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Applying electron paramagnetic resonance spectroscopy to the study of fouling in protein ultrafiltration

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Abstract

Electron paramagnetic resonance (EPR) spectroscopy has been investigated as a method for exploring the transient development of fouling in single protein ultrafiltration and as a potential method to determine protein–membrane interaction. Preliminary research showed that EPR was an ideal method for analyzing short-time solute uptake in ultrafiltration. Studies were performed using spin-labeled BSA (65,000 Da) in solutions of pH 5 or 7, and a total salt concentration of 0.05 or 0.15 M. Polysulfone membranes of 100,000 MWCO were used. The results showed that a substantial amount of protein uptake by the membrane occurred within the first few minutes of ultrafiltration. This implies that pore plugging is a significant step in the overall irreversible adsorption of proteins on and within membranes. Double spin-labeled lysozyme (15,000 Da) was ultrafiltered with 10,000 and 30,000 MWCO polysulfone membranes and the EPR spectra were analyzed. The results showed that the proteins on the membrane surface may have a different conformation than the proteins trapped and lodged in the membrane pores. Preliminary analysis indicates that the proteins within the pores may be more compressed than those on the surface. The resulting spectra did not represent conformations observed for highly constricted proteins or denatured proteins.

Keywords: Fouling; Ultrafiltration; Electron paramagnetic resonance spectroscopy; Protein separation

1. Introduction

Membrane fouling is a significant problem in ultrafiltration, particularly for protein separation. Fouling can severely reduce solvent and solute fluxes from their initial values in a relatively short time. A significant amount of research has been conducted to better understand the dynamics of the fouling process so that preventative methods can be developed. Much of this research has focused on transient solute uptake on the ultrafiltration membrane. Current methods for quantifying solute uptake by ultrafiltration membranes include: ^{125}I, ^{14}C, or fluorescent labeling, gravimetric measurement, and spectrophotometric analysis. However, each of these methods has specific limitations which make short-time solute uptake analysis, on and within membranes, difficult or impossible. Gravimetric and spectrophotometric methods generally are effective only for long-time cases. Fluorescent and ^{14}C labeling are useful for analyzing solute

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uptake on the surface of the membrane, but the signal from solute within the pores can be attenuated by the membrane. While the signal from ^{125}I-labeled solutes is not hindered by the presence of the membrane, radioactive labeling has recently been found to cause preferential adsorption [1]. Little or no work exists on the understanding of the configurational changes of an adsorbed or lodged protein inside a membrane pore.

This work focuses on the use of electron paramagnetic resonance (EPR) spectroscopy to analyze transient fouling caused by nitroxide spin-labeled proteins in ultrafiltration membranes. The EPR technique has the sensitivity to analyze the initial solute uptake in ultrafiltration and has a signal that is transparent to the membrane. Also, the solutes and labels can be selected such that they are not preferentially adsorbed. In addition, EPR spectroscopy can be used to further identify the time-dependent conformation of the protein within and on the membrane matrix.

This research focused on two aspects of the use of EPR spectroscopy in this context. Initially, EPR was used to monitor mass uptake of nitroxide spin-labeled proteins in and on the membrane during ultrafiltration. This will be particularly useful for analysis of solute uptake during the initial phase of ultrafiltration. Also, EPR spectroscopy was used to provide preliminary evidence of its potential in providing information on protein–membrane interactions. Both of these methods represent new applications of EPR technology to study protein interactions with ultrafiltration membranes.

The resulting EPR spectra obtained from adsorbed spin-labeled protein on membranes can be double integrated to provide a very accurate measurement of the concentration of the protein on and within the pores. EPR spectra of nitroxide spin-labeled proteins can also be used to determine potential structural changes in the membrane–protein–solute environment in the vicinity of the spin label. Nitroxides are particularly attractive spin labels due to the large number of different chemistries that are available for these compounds, as well as their potential to provide information on mobility and protein conformation [2]. Specific binding to various amino acid moieties can also be achieved. This has the added advantage of observing the environment around the spin label at different locations in the protein. The analysis of several combined single spin labels can provide insight on the conformation of the protein both on the membrane surface and within the membrane pores.

