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Relationship of rotational correlation time from EPR spectroscopy and protein–membrane interaction

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Abstract

A study was carried out to determine if rotational correlation time of spin-labeled hen egg lysozyme (HEL) interacting with ultrafiltration membranes could be used to infer protein–membrane interaction. Polysulfone and cellulosic membranes, which have notably different adsorption properties, and membranes with varying pore sizes were used in this study. Based on this study, it was determined that the rotational correlation time does reflect variations in protein adsorption and pore plugging on membranes. The rotational correlation times for the highly adsorbent polysulfone (2.82×10^{-8} s) were significantly higher than those obtained from proteins on cellulosic membranes (0.62×10^{-8} s) and from those in solution (0.17×10^{-8} s). Rotational correlation time was also increased due to steric hindrance associated with pore plugging, although it was not as significant as the adsorption effect. This study indicates that the rotational time constant can be used to infer the type of protein–membrane interaction.

Keywords: Fouling; Ultrafiltration; Protein–membrane interaction; Electron paramagnetic resonance spectroscopy; Rotational correlation time

1. Introduction

Electron paramagnetic resonance spectroscopy (EPR) can be used to examine protein fouling of ultrafiltration membranes. Quantitative information on protein uptake could be obtained even when as little as 1 $\mu g/cm^2$ of spin labeled protein is associated with the membrane. EPR could also be a valuable tool to determine protein conformational changes during the membrane fouling process [1]. Uptake of protein by the membrane could bring about local constriction that would restrict the possible motion of the protein and its associated spin label. These changes in motion of the protein could result in changes in the rotational correlation time (τ_R) of the spin-label associated with the protein. In this work we examine the possible use of spin-label rotational correlation times to infer environmental and or conformational changes of the fouling protein. This would provide new information on the fouling process.

2. Description of rotational correlation time

Knowles et al. [2] describe rotational correlation time, τ_R, as the average time for which the molecule

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moves in any given direction. As an example, let us consider a paramagnetic molecule, such as 3-carboxy proxyl, suspended in a non-viscous liquid. This molecule may rotate in any direction for a given length of time before beginning rotation in some new direction. Such a rapidly tumbling nitroxide will yield an EPR spectrum of three sharp, relatively narrow lines of nearly equal height as seen in Fig. 1.

If the nitroxide spin-label is attached to a much larger molecule, e.g. a protein, then both the rotations of the protein and the spin-label must be considered. If the protein is significantly larger than the spin-label, its overall rotational time will be relatively slow. Thus, the EPR lineshape observed will result from only the motion of the spin-label in its local environment. The rotational correlation time for the attached label is relatively longer when attached to a protein because the surrounding protein makes it more difficult for it to change rotational direction. However, the rotation of the label may also be restricted by the surrounding protein and its environment, thereby limiting potential rotational orientations [3]. The resulting EPR spectrum of the spin-labeled protein is distorted in the high field line (as noted by the arrow in Fig. 2) due to the increase in τ_R.

Thus, changes in the protein environment and protein–membrane interaction are reflected in spectral lineshapes changes and the associated value of τ_R. Previous work shows that τ_R is sensitive to the

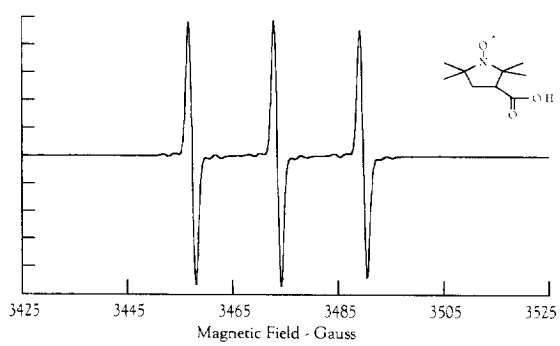

Fig. 1. EPR spectrum of 3-carboxy proxyl in water at room temperature. Parameters: modulation amplitude, 0.6 G; time constant, 164 ms; receiver gain, 6.30×10^4; scan width, 100 G; power 20 mW; frequency, 9.76 GHz; scan center, 3475 G; number of scans, 10; cavity TM_{110}; temperature, 293 K. Inset: Structure of 3-carboxy proxyl.

