

Evaluation of Protein Adsorption on Chitosan Surfaces with Reflectometry Interference Spectroscopy

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Abstract: Using a biomedical sensor setup RIfS we have investigated the kinetic behavior of human albumin (Alb), human fibrinogen (Fib), and human immunoglobulin G (IgG) adsorbed onto surfaces of chitosan. Polystyrene (PS) was used as the control material in this study. The optical thickness of three kinds of proteins measured by RIfS was related to their molecular dimensions and potential orientations on a film surface. According to the operation principle of RIfS and the molecular dimensions of three kinds of proteins, the adsorbed layers of proteins onto the surface of chitosan and PS was calculated by using a newly introduced equation. The microstructure of the chitosan and polystyrene film and the surfaces with adsorbed proteins were imaged by atomic force microscopy (AFM). With AFM analyses the lateral distribution of the protein molecules on surfaces have been recognized. The results show that the number of adsorbed layers of the three proteins on the surface of chitosan are 0.635 for Alb, 0.158 for Fib and 0.0967 for IgG, and of polystyrene are: 0.577 for IgG, 0.399 for Fib, 0.336 for Alb. This study confirmed that RIfS is a useful tool for the analysis of plasma proteins adsorbed on a surface of biomaterials. Results show that at first on the surface of chitosan film much more Alb than Fib was adsorbed which demonstrated that chitosan has a antithrombus function. Secondly, on the surface of chitosan film more Alb and less Fib were adsorbed than on the surface of PS film, which demonstrated that chitosan has a better blood compatibility than polystyrene. Thirdly, the calculated layer number of the three proteins indicated that on both chitosan and PS substrates monolayer coatings form.

Key words: Biomedical sensor, RIfS, chitosan, Protein adsorption, Biomaterials

1. Introduction

Optical interferometry is well known for the measurement of physical parameters such as speed and distance. Further, light interference was used for visual detection of antigen-antibody reactions [1]. Spectral interferometry has been considered as an interesting approach to detecting sensitively and rapidly any changes of optical path length in thin polymer films caused by antigen-antibody interaction in immunochemical sensing [2]. After the development of surface plasmon resonance (SPR) [3,4], another successful optical approach, Reflectometry Interference Spectroscopy (RIfS), was introduced as a highly sensitive and robust technique for direct label-free monitoring of the interaction of biomolecules [5,6,7].

RIfS is based on the spectral distribution of white-light reflectance from transparent thin layers. A distinct reflectance pattern with alternating maxima and minima results from the interference of beams partially reflected from each interface of the interference layer. Binding detection with RIfS shows little temperature dependence, allows avoidance of gold layers, and is promising for high throughput screening. The simple test format and high sensitivity makes RIfS attractive for detecting and characterizing the interaction of biomolecules. Biological material deposited at the surface during a binding event increases the optical thickness of the interference layer, leading to a shift in the interference pattern. This approach allows on-line monitoring of binding reactions with high resolution [8]. The main advantages of the RIfS transducer are its ruggedness, the small active area, and the simple construction [9].

A sensitive dynamic determination made on gaseous or liquid hydrocarbons as well as immunoreactions with RIfS was reported in 1993 [6]. Then, an investigation of the specific binding of low molecular weight ligands to immobilized receptors was carried out by using RIfS; two model systems were studied in which the binding event was successfully detected [10]. Next, RIfS was utilized for studying the hybridization of antisense with its complementary target sequences and especially to investigate the interaction of terminal- and backbone-modified antisense ONs targeted to multidrug resistance1b-mRNA [8]. Other applications with RIfS were in a dyeless optical detection of ammonia in the gas phase, by using pH-responsive polymers. Also, clinical detection of HbsAGb was accomplished [11, 12]. Recently, the application of RIfS for the evaluation of the biocompatibility of biomaterials has been described [13].

Since the protein adsorption on the surface of biomaterials is an important factor for the adhesion of platelet and biocompatibility, the characterization of adsorbed proteins on the surface of various biomaterials, such as biopolymers, alloys, ceramics etc., has been extensively investigated by means of different techniques [14-26].

Chitosan is a natural source polymer prepared by N-deacetylation of chitin, which is the main structural component of crab and shrimp shells [27]. Intensive investigations have been carried out for the structure, the chemistry, the biological properties, and the applications of chitosan [28].

