic increase in the β-structure of BSA adsorbed on PHBV surface might be due to the aggregation of BSA upon its interaction with the surface [20]. However, the amount of BSA adsorbed on the PHBV surface is lower than the amount adsorbed on PHB. This result might be due to BSA aggregation being more predominant than BSA adsorption on the PHBV surface [20].

The BSA adsorption on the PHB fits the Freundlich isotherm better than the Langmuir isotherm based on the R^2 values obtained from the fitting. Still, the R^2 value for Langmuir fitting is also significant. And for BSA adsorbed on PHBV the R^2 values for the Langmuir and Freundlich isotherms are the same (0.99). The seemingly good fit of the Langmuir isotherm phenomenon is explained in detail in a paper by Latour and can be summarised as follows [4]. The shape of the protein adsorption isotherms frequently appears to fit the Langmuir isotherm without satisfying the conditions required for a Langmuir adsorption process. Conditions required for a Langmuir adsorption process are; the adsorption represents a dynamic reversible process where all the adsorption sites can be considered equivalent, independent and distinguishable from each other; one solute molecule is bound by one adsorption site; the adsorbed solutes don't interact with each to affect their adsorption behaviour. However, proteins can actually undergo conformational changes upon surface adsorption that can induce spreading, reorientation on the surface, clustering, and aggregation as a function of concentration [7], resulting in a Langmuir-shaped isotherm without satisfying the requirements of a Langmuir adsorption process [4]. Generally, for protein adsorption, the change in the surface adsorbed protein's conformation causes a difference in adsorption sites' size and energy at different solution concentrations, and protein aggregation can occur, which may cause multilayer adsorption. These phenomena violate the conditions necessary for assuming a Langmuir type adsorption [4]. As a result, the adsorption isotherm might resemble a Langmuir type isotherm. However, the points in the isotherm represent the saturation of the surface due to the irreversible adsorption with differing degrees of protein spreading, orientation, and packing arrangements instead of sub-monolayer equilibrium adsorption with a coverage building monolayer with the increasing solution concentration [4]. When the drastic changes of the surface adsorbed BSA's conformation on PHB and PHBV are considered in the light of the characteristics of protein adsorption described by Latour, it can be concluded that the Freundlich isotherm would be more suitable for describing the protein adsorption. The Freundlich isotherm, on the other hand, can be used to fit various

adsorption processes because it considers the interactions between the surface adsorbed proteins and the interactions between the adsorbed proteins and the surface. Although not being a good fit for BSA adsorption on PHB and PHBV, the Langmuir isotherm can still be used to get an estimate of the maximum amount of protein adsorbed on the surface at full surface saturation [4]. According to our results no correlation between the WCA and the amount of protein adsorption was observed. It is known that the amount of surface adsorbed proteins increases as the polar component of SFE decreases due to the electrostatic and hydrophobic interactions between the surface and the protein [24]. The lower polar SFE component of the PHB surface might be responsible for the higher amount of BSA adsorption compared to the PHBV surface but it should be noted that the difference between the polar components of PHB and PHBV are not drastic.

CONCLUSION

Protein adsorption is an important phenomenon affecting the performance of biomaterials in medical applications. To the best of the author's knowledge, this paper reports the adsorption behaviour of a protein and the changes in its secondary structure upon adsorption on the surface of PHB and PHBV for the first time. BSA adsorbed on PHB and PHBV surfaces undergo significant conformational changes favouring increasing amount of β-structure, and although yielding similar R^2 values, its adsorption behaviour can be better explained with the Freundlich isotherm rather than the Langmuir isotherm. Still, at full surface saturation, the Langmuir isotherm can be used to estimate the maximum amount of surface adsorbed protein.

Although this paper provides valuable information for the understanding and modulation of the protein adsorption behaviour on PHB and PHBV surfaces, the adsorption of different proteins including the human serum albumin, fibrinogen, fibronectin, and the changes on their secondary structures upon surface adsorption should also be analysed. For instance, BSA is a globular protein that is classified under the soft protein class. Soft proteins are known to easily change their conformation and structure upon adsorption. Fibrous proteins, on the other hand, are less sensitive to environmental changes and this relative increase in stability makes them less susceptible to undergo conformational changes upon adsorption [23]. In addition, other protein adsorption models such as random sequential adsorption should also be employed to elucidate the protein adsorption behaviour on these biopolymers. The implication of the different conformations of adsorbed protein on cellular response to PHB and PHBV should also be investigated.

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