

PRELIMINARY STUDY OF EXTRACTABLE PROTEIN BINDING USING MALEIC ANHYDRIDE COPOLYMER

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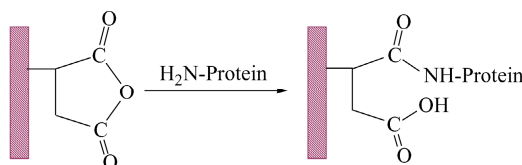
Abstract A preliminary study of using maleic anhydride copolymer for protein binding has been carried out. The polymeric films were prepared by compression of the purified resin and annealing the film to induce efficient back formation of the anhydride groups. The properties of the film surface were analyzed by attenuated total reflection Fourier transforms infrared spectroscopy and water contact angle measurements. The protein content was determined by Bradford assay. To obtain optimum conditions, immersion time for protein binding was examined. Results revealed that proteins can be successfully immobilized onto the film surface *via* covalent linkage. The efficiency of the covalent binding of the extractable protein to maleic anhydride-polyethylene film was estimated at 69.87 $\mu\text{g}/\text{cm}^2$, although the film had low anhydride content (3%) on the surface.

Keywords: Maleic anhydride copolymer; Extractable protein; Protein-binding; Skim rubber.

INTRODUCTION

Natural rubber (NR) has been available commercially for more than a century. Its latex-dipped products, particularly gloves, have been used preferentially as effective barriers to transmitted diseases. However, proteins retained in the latex products are reported to cause serious allergy for some people. Several methods for allergenic protein reducibility include the use of low protein latex, leaching during processing, enzyme treatment, chlorination of the finished product, addition of fumed silica and irradiation method. These methods are difficult to process and need a long incubation time for processing. New technique of protein removal from natural rubber should be developed. It is a simple and rapid technique by immersion of functionalized support into natural rubber latex under optimum conditions. The proteins can be immobilized on support surface *via* covalent linkage.

Maleic anhydride copolymers have already been applied for covalent protein immobilization. They allow the direct coupling of proteins by adsorption due to their highly negatively charged groups. Moreover, the anhydride group is very reactive to primary amine group and slightly less reactive to alcohol. Studies on protein removal have shown that amino groups of proteins spontaneously react in neutral and mildly alkaline media with the anhydride groups to form amide bonds, as shown in Scheme 1.



Scheme 1 Coupling reaction between maleic anhydride and protein

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In the case of commercial copolymers, grafting of maleic anhydride onto olefin polymers was performed through reactive processing. It was found that purification was necessary for the removal of unreacted maleic anhydride in polymer mass^[1-7]. Several purification methods have been proposed such as dissolution of sample in xylene followed by precipitation in acetone^[1-3], soxhlet extraction with acetone^[4-6] and supercritical carbon dioxide extraction^[7]. In general, the hydrolysis of anhydride group could occur in the producing process. This led to the appearance of carboxylic acid on the graft copolymer film. Thermal annealing for sufficient time is necessary to lead to a back-formation of the anhydride^[8, 9].

Bovine serum albumin (BSA) has been generally used as a model protein to investigate behaviors of protein binding on functionalized supports. The effect of immersion time on the amount of immobilized protein on the substrate has appeared in many reports^[10-14]. Furthermore, extractable protein in skim rubber was prepared under the extraction conditions (such as solution type, number of extractions and sample size) determined in our previous work^[15].

The application of Fourier transform infrared spectroscopy in the attenuated total reflectance mode (ATR-FTIR) to characterize surface functionality and investigate protein immobilization has been presented in a number of publications. Some examples have been illustrated here. The ATR technique has been used to study clean and BSA fouled polysulphone ultrafiltration membranes by Pihlajamäki *et al.*^[16]. Belfer *et al.*^[17] used ATR spectroscopy to detect chemical changes during modification and BSA adsorption on polyethersulfone membranes. Ma *et al.*^[18] have successfully applied ATR-FTIR to study covalent immobilization of gelatin and collagen on the surface of poly-L-lactic acid films. Surface wettability of the modified films and the protein immobilized films was evaluated using contact angle measurements.