Chitosan has been widely used as a new biomaterial since it is safe and has a good biocompatibility with the human body [29-35]. Chitosan also has a haemostatic function [36] and may have an antithrombogenic function. Some investigators reported the platelet adhesion on the surface of modified chitosan [37,38]. However, the protein adsorption occurs before the platelet adhesion and is the first step of thrombosis. The aim of this study is to evaluate the protein adsorption behavior of

human albumin (Alb), human fibrinogen (Fib), and human IgG on chitosan, by using RIfS. The kinetics of the adsorption process is investigated. Polystyrene is used as the control material, since its surface chemistry is well defined and it is frequently used as a model system in protein adsorption studies [39].

2. Materials and Methods

2.1 RIfS Apparatus

The RIfS setup used has been developed in our laboratory [40]. The main elements of the setup were a CCD spectrometer with a bifurcated optical fiber and an adsorption cell with a volume of about 30 μ L. The bifurcated optical fiber was used to illuminate the transducer chip and to collect the light reflected from the chip. The chip is a glass substrate on which a 20-nm Ta₂O₅ film was deposited in order to enhance the interference efficiency. An original software was used to control the collection of full spectra, to analyze the experimental data in real-time, and to draw the experimental curves on-line.

2.2 Preparation of Chitosan and PS films

Chitosan with a degree of deacetylation of 93.2% and of an average molecular weight of 750,000 Da was purchased from Ocean Institute of Jiangsu, China. The material is powder form with 80-100nm granularities and has a white color. The thin films of chitosan exposed to proteins were prepared by the spin-coating method. At first, the chitosan was immersed in 0.5% acetic acid (Factory of Chemical Reagents of Nanjing, China) with 10mg/mL. After the chitosan was dissolved completely, the solution was filtered with a middle speed qualitative filter paper (Factory of Fuyang Special Paper of Hangzhou, China). Then, 50- μ L chitosan solution was pipetted out and dropped onto the surface of the transducer chip (1.2cm*1.2cm). A chitosan film was spin-coated on the transducer chip using a spinning-machine at 1700 rpm. Typically, the film thickness was ~650 nm.

Polystyrene (Nunc Inc. Denmark) was dissolved in toluene (Factory of Chemical Reagents of Nanjing, China) at a concentration of 40mg/mL. The coating process of polystyrene film on the transducer chip is same with the chitosan film. All materials and reagents as well as chemicals were of analytical grade or better.

2.3. Proteins

Three kinds of proteins were used : human albumin (Alb), human fibrinogen (Fib), and human IgG (Sigma, Dorset, UK). For each measurement a protein solution was prepared freshly in 10mM phosphate buffer solution (PBS, pH=7.4) of a concentration of 0.1mg/mL. Deionized water was used to prepare the aqueous solutions.

2.4 Protein Adsorption Measurement

The protein adsorption measurement has been performed as follows: the sensor chip coated with chitosan/PS film was mounted into the adsorption cell carefully and fixed with two clips. Then, the

peristaltic pump was started to drive PBS (pH=7.4) flow into the adsorption cell. The PBS cleaned the surface of the chitosan/PS film, drove out air bubbles that may be present in the adsorption cell. Most importantly, it produced a physiological pH-environment for the adsorption of proteins. After this treatment, the protein solution flowed into the adsorption cell at a rate of 200 $\mu\text{L}/\text{min}$ and the kinetic adsorption process of proteins on the surface of the chitosan/PS film can be observed in situ on the monitor of the computer. Until the kinetic adsorption curve of proteins became stable, PBS was pumped into the adsorption cell again to wash the proteins which did not adsorb tightly onto the material surface. The washing would get a response on the adsorption curve. The mark on the Y axis of the Figs.1-4 give the thickness of the proteins adsorbed on the chitosan/PS film. For each kind of proteins, at least three tests were performed ($n=3$).

2.5 Calculation of the Adsorbed Layer of Protein

Since the molecular dimensions of various proteins are different, a normalized treatment of the experimental data was performed using a previously defined equation [13] in order to obtain the effective adsorbed layer number L_p (Eq.1):

$$L_p = \frac{\overline{x_p}}{D_p} \quad (1)$$

where D_p is the molecular dimension of a single protein and $\overline{x_p}$, the average thickness of each type of proteins on the surface of the material intended to be probed with RIFS.