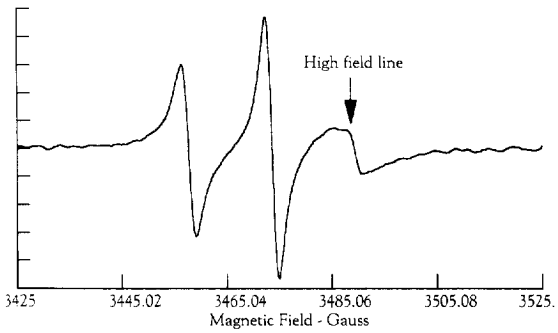

Fig. 2. Spectrum of hen egg lysozyme labeled at His-15 with 3-(2-bromoacetamido) proxyl. Parameters: modulation amplitude, 0.6 G; time constant, 164 ms; receiver gain, 8×10^5; scan width, 100 G; power 20 mW; frequency, 9.76 GHz; scan center, 3475 G; number of scans, 10; cavity TM_{110}; temperature, 293 K.

environment in which the EPR responsive material is contained [2–6]. Therefore, the rotational correlation time can be altered by either changes in the protein surroundings or in solution properties. These changes in the rotational correlation times may reflect the type of protein–membrane interaction that occurs during the fouling process.

This research focuses on analyzing the significance of τ_R in inferring interaction of proteins with ultrafiltration membranes. Rotational correlation times of a spin-labeled protein in solution are compared to spin-labeled proteins that are adsorbed onto or constricted by membrane pores. If the fouling protein has very little interaction with the membrane and only minor configurational changes occur, then no significant changes in τ_R would be expected. However, if the spin-label motion becomes sterically hindered during the fouling process, then an increase in τ_R would be expected. When substantial protein–membrane interaction exists, profound increases in τ_R are anticipated. Thus, relative values of rotational correlation time can infer general protein–membrane interaction mechanisms.

3. Evaluation of τ_R

Several methods are available for evaluating τ_R from EPR spectra [2,7–11]. The choice of method is dependent on its expected value, the spectral signal-to-noise ratio, and experimental data available. The

methods generally fall into empirically based or simulation-based categories. The two methods summarized here are of the empirical type.

Method 1: $10^{-10}\text{s} < \tau_R < 10^{-9}$ s [2].

In this method, the rotational correlation time is determined from the lineheight and linewidth of the EPR spectrum of the labeled protein in solution. For this case, τ_R is given by

$$\tau_R = 6.5 \times 10^{-10} \Delta H_0 \left(\sqrt{\left(\frac{w(0)}{w(-1)} \right)} - 1 \right) \quad (1)$$

where ΔH_0 (in Gauss) is the linewidth of the central line and $w(0)$ and $w(-1)$ are the lineheights of central and high field lines, respectively. This method is appropriate for weakly and mildly immobilized spin-labels.

Method 2: $\tau_R > 10^{-9}$ s [8,10].

This method is useful for determining slow motional τ_R values. EPR spectra of the labeled protein in solution and at its rigid limit, e.g. frozen solution at 100 K (Fig. 3), are required. τ_R is evaluated from the expression,

$$\tau_R = a(1 - S)^b \quad (2)$$

where

$$S = A'_{zz}(\text{G}) / A^R_{zz}(\text{G}) \quad (3)$$

A^R_{zz} is identical to the magnetic tensor A_{zz} and A'_{zz} is the generic room-temperature general magnetic tensor. The values of a and b are determined by evaluating the peak-to-peak derivative Lorentzian linewidths (δ) such that

$$2\Delta^r_l = 1.59\delta \quad (4)$$

and

$$2\Delta^r_h = 1.81\delta \quad (5)$$

where $2\Delta^r_m$, in Gauss (m = l or h), is determined from the EPR spectrum, Fig. 3. Thus, the values for a and b in Eq. (2) are established. Table 1 shows some of these values.