The aim of this research was to investigate the feasibility of using maleic anhydride copolymer to bind extractable protein from natural rubber (NR). The functionalized film was prepared under optimal conditions and the protein immobilization was determined.

EXPERIMENTAL

Materials

Polyethylene grafted with maleic anhydride (Fusabond® E MB226D) was supplied from Chemical Innovation Co., Ltd. All chemicals and solvents used were of analytical grade. Acetone was purchased from Labscan. Phosphate buffered saline (PBS) was purchased from Merck. Sodium dodecyl sulfate (SDS) and xylene were purchased from BDH. Bovine serum albumin (BSA) as a model protein and Bradford reagent were purchased from Sigma.

Preparation and Characterization of Maleic Anhydride-Polyethylene Films

A commercial maleic anhydride grafted polyethylene (PE-g-MAH) with 0.90 wt% MAH was used for the film preparation. About 3 g of polymer resin was purified by dissolving in 300 mL xylene under reflux at 130°C for 1 h. Then, the temperature was lowered to 45°C with continuous stirring. The precipitate was vacuum filtered, washed several times with acetone and dry in an oven. The efficiency of purifying of the samples was examined by Fourier transform infrared spectroscopy (Equinox 55, Brüker Corporation).

The purified sample was prepared into a sheet by using a hydraulic hot press at 15 ton and under constant temperature of 140°C for 5 min. Then, the film was annealed in an oven to obtain high content of anhydride groups on the surface. The surface wettability of sample was evaluated using contact angle measurements. Static distilled water contact angle was measured on the film with a Krüss Goniometer using the sessile drop technique. The contact angles reported in this paper were the average of at least 7 measurements, placed at different positions on the specimen. Fourier transform infrared spectroscopy in the attenuated total reflectance mode (ATR-FTIR) was used to determine the chemical changes of the surface. Using a single reflection Ge ATR crystal at a 45° incident angle, the estimated analyzing depth was approximately 1 µm.

Quantification of maleic anhydride in samples was tested by FTIR. The wavenumbers of interest were 1787 cm⁻¹, characteristic of carbonyls from anhydride groups and 1460 cm⁻¹, characteristic of CH₂ bending of

PE. Using these bands, the anhydride carbonyl index (ACI) was calculated:

$$ACI = \frac{A_{1787}}{A_{1460}}$$

where A_{1787} is absorbance at 1787 cm^{-1} and A_{1460} is absorbance at 1460 cm^{-1} .

Extraction of Protein from Skim Rubber

About 5 g of small NR pieces was immersed in 2% SDS solution and then shaken at room temperature for 12 h in 3 times. The extract solution was then immediately subjected to protein precipitation. 1 mL of the filtrate was treated with 3 mL of 2-mercaptoethanol (0.07 vol%) and trichloroacetic acid (TCA, 100 g/L) in cooled acetone. The mixture was allowed to stand at -20°C for 24 h. The resulting precipitated proteins were collected by centrifugation of speed 10000 r/min at 4°C for 10 min, and were rinsed with acetone to remove acid. Finally, the extractable proteins (EP) were redissolved in lysis buffer solution and stored at -20°C for further experiment. The complete details of the protein extraction and characterization are described in our previous work^[15].

Protein Binding Studies on PE-g-MAH Films

Extractable proteins of 1 mg/mL were used to study the protein binding on the film. Circular samples having a diameter of 0.6 cm were immersed in 0.1 mol/L PBS at pH 7 for 2 h before binding. The films were then placed into microtube to which protein solution was added. The mixture was incubated at room temperature for defined time on shaker. After that, the films were removed from the protein solution and washed with PBS and SDS solutions, respectively. Bradford Micro-Assay^[19] was utilized to determine the protein content by measuring the absorbance at 592 nm.

The amount of immobilized protein on the film was calculated from the protein content in solution before and after binding. The binding capacity was defined as the amount of immobilized protein on the film surface in $\mu\text{g protein/cm}^2$ surface area. The reported value is based on the average value of 5–10 samples.

RESULTS AND DISCUSSION

Preliminary Study on Using PE-g-MAH Resin to Bind Protein

Prior to the main study, the ability of maleic anhydride group to bind protein was investigated. Milk was selected as a sample solution because it has high protein content, especially casein proteins and lower interference than other materials. The protein binding was studied in milk by using PE-g-MAH granule as a solid support.