2.6 AFM Imaging

The microstructure of the chitosan and polystyrene films and the surfaces with adsorbed proteins were imaged with a commercial atomic force microscope (AFM, Nanoscope IIIa, Digital Instrument, Santa Barbara, USA). The instrument was operated in Tapping Mode using silicon cantilevers oscillating with the average amplitude of 100 nm and a resonance frequency between 200 and 450 kHz. The scanning rate selected was 1 Hz. All images presented in this paper were obtained repeatedly and were stable under the experimental conditions.

3. Results and Discussion

3.1 Kinetics of Protein Adsorption onto PS Films

In Fig.1 the kinetic adsorption curves of human Alb, IgG and Fib onto the surface of a PS film are shown. The curves evidence that the adsorption behavior of the three proteins onto PS film are similar: initially, the processes of all three proteins increase quickly in the initial stage and reach their equilibrium values after about 500 s. Secondly, after washed with PBS the adsorption curves have different tendencies: the curve of IgG falls about 0.2nm, which means some loosely adsorbed molecules were removed from the PS film. Nevertheless, the curves of Alb and Fib show almost no

response, which shows the adsorption process of albumin and fibrinogen onto the surface of PS film have not been affected.

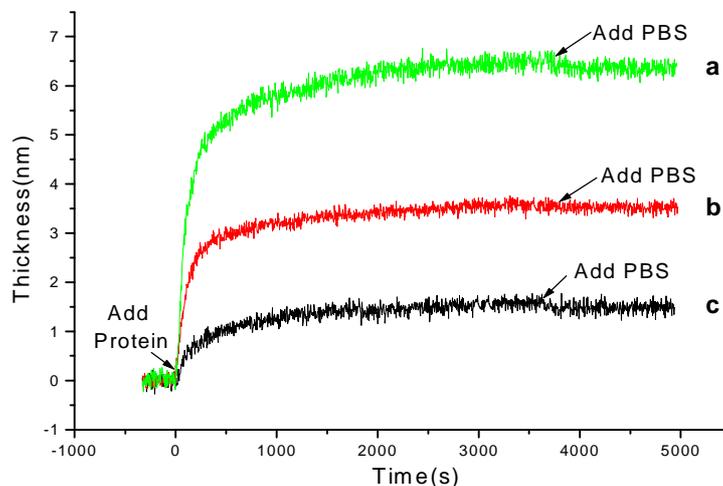


Figure 1. Kinetic curves of protein adsorption onto the surfaces of PS film: a) IgG, b) Fib, c) Alb.

This result can be derived from the difference of sizes and structures of three kinds of proteins. The adsorbed process of proteins onto the surface of biomaterials is pictured schematically in Fig. 2. According to Green et al. [39], albumin is a globular protein and the potential orientation on a surface is ca. 4 nm. Fibrinogen has a rod-like structure with approximately 10-nm width and the molecules may lie planar on the biomaterials interface. IgG has a relatively globular Y shaped structure with a height of ~12 nm. Therefore, the different molecular dimensions of the three proteins are responsible for the difference of the thickness. The smallest height of Alb molecules, for example, yields the thinnest film. The intermediate molecular dimension of Fib results in an intermediate optical thickness between Alb and IgG. The random orientation of IgG molecules adsorbed on a PS film results in a thickest adsorptive layer as measured by RIfS. The curve family is similar to that reported by Green et al. for PS material by an analysis using SPR [39] and by us for hydroxyapatite using RIfS.

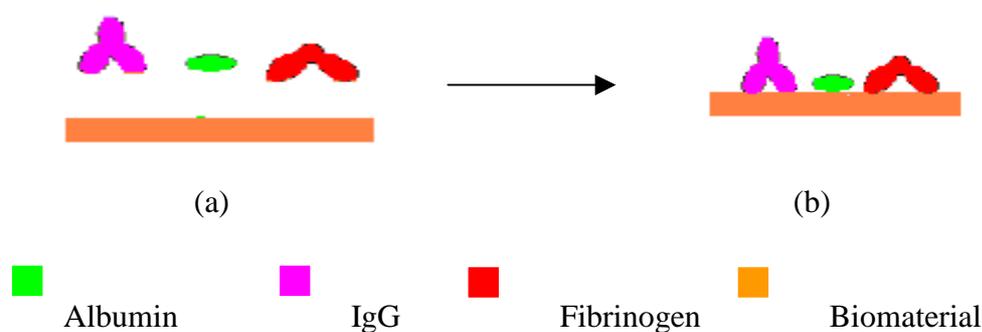


Figure 2. Sketch map of adsorbed process of proteins onto the surface of biomaterials: (a) before and (b) after adsorption.