Doubly-labeled proteins may also be of potential use in analyzing protein structure during fouling. Spin–spin interaction, that results from doubly-labeled macromolecules could create changes or add lines in the continuous wave EPR spectrum, provided the spin labels are within 13 Å [3,4]. The resulting spectra could provide insight into the changes in the protein's conformation which may take place during fouling.

Hen egg lysozyme (HEL) and bovine serum albumin (BSA) were the model proteins used in this study. HEL is ideal since it has been analyzed by spin labeling [5–7] and likewise, it has been used as a sample molecule in ultrafiltration studies [8,9]. HEL has a molecular weight of ~15,000 and dimensions of $45 \times 30 \times 30$ Å. HEL can be labeled selectively with single or multiple labels at specific sites [5]. Some of these sites are Lys-1, His-15, and Asp-52. These sites can be labeled with spin labels such as 3-(2-bromoacetamido)-proxyl, diazo-methyl-carboxy-proxyl, and maleimido-proxyl.

BSA was used because it is well documented in the membrane separation literature for its adsorption effects on flux. It has a molecular weight of ~165,000 and dimensions of $140 \times 40 \times 40$ Å. Our preliminary studies with the 3-maleimido-proxyl spin label have shown that the labeled BSA has three susceptible sites occupied.

2. Experimental

2.1. Preliminary experiments

Since the use of EPR spectroscopy to analyze protein uptake and interaction with ultrafiltration membranes is a novel application, several preliminary experiments were performed to ensure the feasibility of this method. These experiments include: adequate purification of the pro-

tein and preparation of spin-labeled protein; development of the EPR protocol; elucidation of potential background signals from membranes and solution, and interaction of the free spin label with the ultrafiltration membrane; physical property differences between the spin-labeled protein and the non-labeled protein; preferential adsorption of spin-labeled protein to the membrane during ultrafiltration; and determination of the effective wash time needed to remove surface deposition of proteins that are loosely bound.

HEL was obtained from Sigma Chemicals (St. Louis, MO, L-6876) and purified by size exclusion chromatography using polyacrylamide size exclusion gel (5×30 cm Bio Rad column, Bio Rad, Richmond, CA, P-30) with 10 mM NH_4Cl as the eluent. The collected fractions were adjusted to pH 8, using NH_4OH, and lyophilized. Electrophoresis (Hoefer® SE-250) was performed to ensure that the HEL fraction was free of impurities. Sample compositions of each fraction were also examined using CM-silica ion exchange HPLC. The purified HEL was stored in an amber bottle at −20°C.

The spin labeling of lysozyme has been developed by others [5–7]. The labels used for singly-labeled and doubly-labeled lysozyme were 3-(2-bromoacetamido)-proxyl and 3-maleimido-proxyl, respectively.

Analytical HPLC analysis, using a CM-silica column, revealed the presence of at least four species in the doubly-spin labeled lysozyme solution as expected [5]. Unreacted label was removed by a process incorporating centrifugation, ultrafiltration, and lyophilization.

Ultrapure BSA (Miles Laboratory, Kankakee, IL, Pentex* Bovine Albumin Monomer Standard, 81-028-2, Lot No. P343) was mixed in 7 ml of 0.1 M phosphate buffer. It was labeled with 3-maleimido-proxyl in the same manner as described above for labeling of lysozyme, for 6 h at 40°C. All other processing was handled in a similar manner.