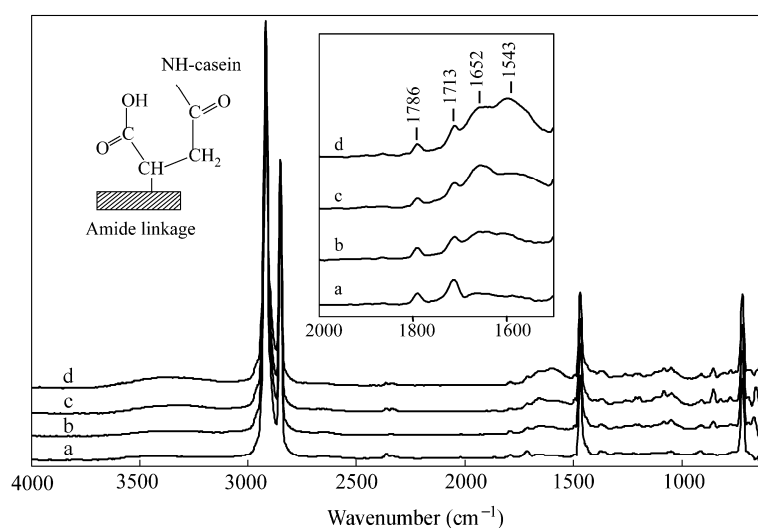


Fig. 1 ATR-FTIR spectra of resin before (a) and after binding protein in milk for (b) 5 h, (c) 7 h and (d) 9 h

The covalent coupling of protein molecules to PE-g-MAH resin involves mild reaction. The reaction mixture was stirred in varied times from 5 h to 9 h at a temperature of about 10°C to maintain a stability of protein and its coupling reaction mostly occurred on the surface. After binding, the ATR-FTIR technique was used to analyze changes of the chemical structure on the surface.

ATR spectra in Fig. 1 present the evidence of the protein immobilization on the polymeric substrate. Two characteristic peaks, 1652 cm^{-1} (amide I) and 1543 cm^{-1} (amide II), are inspected as proof of the presence of protein on the surface. The amide I band originates predominantly from the C=O stretching vibration of the peptide groups. Furthermore, it was found that the ability of protein binding increased with increasing time to 9 h of binding reaction. From the obtained result, it is possible that the protein could be immobilized onto PE-g-MAH under mild condition.

Preparation and Characterization of Copolymer Films

Bettini and Agnelli^[1] reported that the purification methodology was efficacious in the removal of unreacted maleic anhydride. Several techniques, such as infrared spectroscopy (FTIR) and thermogravimetric analysis, were used to check the conversion of the acid groups to anhydrides. In this work, the commercial PE-g-MAH granules were purified by dissolving in xylene and precipitating in acetone. The chemical structure of purified sample in powder form was examined by ATR-FTIR. As seen in Fig. 2, the amount of carboxylic group (1712 cm^{-1}) and impurity substance (600–1350 cm^{-1}) decreased significantly due to removal of moisture and unreactant. However, the purified material also contained the hydrolyzed anhydride groups. The anhydride content was calculated from IR data. Unlike the earlier result^[1], the difference in carbonyl index was not significant for the samples with and without purification (Fig. 3). This may be explained by the fact that, the samples used in our work have a very low degree of maleic anhydride.