3.2 Kinetics of Protein Adsorption onto Chitosan Films

The kinetic adsorption curves of human Alb, Fib, and IgG on the surface of a chitosan film are shown in Fig. 3–5. According to these curves the adsorption behavior of three kinds of proteins onto chitosan is dissimilar. For Alb, the adsorption processes amplifies slowly in the initial stage and reaches its stable values after about 8000 s. This means that during this period the adsorption of albumin on chitosan continued. Nevertheless, for Fib and IgG the adsorption processes is fast in the initial stage and reaches its stable values after about 1500 s, which means that the adsorption process of Fib and IgG on chitosan films reaches saturation fast. After washed with PBS, the curves of Alb, Fib, and IgG fall apparently, which indicates more loosely adsorbed molecules were removed from the chitosan film.

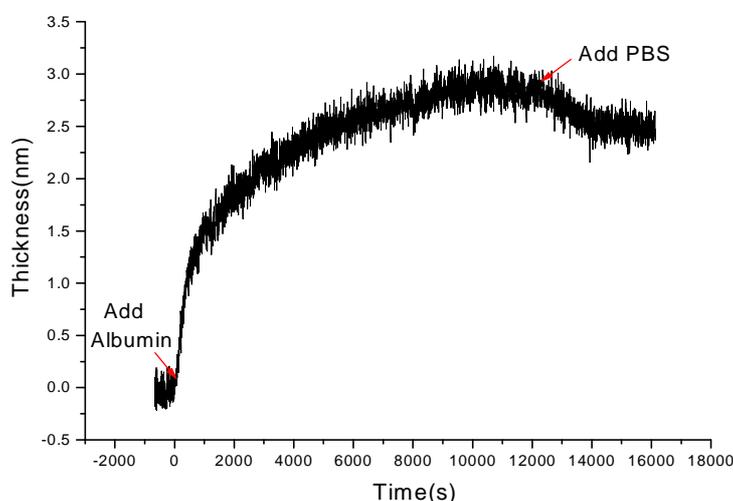


Figure 3. Kinetic curves of adsorbed Alb on the surface of chitosan film.

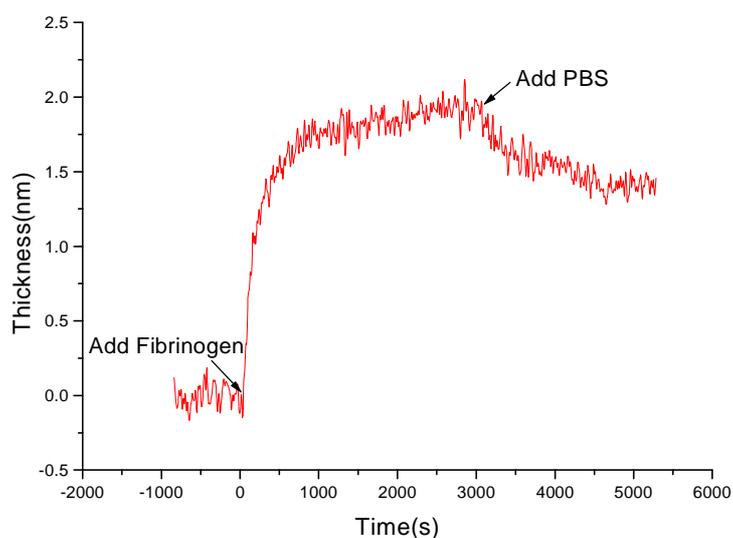


Figure 4. Kinetic curves of adsorbed Fib on the surface of chitosan film.