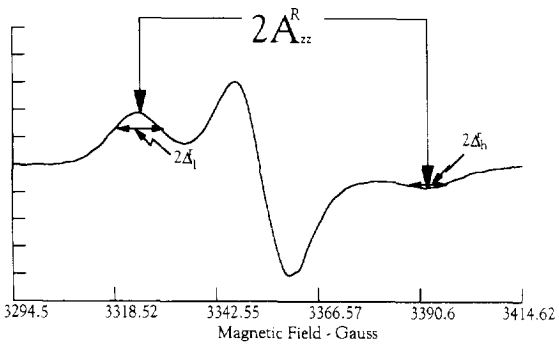

Fig. 3. Spectrum of frozen membrane PTTK exposed to hen egg lysozyme. Parameters: modulation amplitude, 1 G; time constant, 82 ms; receiver gain, 2.5×10^4; scan width, 120 G; power 20 mW; frequency, 9.42 GHz; scan center, 3475 G; number of scans, 50; cavity, standard; temperature, 100 K.

Table 1
Parameters for evaluating rotational correlation time [10]

Diffusion model	Linewidth (G)	a (10^{-10}) s	b (10^{-10} s)
Brownian	0.3	2.57	−1.78
	3.0	5.4	−1.36
	5.0	8.52	−1.16
	8.0	1.09	−1.15
Free	0.3	6.99	−1.20
	3.0	1.10	−1.01
Strong	0.3	2.46	−0.589
	3.0	2.55	−0.615

4. Experimental

Hen egg lysozyme (HEL, 15 kDa, Sigma Chemical Company, St. Louis, MO, USA, L-6876) was used as the test molecule for this experiment. The protein was purified by size exclusion chromatography using Toso Haas HW-50 F (Supelco, Bellefonte, PA, Lot 50HWF79R) and freeze dried. This method produced a salt free powder.

The purified HEL was labeled with 3-(2-bromoacetoamido) proxyl by the methods of Schmidt and Kuntz and Wein et al. [12,13]. The label reacts at His-15 and adds 198.26 MWU weight to the total protein mass, only a 1.3% increase in mass over the unlabeled protein. It was previously shown that this label does not result in preferential adsorption on the membrane materials used in this study [1].

Ultrafiltration tests were performed in a batch cell apparatus (UHP-43, Cole Parmer Scientific, Vernon Hills, IL). The test solution was composed of a citrate–phosphate saline buffer at pH 4.5 that contained 0.02% NaN_3 as a preservative. Total salt

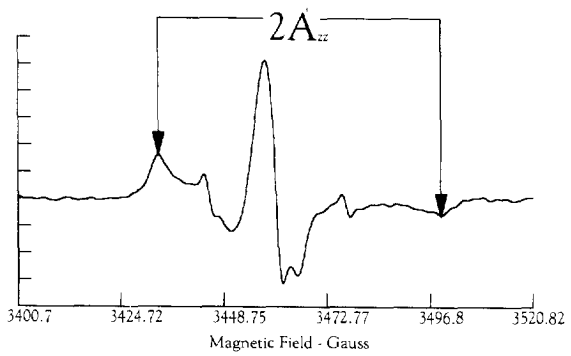

Fig. 4. Spectrum of PTGC membrane exposed to hen egg lysozyme. Parameters: modulation amplitude, 1 G; time constant, 82 ms; receiver gain, 2.5×10^5; scan width, 120 G; power 20 mW; frequency, 9.73 GHz; scan center, 3460 G; number of scans, 50; cavity TM_{110}; temperature, 293 K.

Fig. 6. Spectrum of PTMK membrane exposed to hen egg lysozyme. Parameters: modulation amplitude, 1 G; time constant, 82 ms; receiver gain, 2.5×10^5; scan width, 120 G; power 20 mW; frequency, 9.73 GHz; scan center, 3461 G; number of scans, 50; cavity TM_{110}; temperature, 293 K.

content was 0.15 M. The solution protein concentration was 0.1% of which 5% was labeled protein.

As mentioned above, membranes were selected that reflect well-known differences in protein–membrane interaction. Thus, polysulfone and cellulosic membranes were used to represent differences in adsorption behavior. It has long been recognized that polysulfone membranes are highly hydrophobic and that cellulosic membranes are generally hydrophilic. As a result, proteins are more readily adsorbed by polysulfone rather than cellulosic membranes. Various pore sizes were also selected to examine the effect of pore constriction on the rotational correlation time. Thus, the membranes used were; 10000, 30000, 300000 MWCO (molecular weight cutoff) polysulfone (Millipore, Bedford, MA, PTGC Lot P3NM8372, PTTK Lot P4BM8442, and PTMK Lot P4PM0209, respectively), and 10000 and 30000 MWCO cellulosic (Millipore, PLGC Lot P4BM8991 and PLTK Lot P3EM7199, respectively). The system operated with a transmembrane pressure of 34.5 kPa, and a stirrer speed of 500 rpm for 1 h. All experiments were done in duplicate.