A Bruker ESP-300E EPR spectrometer was used throughout this study. Based on the S/N ratio seen in these experiments (9.25/1) we are able to detect 10^{13}–10^{14} spins. This translates into a limit of detectability of protein of 4 μg of labeled BSA (L-BSA). This is more sensitive than weighing methods of these applications. The membranes samples were examined in the spectrometer using a Wilmad quartz tissue cell. Liquid samples were examined using a Wilmad quartz flat cell. Both liquid and membrane samples used 3-carboxy-proxyl as a standard for quantification. Since the 3-carboxy-proxyl samples have a line width of ~1 G and the labeled proteins have a line width of ~8 G, the instrument has a much greater sensitivity for the standard and, thus, this was not a limiting factor in our quantification. The same respective sample cell was used for both the standard and the samples.

Possible background signals for the cellulosic and polysulfone membranes, buffer solution, and the non-labeled proteins were investigated. Essentially no background signals were observed from the cellulosic membranes or the buffer solution. A small background signal was observed for the polysulfone membranes, however, this signal can be easily subtracted from the EPR spectra from other samples. The retention of free spin label with membranes was determined to be negligible.

The pIs of the labeled and unlabeled BSA were determined by isoelectric focusing to evaluate whether spin labeling altered this property of the protein. The gels were run using Servalyt 3–6 of a 5% gel using p*I* standards (Pharmacia). The pH gradient of the gel was determined using a surface pH probe. The overall p*I* ranges were 4.3–4.5 and 4.2–4.4 for the unlabeled and labeled BSA, respectively. This indicated that the labeling process did not significantly effect the pI of BSA.

The overall mass of the labeled BSA was determined to be only 0.9% greater than the mass of unlabeled BSA.

To test protein adsorption on the membranes, mass uptake analysis was performed using both gravimetric and EPR methods. The gravimetric method was a modification of that of Robertson and Zydney [1]. Fourteen 100,000 MWCO polysulfone membranes (Millipore, No. PTHK0MS10, lot No. H3EM99295) were used in this study, seven for each method.

A preferential adsorption factor was used as follows [1]:

$$y=\frac{p_1^*/p_2^*}{p_1/p_2} \quad (1)$$

where p^* is the mass of protein per area and the subscripts 1 and 2 represent the labeled and unlabeled material, respectively. The value p represents the solution concentration.

From the samples, the value of y was calculated to be 1.15 ± 0.52. Since values near unity indicate no preferential adsorption, the labeled BSA had no unique affinity for the polysulfone membranes. It should be noted that ^{125}I-labeled proteins have a y value on the order of 55, clearly indicating strong adsorption to the polysulfone membrane [1].

2.2. Effective wash time

The effective wash time necessary to obtain a stable signal from the protein-exposed membrane was determined using saline buffer solution in a crossflow ultrafiltration cell (Millipore, Minitan S) with negligible transmembrane pressure and a crossflow rate of 170 ml/min. The effective wash time was determined to be 10 min.

The results of these preliminary experiments demonstrated that the EPR technology is a viable method for analyzing protein uptake within and on the fouling membrane and protein–membrane interaction.

2.3. Transient uptake

Preliminary studies were performed with a 1% BSA solution (of which 1% was 3-maleimido-proxyl-labeled BSA) in solution to determine its rate of uptake on fresh polysulfone membranes (100,000 MWCO). Four solutions were prepared that had a pH of 5.0 or 7.0 and a total salt concentration of 0.05 or 0.15 M. The run times were 5, 10, 30, and 120 min. The pH 7, 0.15 M salt run was also operated for a time of 240 min. After each run the membranes were washed as described. A standard crossflow ultrafiltration apparatus (Millipore, Minitan S) was used with an exposed membrane area of 13×60 mm. The crossflow flowrate, controlled with a peristaltic pump, was 170 ml/min and the transmembrane pressure was 35 kPa.