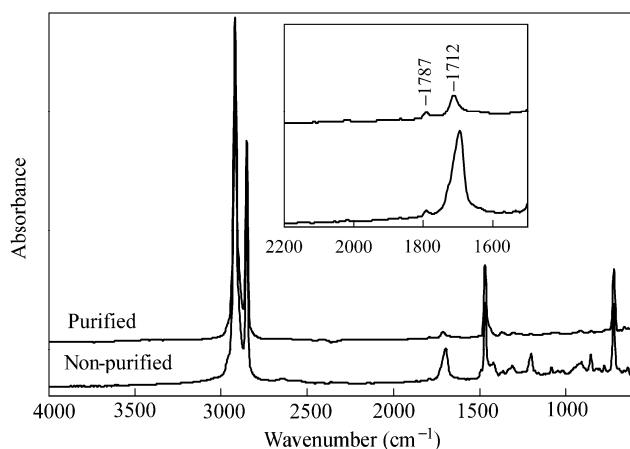


Fig. 2 ATR-FTIR spectra of non-purified and purified PE-g-MAH samples

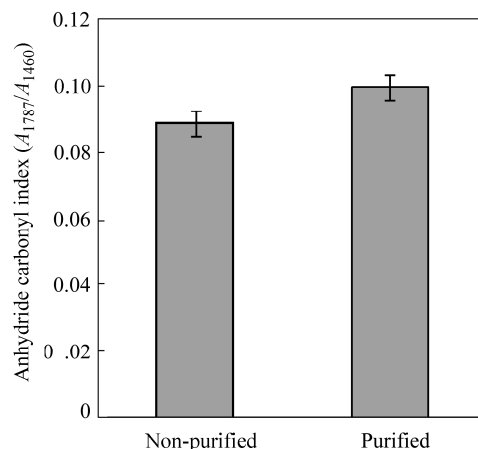


Fig. 3 Effect of purification on the anhydride carbonyl index of purified and non-purified samples

It was known that the re-formation of maleic anhydride groups can be affected by simply heating a sample containing maleic acid groups. In general, conditions of annealing near the melting point of sample lead to an increase in polymer-chain mobility and therefore to changes in the surface properties of films. Some polymer chains embedded in the film may unfold to the surface layers of the film. In this work, the melting temperature (T_m) of prepared sample was measured by using differential scanning calorimetry (DSC). The results indicate that the T_m of PE-g-MAH is about 120°C. Schmidt and his group^[9] found that the annealing at 120°C for 3 h of the modified maleic anhydride copolymer films induced efficient back-formation of the anhydride groups. It was also reported that the annealing at 120°C in a dry atmosphere was used to convert the acid groups to anhydrides in various polymers such as poly(ethylene-*alt*-maleic anhydride)^[20] and poly(octadecene-*alt*-maleic

anhydride)^[21,22]. In addition, vacuum annealing can also be used to generate the anhydride groups. The heat treatment in vacuum oven at a temperature of about 100°C has been suggested by Rim^[8]. Cyclic anhydride could be formed by vacuum annealing maleic anhydride-grafted polypropylene for various times from 24 h to 96 h at 130°C^[1]. Alberti and coworkers^[23] annealed poly(octadecene-*alt*-maleic anhydride) film for 18 h at 90°C under vacuum. However, it was found that the styrene-maleic anhydride copolymer started to degrade above 100°C^[24]. The dehydration of maleic acid is the main reaction of decomposition.

Therefore, to generate anhydride groups and avoid product degradation, we annealed the PE-g-MAH film at high temperatures for short time and low temperatures for long time in a common oven. Two annealing conditions, high temperature at 120°C for 3 h and low temperature at 110°C for 24 h were chosen in this work for determining the best condition to obtain high MAH content in the material. It was expected that this simple method could give enough MAH content to be used for further experiments.

When the samples were submitted to heat treatment, the intensity of carbonyl bands located at 1712 and 1787 cm^{-1} changed significantly (Fig. 4). This observation might be justified by the conversion of acid groups to anhydrides. The result for high temperature heating (120°C) showed the increase in the cyclic anhydride carbonyl band at 1787 cm^{-1} and the reduction of the carboxylic acid carbonyl band at 1712 cm^{-1} . Furthermore, as can be seen in Table 1, the anhydride carbonyl index maximized when the annealing was carried out at 120°C for 3 h. Therefore, it was concluded that the treatment at this condition led to high content of anhydride group in the polymer bulk.