Prior to the ultrafiltration run, a hydraulic permeability test was performed using distilled water. Afterwards the ultrafiltration was carried out. After 1 h, the protein solution was removed and replaced with distilled water. The membrane was exposed to the water for 10 min at 500 rpm and no pressure. Upon

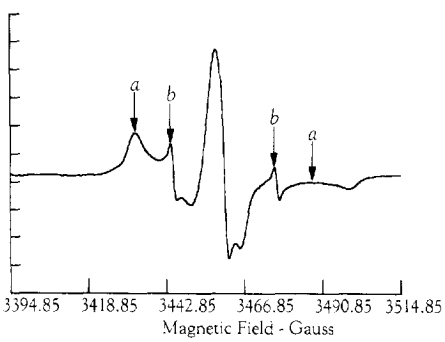

Fig. 5. Spectrum of PTTK membrane exposed to hen egg lysozyme. Arrows indicate the presence of two species. Species *a* is the predominate, slower species; while *b* marks the less predominate, faster species. Parameters: modulation amplitude, 1 G; time constant, 82 ms; receiver gain, 2.5×10^5; scan width, 120 G; power 20 mW; frequency, 9.73 GHz; scan center, 3455 G; number of scans, 50; cavity TM_{110}; temperature, 293 K.

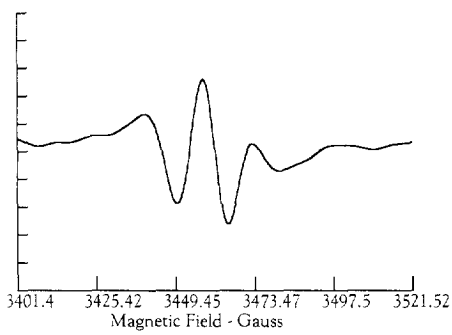

Fig. 7. Spectrum of PLTK membrane exposed to hen egg lysozyme. Parameters: modulation amplitude, 1 G; time constant, 82 ms; receiver gain, 2.5×10^5; scan width, 120 G; power 20 mW; frequency, 9.73 GHz; scan center, 3461 G; number of scans, 50; cavity TM_{110}; temperature, 293 K.

Table 2
Rotational correlation time (τ_R) for spin-labeled HEL when ultrafiltered with specific membrane types and in solution

Protein interaction environment	Membrane MWCO	τ_R Evaluation method no.	τ_R (10^{-8} s)
Polysulfone membrane	300000	2	2.12 ± 0.09
Polysulfone membrane	30000	2	2.82 ± 0.11
Polysulfone membrane	10000	2	2.18 ± 0.61
Cellulosic membrane	30000	1	0.62 ± 0.07
Solution	N/A	1	0.17 ± 0.09

completion, the membrane hydraulic permeability was again determined. Next, a 12×39 mm^2 membrane sample was excised from the membrane and stored in distilled water for not more then 6 h before EPR examination.

EPR spectroscopy was carried out using a Bruker ESP 300 spectrometer (Billerica, MA). The room temperature runs were performed in the same manner as that of Oppenheim et al. [1]. Frozen samples were examined at 100 K using a Bruker variable temperature accessory (ER411VT) and standard cavity.

Unique to this analysis was the method by which the samples were introduced into the cavity. Generally, semi-rigid samples are powdered or homogenized when analyzed at low temperatures. However, due to the physical nature of UF membranes, they do not lend themselves to these methods and would result in significant error. To circumvent this problem, membrane samples were carefully wrapped around the exterior of a Wilmad, 3 mm o.d., quartz, tube secured in place with Parafilm®. Note: repeated tests of Parafilm® show that it does not produce an EPR signal. The sample and tube were placed into the cavity and frozen. Frozen solution samples were analyzed in a Wilmad, 3 mm i.d., quartz, tube in the same cavity. In cases where noise was significant the Bruker polynomial filter, which is a standard filter, was used to reduce background noise.