The membranes were examined in the Bruker ESP 300E spectrometer using a Wilmad quartz tissue cell. Samples were scanned 50 times and time averaged. All samples were taken at room temperature. Typical instrument settings were: power, 20 mW; frequency, 9.7135 GHz; receiver gain, 2.5×10^5; modulation frequency, 100 kHz; modulation amplitude, 1.056 G; sweep width, 100 G; time constant, 20.972 ms; and sweep time, 81.92 s. Duplicate runs were performed and averaged. It has previously been shown that at 20 mW power in the TM cavity, no significant saturation of nitroxide samples in aqueous samples was observed [10].

2.4. Protein–membrane interaction

Doubly-labeled HEL was prepared as described in Section 2.1. The ultrafiltration apparatus was operated for 120 min for each run; 10,000 and 30,000 MWCO polysulfone membranes were used. Membrane samples were analyzed using EPR spectroscopy with essentially the same settings as used above. The solution sample were analyzed with a Wilmad quartz flat cell in a Bruker TM cavity. The instrument settings for the liquid sample were: power, 20 mW; frequency, 9.765 GHz; receiver gain, 1.0×10^6; modulation frequency, 100 kHz; modulation amplitude, 0.594 G; sweep width, 100 G; time constant, 163.84 ms; and sweep time, 83.886 s. The liquid samples were scanned 20 times.

3. Results and discussion

3.1. Transient uptake

Table 1 compares the mass uptake of a solution of pH 7 and 0.15 M salt, after 2 h of operation, with other literature values. The amount of BSA measured on the nominal surface of the membrane was 0.33 ± 0.1 mg/cm^2. Although no case is directly representative of this study, nevertheless, it appears that the amount of mass

Table 1
Comparison of BSA uptake studies

	This study	Refs.				
		[4]	[1]	[10]	[11]	[12]
Detection method	EPR	Spectrophotometric	Mass	Scintillation of ^{14}C	Spectrophotometric	Spectrophotometric
Amount of BSA on membrane (mg/cm^2)	0.33±0.1	0.09–0.15	0.066	0.00012–0.0002	0.002	24
Membrane polymer	Polysulfone	Dynel	Polyethersulfone	Polysulfone and cellulose acetate	Polysulfone	Polyacrylonitrile
Molecular weight cut-off	100 000	100 000	100 000	6 000–12 000	25 000	10 000
Conditions	pH 7 0.15 M salt 2 h Crossflow	pH 7.4 0.1 M salt 3 h Stirred cell	pH 7 0.15 M NaCl 24 h Static	pH 7 0.15 M NaCl 2 h Crossflow	pH 6 0.15 M salt 1 h Static	pH 7.4 0.15 M salt Crossflow

uptake we observe is in reasonable agreement with other reported values, when different conditions and membrane types are taken into consideration.

Fig. 1 shows the transient protein uptake for the membranes analyzed. The relatively large error bar is most likely related to the large variations in the pore size distribution of the membrane samples for the repeated runs. This has been observed by others [1]. As can be seen, a significant amount of solute uptake occurs within

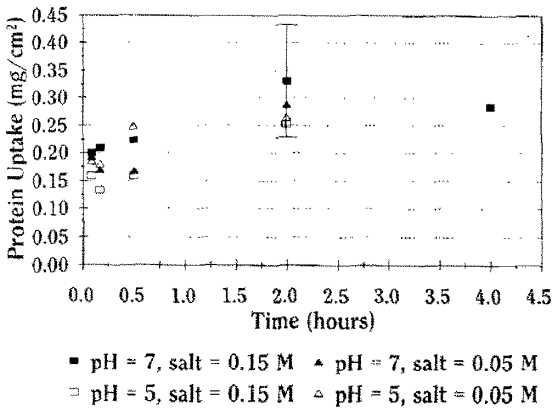

Fig. 1. Plot of protein mass uptake on membranes versus ultrafiltration time. Error bar based on standard deviation for 3 repeated studies for the same case.

the first 10 min of ultrafiltration. The relatively sharp increase of mass in the initial 5 and 10 min, coupled with the apparent independence with respect to the solution properties, indicates that the initial solute build-up is most likely due to internal pore plugging rather than adsorption. After some time, the solute uptake increases, but the rate is substantially reduced. It is expected that the lodged macromolecules begin to adsorb, which further occlude pores, resulting in an increased mass uptake. Future studies will utilize transmembrane pressure pulsed ultrafiltration to reduce the amount of solute lodged in the pores. The results of these studies can then be compared to discern the significance of pore plugging in the initial mass uptake.