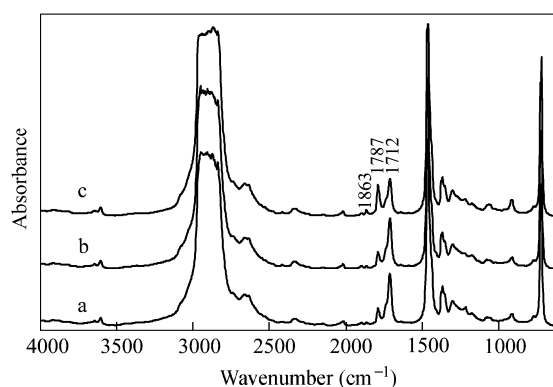


Fig. 4 Transmission FTIR spectra of PE-g-MAH film (a) before and after annealing at (b) 110°C, 24 h and (c) 120°C, 3 h

Table 1. Effect of annealing conditions on bulk and surface properties of PE-g-MAH films

PE-g-MAH sample	Anhydride carbonyl index (A_{1787}/A_{1460})	Contact angle ($^{\circ}$)
Non-annealed	0.170	83 ± 2
Annealed (110°C, 24 h)	0.412	96 ± 2
Annealed (120°C, 3 h)	0.501	101 ± 2

In order to examine the surface properties of the specimen, PE-g-MAH films before and after annealing were characterized by the measurement of water contact angle (WCA). Table 1 shows that the contact angle of non-annealed film is lower than that of annealed films. This indicated that the annealing of PE-g-MAH film increased the hydrophobicity of film surface. Changes of air-water contact angles for the annealed films were due to the increasing of anhydride groups on the film surface. In addition, the annealing at temperature 120°C for 3 h gave higher contact angle than the annealing at temperature 110°C for 24 h.

From all results, it can be concluded that, for the system under investigation, the commercial PE-g-MAH granules were first purified by dissolving in xylene and precipitating in acetone. Then, the film of polyethylene-maleic anhydride copolymer was prepared by compression at 140°C for 5 min. Finally, the sample was annealed at 120°C for 3 h in order to obtain high content of anhydride groups on the surface.

Protein Binding Studies on PE-g-MAH Film

Determination of optimum immersion time

It was reported that the amount of immobilized protein on the substrate is related with the change in immersion time for protein binding^[10–14]. To determine the suitable time for protein immobilization, the commercial PE-g-MAH film having a size of approximately 2 cm × 2 cm was used for BSA binding. BSA is selected for this experiment because it is readily available. In addition, it was reported that the chemical composition of BSA was similar to that of the extractable protein^[15]. The impurities, particularly non-protein components, were found in minor quantities in extractable protein from skim rubber. The reaction was kept at room temperature and the immersion time was varied from 2 h to 8 h. Figure 5 shows the kinetics of protein binding on the sample.

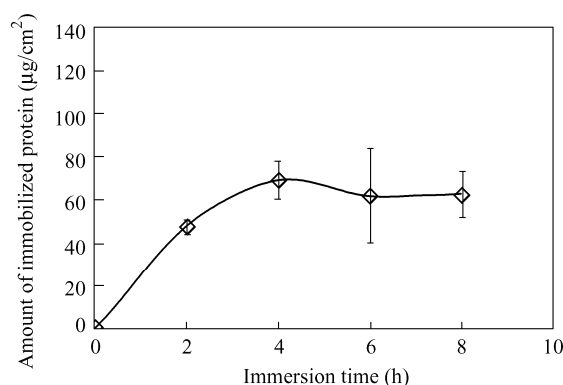


Fig. 5 Effect of binding time on the BSA immobilization

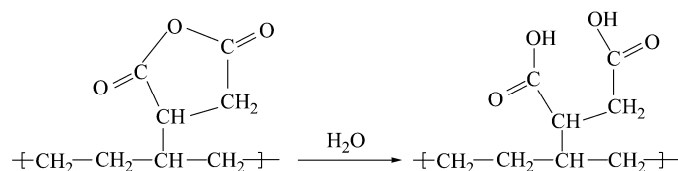
It was found that as the immersion time increased, the amount of immobilized protein increased in the first 4 h of protein binding. After this time, the protein immobilization on polymeric surfaces is completed. This may be because the binding reaction is controlled by a steric effect. When a longer immersion time was used, the residue amino groups of protein are difficultly immobilized onto the film. So, the optimum immersion time at 4 h was selected for protein binding study. At this time, the immobilized BSA on commercial PE-g-MAH was 72.34 µg/cm². A similar result was found earlier for BSA-coupling on ethylene vinyl alcohol copolymer (EVAL) membranes activated by CO₂-plasma treatment^[12]. The amount of protein bound onto the EVAL membranes was presented as a function of the reaction time. The curve of binding time shows high-protein binding rate at the initial time and constant rate in the next time, in which the amount of immobilized BSA is completed after 8 h. Furthermore, Can and Güner^[13] explained that the constant binding rate at the longer time may be because of the decrease in BSA concentration in the container with time due to protein binding.