5. Results and discussion

Comparison between solution and membrane spectra (Figs. 2, 4–7) show a dramatic change in spectral lineshape and lines present. Examination of the solution (Fig. 2) and cellulosic (Fig. 7) spectra show similar lineshape characteristics. In contrast, the polysulfone samples show an increase in the number of lines present (as noted by the *b* arrows in Fig. 5). These lines indicate the presence of a small amount of a rapidly rotating species. This species is not seen in the cellulosic sample. Since free label does not interact with the membrane, it is unlikely that the faster species is free label [1]. Because the faster species center and high field lines coincide with spectral lines from the slower tumbling species, the methods described above could not be used to determine its rotational correlation time. However, the slower tumbling species is quantitatively the dominant component in the spectrum and is representative of the spin-labeled protein. Table 2 summarizes the value of τ_R for the dominant species.

Table 2 also shows the results of the τ_R evaluation. The spectra were evaluated by both Methods 1 and 2. When the result of a method was well outside the suggested range, its value was not used. Also, guidelines found in the literature [2,7,8,10,11] helped in method choice. As part of Method 2, δ was evaluated using the values for *a* and *b* from Table 1. The values in Table 2 are for a δ of 5. Evaluation of δ for this set of experiments was found to be 5.96 ± 0.49 (G) where the error presented is the sample standard deviation. Values of τ_R were also determined for $\delta = 8$. No significant differences from the results for $\delta = 5$ were observed. No results for 10000 MWCO cellulosic membranes could be obtained as the EPR signal was below the limit of detection.

As can be seen, the polysulfone, for all pore sizes, increases the rotational correlation time substantially for the labeled HEL protein as compared to the cellulosic and solution cases. It is generally accepted that adsorption on polysulfone membranes is substantially higher than that of cellulosic membranes due to increased hydrophobicity of the polysulfone membrane in aqueous medium. Thus, it can be inferred that the increased rotational correlation times for the polysulfone membranes are the results of protein adsorption. The cellulosic membranes have τ_R values on the order of those of the solution. This infers that the molecule is not as tightly held and that the molecule is less restricted by the cellulose polymer.

The restrictive 30 000 MWCO polysulfone membrane had an additional increase in τ_R of approximately 0.67×10^{-8} s, over the rejecting 10 000 MWCO polysulfone and less restrictive 300 000 MWCO membrane. This additional rotational correlation time is equivalent to the 0.62×10^{-8} s value of τ_R for the 30 000 MWCO cellulosic membrane. This implies that the pore restriction for the 15 000 Da molecule in 30 000 MWCO adds an additional 0.4 to 0.5×10^{-8} s to the rotational time and that hydrophobic adsorption adds an additional time of approximately 2×10^{-8} s. Clearly adsorption has a more significant effect on correlation time than pore plugging.

6. Conclusions

An examination was carried out to determine if the rotational correlation time (τ_R) of spin-labeled proteins would change when ultrafiltered through membranes. The test encompassed the use of polysulfone and cellulosic membranes of various pore sizes. This study clearly indicates that τ_R is sensitive to changes in material and pore sizes. Rotational correlation times on polysulfone were significantly higher than those for cellulosic membranes and solution. Correlation time increased due to steric hindrance associated with pore plugging, however, this effect is small when compared to changes in τ_R due to hydrophobic adsorption. These results show promise in using the rotational correlation time of bound species to membranes to determine the mechanism of interaction.

7. List of Symbols

A_{zz}	magnetic tensor (G)
H_0	linewidth of the central line of a nitroxide EPR spectrum (G)
S	ratio of magnetic tensors
a	coefficient for evaluation of rotational correlation time (s)
b	exponent for evaluation of rotational correlation time
$w(n)$	lineheight of specific line in a nitroxide EPR spectrum
$n = 0$	central line
$n = -1$	high field line

7.1. Superscripts and subscripts

R	rigid limit value
h	high field
l	low field
′	solution value

7.2. Greek

Δ^r	half linewidth at half lineheight in a ridged limit spectrum (G)
δ	derivative Lorentzian linewidth (G)
τ_R	rotational correlation time (s)

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