3.2. Protein–membrane interaction

The EPR spectra of doubly-labeled HEL in saline buffer solution is shown in Fig. 2. Ultrafiltration of the doubly-labeled HEL with 10,000 and 30,000 MWCO polysulfone membranes for 120 min resulted in the EPR spectra shown in Figs. 3 and 4, respectively. The lineshapes from the exposed 10,000 and 30,000 MWCO membranes are significantly different. Since lysozyme (15,000 Da) is essentially rejected by the

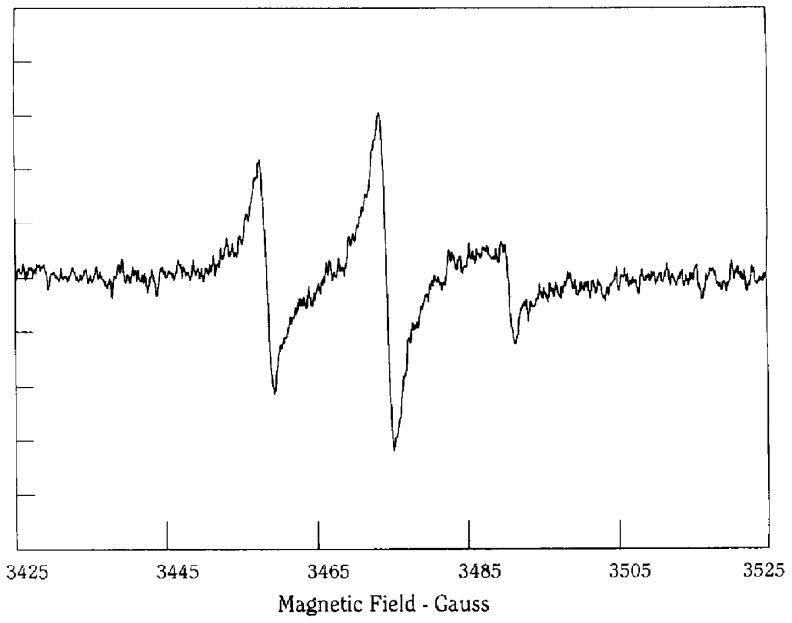

Fig. 2. ESR spectrum of labeled lysozyme in buffer solution. Conditions: receiver gain, 1×10^6; modulation amplitude, 0.594 G; time constant, 163.84 ms; sweep time, 83.886 s; sweep width, 100 G; frequency, 9.766 GHz; power, 20 mW; cavity, tm; temperature, 293 K.