Protein-binding capacity of PE-g-MAH

Many studies have been reported on the effect of reactive group content on the amount of immobilized protein^[25, 26]. Therefore, maleic anhydride concentration on the sample surface was determined by ATR-FTIR measurements. In this work, the anhydride content on sample surface could be quantitatively analyzed from the intensity ratio of C=O stretching of anhydride group (1787 cm⁻¹) to CH₂ bending of PE (1460 cm⁻¹) multiplied by 100. It was found that the commercial PE-g-MAH film had low anhydride content (3%) on the surface.

The immobilization reaction onto the PE-g-MAH occurs by nucleophilic attack of the reactive anhydride moieties by the primary amine groups of the proteins. Upon nucleophilic attack, the anhydride yields one carboxylic acid for every acylated product. In this study, the hydrolysis of anhydride with water molecule under aqueous condition, as shown in Scheme 2, was examined (Experimental detail is not shown here). It was found that the side reaction from hydrolysis by water rarely occurred under this mild condition of protein-binding process. Therefore, the majority of carboxylic acid content on protein-immobilized film was obtained from protein coupling reaction. The contact angle of annealed film decreased from 101° to 63° after BSA-binding.

The reduction of contact angle was believed to be the result of formation of carboxylic acid groups on the film surface.



Scheme 2 Hydrolysis of PE-g-MAH surface

For analysis of protein-binding, the volume of protein solution may influence the protein content, *e.g.*, higher absorbance was observed with increasing sample volume^[27]. In this work, to avoid this possible effect in protein-binding analysis, two volumes of sample solution, 10 and 15 μL , were used in all experiments. However, the amount of immobilized protein did not vary significantly with sample volume (Table 2). This indicates that the protein-binding efficiency was not greatly influenced by volume changes within 10–15 μL range in our samples. The immobilized EP on commercial PE-g-MAH was $69.87 \mu\text{g}/\text{cm}^2$.

Table 2. Amount of immobilized EP on annealed PE-g-MAH film for different sample volumes

Volume of protein solution for analysis (μL)	Amount of immobilized protein ($\mu\text{g}/\text{cm}^2$)
10	66.68 ± 10.10
15	72.41 ± 6.66
Average	69.87 ± 7.90

Protein concentration = 1.0 mg/mL

Immobilization of proteins can principally occur through (i) electrostatic and/or hydrophobic attraction, (ii) entrapment or encapsulation and (iii) covalent binding to polymeric supports by reaction of the functional groups with the protein. A number of studies have been performed on the adsorption and immobilization of bovine serum albumin (BSA) on a variety of different substrates. Ladaviere *et al.*^[28] studied the immobilization of BSA onto maleic anhydride-alt-methyl vinyl ether copolymer under aqueous conditions. They established that the main factors ruling the coupling reaction were the presence of electrostatic forces attracting the macromolecules close enough for chemical reaction. Tangpasuthadol *et al.*^[29] found that BSA, a carboxylic acid-rich protein, was significantly less adsorbed onto the carboxylic acid-rich chitosan surface possibly due to charge-charge repulsion. Can and Güner^[13] proved that BSA was adsorbed on crosslinked *N,N'*-diethylaminoethyl dextran by electrostatic attraction, ion-exchange, hydrophobic interaction, and/or hydrogen bonding. Dai *et al.*^[30] immobilized BSA proteins covalently to poly(acrylic acid) surfaces *via N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide activation and amide formation. Shmanai and Bylina^[31] used formylated polystyrene beads as a solid phase support for immunoassay and presented BSA immobilization through covalent coupling. Yang *et al.*^[32] found that BSA was covalently immobilized onto multifunctional epoxy groups containing magnetic spheres. Avramescu *et al.*^[12] reported the covalent binding of BSA onto cellular-type poly(ethylene vinyl alcohol) microfiltration membranes. From these studies, it is clear that the type of immobilization mainly depends upon reactive functional groups on the surface of support.