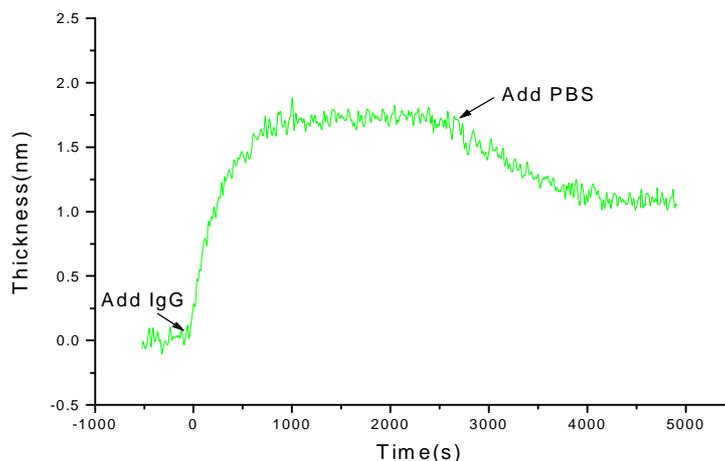


Figure 5. Kinetic curves of adsorbed IgG on the surfaces of chitosan film.

3.3 Comparing of the Kinetic Curves on Chitosan and PS

Fig. 6-8 compare the adsorption of the three kinds of proteins onto the surface of chitosan and PS films. The curves reveal that on the surface of chitosan film more Alb was adsorbed than on the surface of PS film, and conversely, on the surface of PS film more Fib and IgG were adsorbed than on the surface of chitosan films.

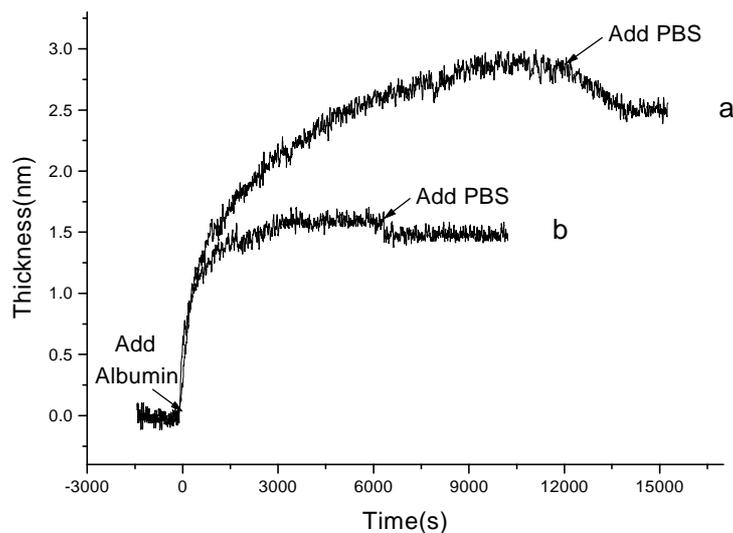


Figure 6. Adsorbed Alb on the surfaces of chitosan and PS: a) chitosan, b) PS.

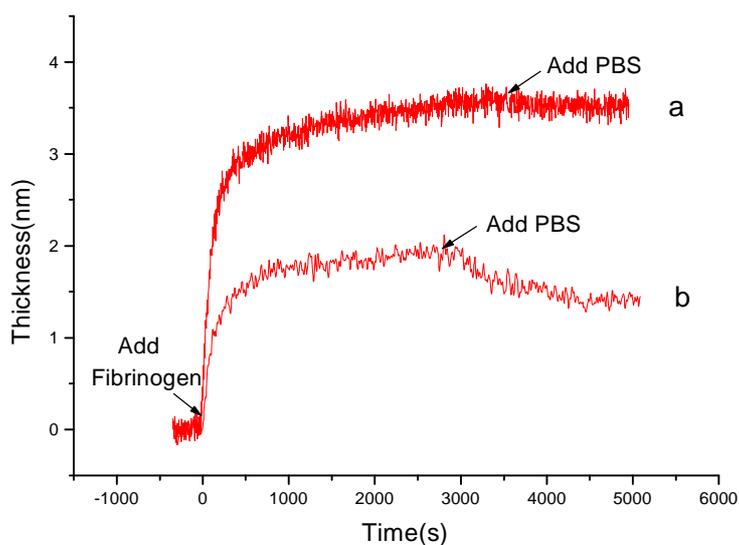


Figure 7. Adsorbed Fib on the surfaces of chitosan and PS: a) PS, b) chitosan.