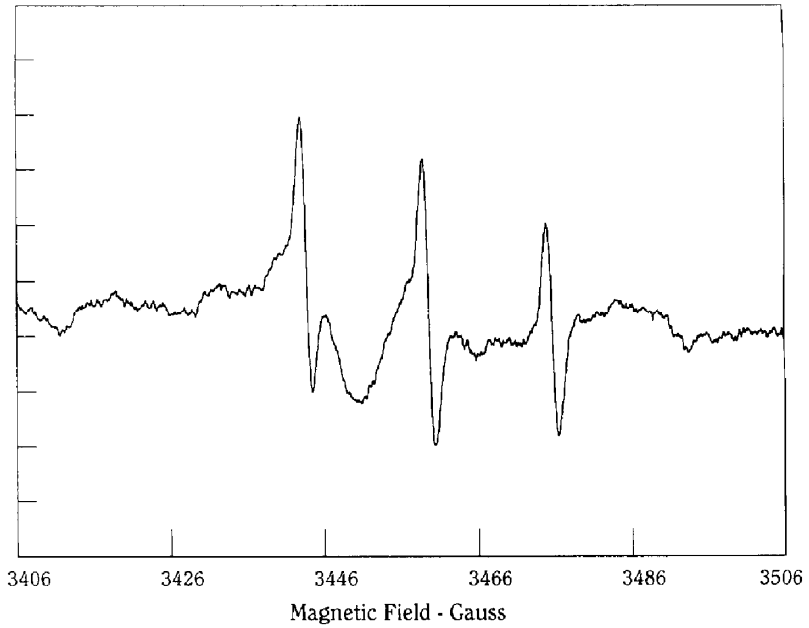

Fig. 3. ESR spectrum of labeled lysozyme on 10,000 MWCO polysulfone membrane. Conditions: receiver gain, 1×10^6; modulation amplitude, 1.056 G; time constant, 81.92 ms; sweep time, 20.972 s; sweep width, 100 G; frequency, 9.724 GHz; power, 20 mW; cavity, tm; temperature, 293 K.

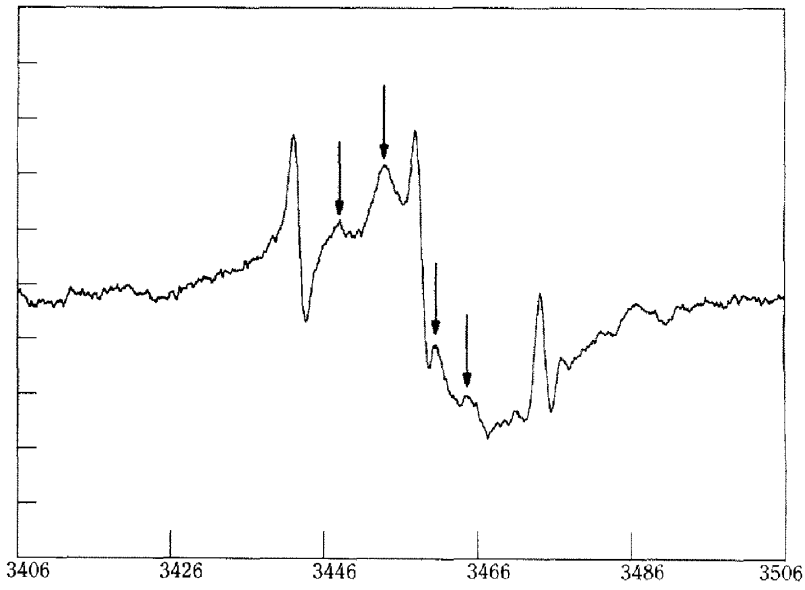

Fig. 4. ESR spectrum of labeled lysozyme on 30,000 MWCO polysulfone membrane. Conditions: receiver gain, 1×10^6; modulation amplitude, 1.056 G; time constant, 81.92 ms; sweep time, 20.972 s; sweep width, 100 G; frequency, 9.722 GHz; power, 20 mW; cavity, tm; temperature, 293 K. Arrows indicate the presence of additional lines.