After BSA-coupling, the PE-g-MAH films were washed with PBS to remove the unreacted protein from their surfaces. Then, the protein that was not covalently immobilized but physically adsorbed on the film surface was desorbed by washing with SDS. Therefore, the electrostatic and hydrophobic forces in the binding process of BSA were eliminated. The immobilized protein onto PE-g-MAH film was investigated by ATR-FTIR. Figure 6 shows that the intensity of anhydride peak (1787 cm^{-1}) decreased after protein binding experiments. Furthermore, the appearance of amide bands at 1543 and 1652 cm^{-1} confirms the protein binding of the prepared

film *via* covalent bond. The similar result was found in the albumin adsorption of modified polyethersulfone ultrafiltration membranes^[17].

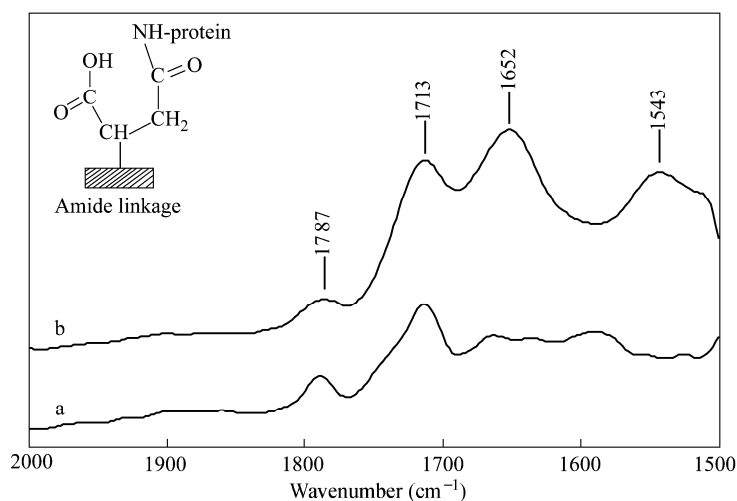


Fig. 6 ATR-FTIR spectra of PE-g-MAH film (a) before and (b) after binding extractable protein

Table 3. Comparison of maximum binding capacity of BSA protein onto various supports

Materials	Maximum BSA-binding capacity ($\mu\text{g}/\text{cm}^2$)	References
Maleic anhydride-grafted polyethylene film	72.34	In this work
<i>N</i> -stearylchitosan film	4.86	[29]
Derivatives of poly(acrylic acid) brushes	5.80	[30]
Formylated polystyrene bead	49.0	[31]
Glutaraldehyde-activated poly(ethylene vinyl alcohol) membrane	80.0	[12]

There is a wide variety of substrates with a wide range of immobilization capacities reported in the literature for BSA immobilization and some of them are listed in Table 3. The maximum BSA-binding capacity that we achieved with the PE-g-MAH film in this study was $72.34 \mu\text{g}/\text{cm}^2$. This result was quite comparable with those previously reported. It should be noted, however, that the variation in conditions for protein binding (such as ionic strength, pH and initial protein concentration) has substantially influenced the amount of immobilized protein. For example, the BSA-binding capacity of PE-g-MAH film is significantly higher than that of formylated polystyrene bead. The used conditions for PE-g-MAH (1 mg/mL BSA, pH 7.0) were different from those for formylated PS (0.1 mg/mL BSA, pH 4.5). Using the same binding conditions, it is shown that the amount of protein immobilization for PE-g-MAH film is similar to that observed for glutaraldehyde-activated poly(ethylene vinyl alcohol) membrane.

CONCLUSIONS

Our results demonstrate the possibility of using commercial maleic anhydride-polyethylene copolymer for binding the proteins in natural rubber. Purification of the commercial resin and annealing of the film at 120°C for 3 h are proven to be successful conditions to prepare the sample. The efficiency of the covalent binding of the extractable protein to PE-g-MAH was estimated to be at $69.87 \mu\text{g}/\text{cm}^2$, although the film had low anhydride content (3%) on the surface. The modification method on the film surface will be developed in further studies to increase the protein binding ability under optimized conditions.

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