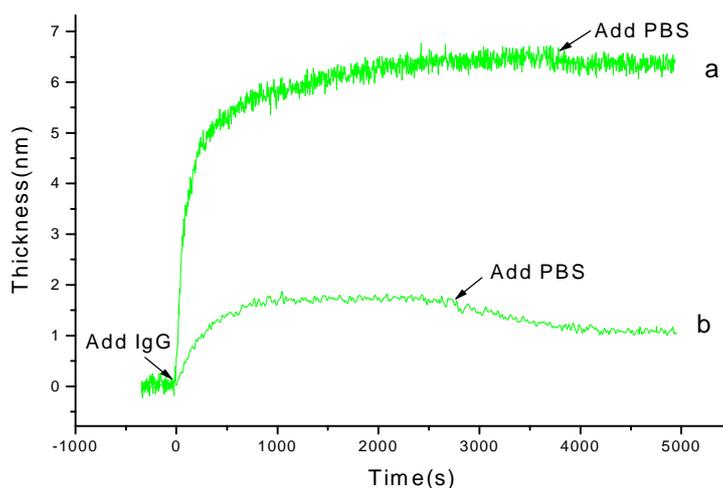


Figure 8. Adsorbed IgG on the surfaces of chitosan and PS: a) PS, b) chitosan.

3.4 Adsorbed Layers of Alb, Fib, and IgG on Chitosan and PS Films

As discussed above, each adsorption curve indicates the thickness of one particular protein adsorbed onto the surface of one kind of material. Thus, the adsorptive thickness of one kind of proteins on films of different materials can be compared directly according to the curve family as in Figs. 6-8. But with the RIfS thickness a comparison of the adsorbed amount of different proteins on the same materials cannot be carried out directly. Since the structure and size of three proteins are very different. After a normalized treatment of the experimental data using the equation, the thickness of the adsorbed protein layers was calculated, which indicates the effectively adsorbed amount of different proteins on one particular material.

RIfS thickness of three kinds of proteins on the PS surface decreases in the order (Tab.1): IgG (6.35nm) > Fib (3.59nm) > Alb (1.34nm). The adsorbed layer number, calculated using the equation, PS has same series: IgG (0.577) > Fib (0.399) > Alb. (0.336).

The thickness determined by RIfS for the three kinds of proteins on the chitosan surface decreases in the order (Tab.2): Alb (2.54nm) > Fib (1.42nm) > IgG (1.06nm). By this order a special phenomena for albumin adsorbed onto chitosan film is recognized. Despite the smallest structure the adsorbed RIfS thickness of Alb is greatest among the three kinds of proteins. This result is very different from the results of PS and of another studies [13, 39]. It indicates that chitosan adsorbed much more Alb than Fib and IgG. In this case the adsorbed layer number of three kinds of proteins on chitosan decreases in the same order: Alb (0.635) > Fib (0.158) > IgG (0.0967).

The calculated layer numbers of three kinds of proteins show that the total amount of each kind of protein adsorbed on chitosan and PS occurs as monolayer adsorption.

According to previous studies, albumin has a thrombus-resistant ability, whereas fibrinogen promotes platelet adhesion on a polymer surface [41]. The calculated number of adsorbed layers of the three proteins on chitosan and PS evidences that the chitosan material shows a higher affinity to Alb and lower affinity to Fib than PS. This means that chitosan has a better antithrombus function relative to PS films.

Table 1. RIfS thickness and adsorbed layer number of three kinds of proteins on the PS film.

Thickness Proteins	\bar{x} (n=3)	SD	Adsorbed Layer
Alb	1.34	0.0868	0.336
Fib	3.59	0.0586	0.399
IgG	6.35	0.0430	0.577

Table 2. RIfS thickness and adsorbed layer number of three kinds of proteins on the chitosan film.

Thickness Proteins	\bar{x} (n=3)	SD	Adsorbed Layer
Alb	2.54	0.0193	0.635
Fib	1.42	0.0220	0.158
IgG.	1.06	0.0505	0.0967

3.5 AFM characteristic

AFM analysis is useful for probing the lateral distribution of the protein molecules with the monolayer and confirmed indirectly the presence of the passively adsorbed protein on the surfaces of biomaterials. The AFM image in Figs. 9(a) and figure10(a) are the microstructure of the chitosan and PS surface, while in Figs. 9(b) and 10(b) the same surfaces with adsorbed protein albumin.