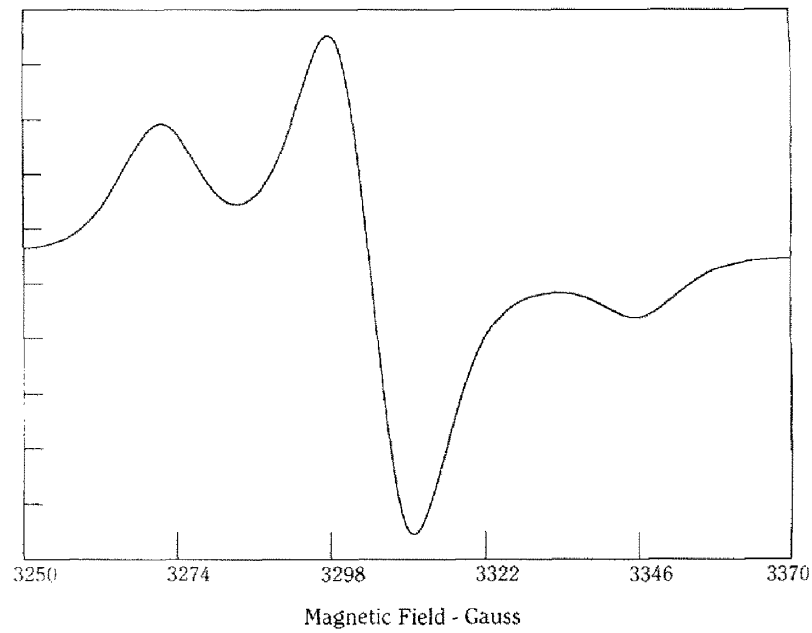

Fig. 5. ESR spectrum of frozen labeled lysozyme in solution. Conditions: receiver gain, 1×10^5; modulation amplitude, 1.011 G; time constant, 327.68 ms; sweep time, 41.943 s; sweep width, 120 G; frequency, 9.302 GHz; power, 20 mW; cavity, st891; temperature, 77 K.

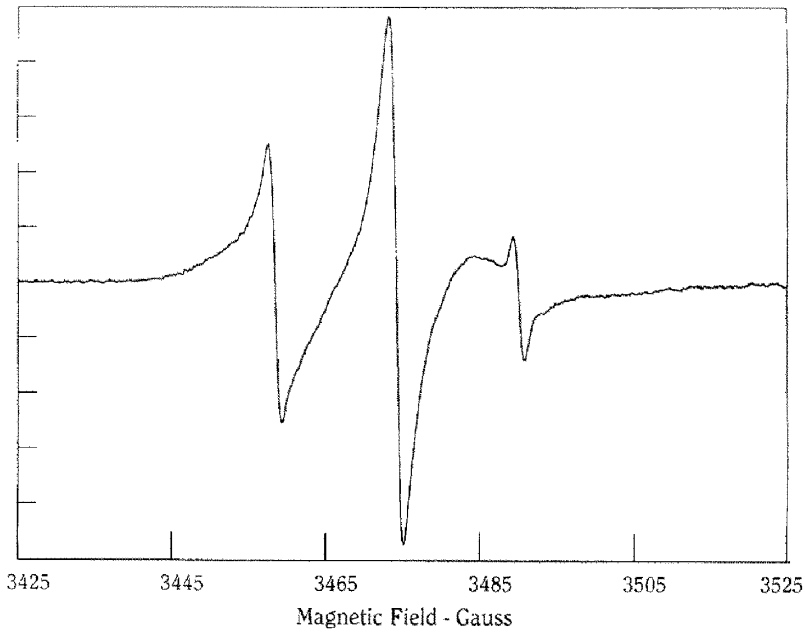

Fig. 6. ESR spectrum of labeled lysozyme in 8 M urea (denatured). Conditions: receiver gain, 1×10^6; modulation amplitude, 0.594 G; time constant, 163.84 ms; sweep time, 83.886 s; sweep width, 100 G; frequency, 9.766 G Hz; power, 20 mW; cavity, tm; temperature, 293 K.

10,000 MWCO membrane, it is assumed that the spectrum for the 10,000 MWCO membrane is associated with surface adsorption only. The spectrum for the 30,000 MWCO membrane is a result of surface adsorption, pore plugging, and pore adsorption.

The spectra observed from the polysulfone membrane are not due to general denaturation nor extensive immobilization. This is because the spectra are not similar to that of the frozen labeled protein spectrum or the urea-denatured labeled lysozyme spectra (Figs. 5 and 6, respectively). The additional lines present in Fig. 4 (indicated by arrows) for the 30,000 MWCO membrane may be due to spin–spin interaction between the two spin labels on the labeled HEL which can occur if the spin labels on the protein are forced closer to one another. An alternate interpretation could be that the new lines may result from a preferential directioning of the adsorbed species. While it is theoretically possible to have intermolecular interaction that can cause these lines, this is highly doubtful since the labeled protein is only 1% of the total proteins available. This implies that the proteins within the pores may be compressed. However, a further analysis of these and other spectra is necessary before a full understanding of these results can be obtained.

4. Conclusions

More detailed analysis of both transient solute uptake and protein–membrane interaction is currently underway. With respect to mass uptake, the intrinsic sieving will be coupled with the observed mass uptake from EPR to provide information that will aid in better understanding the fouling process with respect to mass. In future studies on the solute–membrane interaction aspect, the spin motion will be analyzed for singly-labeled proteins using the method of Schneider and Freed [11]. Successfully analyzing the

ESR spectra may provide insight into the apparent mechanism of protein–membrane interaction within and outside the membrane pores.

This research has shown that EPR spectroscopy has the potential to become a powerful tool in membrane fouling analysis. The methods introduced can be significant in providing an understanding of membrane fouling and can provide important experimental methodologies for future investigations of the membrane fouling phenomenon. EPR spectroscopy offers an effective method for analyzing transient solute uptake and it has the potential for analyzing transient protein–membrane interaction during ultrafiltration.

5. List of symbols

p solution concentration of protein
p^* mass of protein per area
y preferential adsorption factor

5.1. Superscripts and subscripts

1 labeled material
2 unlabeled material

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References

[1] B.C. Robertson and A.L. Zydney, Protein adsorption in asymmetric ultrafiltration membranes with highly constricted pores, J. Colloid Interface Sci., 134 (1990) 563–575.
[2] L.T. Berliner (Ed.), Spin Labeling: Theory and Applications, Academic, New York, NY, 1976.
[3] S.S. Eaton, K.M. More, B.M. Sawant and G.R. Eaton, Use of the EPR half-field transition to determine the interspin distance and the orientation of the interspin vector in systems with two unpaired electrons, J. Am. Chem. Soc., 105 (1983) 6560–6567.
[4] G.R. Eaton and S.S. Eaton, Resolved electron–electron spin–spin splittings in EPR spectra, Biol. Magn. Reson., 8 (1986) 339–397.
[5] G.I. Likhtenshein, Y.D. Akmedov, L.V. Ivanov, L.A. Krinitskaya and Y.V. Kokhanov, Investigation of the lysozyme macromolecule by a spin-labeling method, Mol. Biol., 8 (1975) 40–48.
[6] R.W. Wein, J.D. Morrisett and H.M. McConnell, Spin-label-induced nuclear relaxation. Distances between bound saccharides, histidine-15, and tryptophan-123 on lysozyme in solution, Biochemistry, 11 (1972) 3707–3716.
[7] P.G. Schmidt and I.D. Kuntz, Distance measurements in spin-labeled lysozyme, Biochemistry, 23 (1984) 4261–4266.
[8] K.C. Ingham, T.F. Busby, Y. Sahlestrom and F. Castino, Separation of macromolecules by ultrafiltration: influence of protein adsorption, protein–protein interaction, and concentration polarization, Polym. Sci. Technol., 13 (1980) 141–158.
[9] A.G. Fane, C.J.D. Fell and A.G. Water, Ultrafiltration of proteins through partially permeable membranes – the effect of adsorption and solution environment, J. Membrane Sci., 16 (1983) 211–224.
[10] G. Buettner and K. Kiminyo, Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals, J. Biochem. Biophys. Methods, 24 (1992) 147–151.
[11] D. Schneider and J. Freed, Calculating Slow Motion Magnetic Resonance Spectra: A Users Guide, Baker Laboratory of Chemistry, Cornell University, Ithaca, NY, 1988.