From the AFM image in Fig. 9(a), the pattern in the surface of chitosan material can be revealed. The AFM images of albumin coated chitosan surface in figure9(b) reveal that the most area of the surface of chitosan film was covered with the proteins.

The AFM image of PS film in Fig. 10(a) termed as ultra-flat film [42]. The AFM image in Fig. 10(b) reveal that there is fewer albumin on PS film than on chitosan film in figure 9(b).

AFM analyses are consistent with the calculated adsorbed layer in Tab. 1. and Tab. 2.

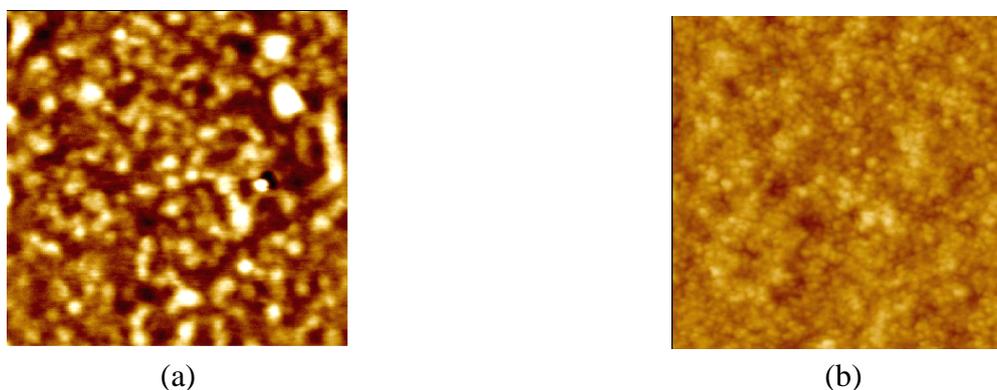


Figure 9. AFM image of microstructure of a chitosan surface (a) and with adsorbed albumin (b).

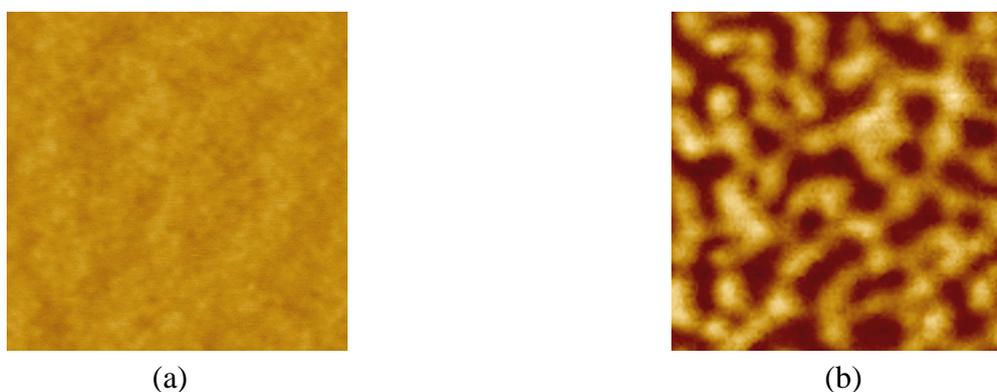


Figure 10. AFM image of microstructure of a PS surface (a) and with adsorbed albumin (b).

4. Conclusions

This study confirmed that RIfS is a useful tool for analysis of plasma proteins adsorbed on a surface of biomaterials. The number of adsorbed layers of three kinds of proteins on the surface of chitosan has a series: Alb > Fib > IgG and PS has a series: IgG > Fib > Alb. Results reported in this paper show that at first on the surface of chitosan film more Alb and fewer Fib adsorbs, which demonstrates that chitosan has a antithrombus function and secondly, on the surface of chitosan film more Alb and less Fib than on the surface of PS film, which demonstrated that chitosan has a better blood compatibility than polystyrene. And thirdly, according to the calculated layer number of the three proteins all the proteins on chitosan and on PS materials represents a monolayer adsorption.

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Sample Availability: Available from the author.