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Electron paramagnetic resonance spin label titration: a novel method to investigate random and site-specific immobilization of enzymes onto polymeric membranes with different properties

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Abstract

The immobilization of biological molecules onto polymeric membranes to produce biofunctional membranes is used for selective catalysis, separation, analysis, and artificial organs. Normally, random immobilization of enzymes onto polymeric membranes leads to dramatic reduction in activity due to chemical reactions involved in enzyme immobilization, multiple-point binding, etc., and the extent of activity reduction is a function of membrane hydrophilicity (e.g. activity in cellulosic membrane ≫ polysulfone membrane). We have used molecular biology to effect site-specific immobilization of enzymes in a manner that orients the active site away from the polymeric membrane surface, thus resulting in higher enzyme activity that approaches that in solution and in increased stability of the enzyme relative to the enzyme in solution. A prediction of this site-specific method of enzyme immobilization, which in this study with subtilisin and organophosphorus hydrolase consists of a fusion tag genetically added to these enzymes and subsequent immobilization via the anti-tag antibody and membrane-bound protein A, is that the active site conformation will more closely resemble that of the enzyme in solution than is the case for random immobilization. This hypothesis was confirmed using a new electron paramagnetic resonance (EPR) spin label active site titration method that determines the amount of spin label bound to the active site of the immobilized enzyme. This value nearly perfectly matched the enzyme activity, and the results suggested: (a) a spectroscopic method for measuring activity and thus the extent of active enzyme immobilization in membrane, which may have advantages in cases where optical methods can not be used due to light scattering interference; (b) higher spin label incorporation (and hence activity) in enzymes that had been site-specifically immobilized versus random immobilization; (c) higher spin label incorporation in enzymes immobilized onto hydrophilic bacterial cellulose membranes versus hydrophobic modified poly(ether)sulfone membranes. These results are discussed with reference to analysis and utilization of biofunctional membranes.

Keywords: Electron paramagnetic resonance; Site-specific immobilization; Enzymes; Biofunctional membranes

1. Introduction

Biofunctional membranes, entities in which a biomolecule, collection of biomolecules or cells are

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immobilized onto polymeric matrices cast in the form of porous membranes, are used in catalysis (membrane-based enzyme bioreactors), separations (affinity membranes), analysis (biosensors; metal ion-specific separations), and artificial organs [1,2]. Although stability of enzymes is enhanced by immobilization [1,3–5], the activity of immobilized enzymes on porous polymeric membranes is often significantly decreased, an annoying problem associated with random immobilization of enzymes in which the active site of the immobilized enzyme points in different directions and orientations. This loss of activity results from a combination of factors, such as blockage of the active site from substrate accessibility, multiple-point binding, or denaturation of the enzyme [6–11] (Fig. 1). In random immobilization, enzymes are either directly attached onto the membrane or via a spacer arm, often through the ε-amino functionality of lysine residues on the protein. However, the presence of numerous lysine residues spread over the surface of the enzyme often leads to different orientations of the enzyme with respect to the membrane and also to the denaturation of active sites due to protein–surface interactions. We have previously shown that only enzymes with accessible active sites are viable enzyme molecules [7].

To circumvent this activity loss upon random immobilization of enzymes, site-specific immobilization using the power of molecular biology is used [8]. For example, we have formed ordered arrays of enzymes on membrane surfaces using molecular biology methods: (i) gene fusion to incorporate a peptide affinity tag at the N- or C-terminus of the enzyme; the enzymes are then attached from this affinity tag to anti-tag antibodies on membranes; (ii) post-translational modification to incorporate a single biotin moiety on enzymes; the enzymes can be attached through a (strept)avidin bridge; (iii) site-directed mutagenesis to introduce unique cysteines to enzymes; the enzymes are attached on thiol-reactive surfaces through the sulfhydryl group on the side chain of the introduced cysteine. In the latter case, the SH group is introduced to the enzyme on the opposite side of the protein from the active site. In all these methods, the active sites of the immobilized enzymes face away from the polymeric surface and, as we demonstrated, a consequent higher activity was retained (reviewed in [8]).

No matter the immobilization scheme, it is necessary to evaluate the efficiency of the immobilized enzyme by determining its activity. However, this can prove problematic, especially if optical methods of analysis are used, since light scattering can occur on the membrane surfaces. Here, we describe a novel approach to measuring enzyme activity of randomly and site-specifically immobilized enzymes on membranes that are hydrophilic or hydrophobic. Electron paramagnetic resonance (EPR), which is not affected by light scattering, is shown to be highly effective in measuring enzyme activity, comparable to traditional methods. The new technique is based on determining the difference in magnetic resonance intensity of an active site-specific spin label before and after reaction with the immobilized enzyme. The difference in intensity is hypothesized to result from the accessibility of the active site of the enzyme to spin label molecules. Further, the results of this study demonstrate that enzyme activity is highest using site-specific immobilization on a hydrophilic membrane.

To gain insight into the interaction of enzymes with the membrane surface, hydrophilic and hydrophobic membranes, bacterial cellulose [12] and modified poly(ether)sulfone (MPS) membranes, respectively, were used in both random and site-specific immobilization techniques. Subtilisin and organophosphorus hydrolase (OPH) were used to generalize our findings. Subtilisin is a commercially available enzyme that contains a serine in the active site [13]. OPH, which has received a great deal of attention due to its

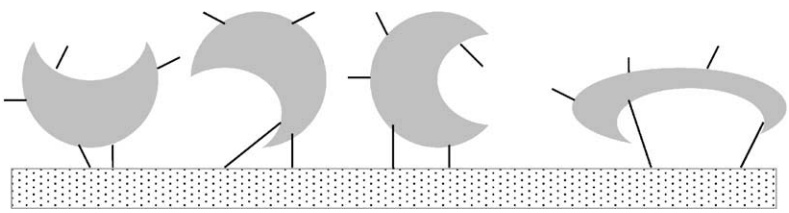

Fig. 1. Random immobilization of proteins. Indentation indicates binding/active site of the protein.

unique ability to hydrolyze and detoxify organophosphorus nerve agents [14–17], has two divalent metal ions located in its active site [18].

Two types of immobilization were studied, random and site-specific immobilization. Random immobilization is a less complicated immobilization technique and, as noted above, results in an enzymatic activity significantly lower than that of the enzyme in solution [3,5,7,19]. Site-specific immobilization is a more involved process, and it is possible that the resulting enzymatic activity approaches that of the enzyme in solution [8]. Previous EPR studies showed that random immobilization onto membrane surfaces resulted in two environments for the enzyme [7,20,21]. One of the enzyme environments had a much higher activity than the other. The ability to know the exact amount of enzyme that is on the membrane surface, would be an invaluable tool in studying the different techniques of enzyme immobilization and the effects of the membrane on the activity of the enzyme. This was the motivation for the present study.

2. Materials and methods

Random immobilization was accomplished by formation of a covalent bond between amino groups of lysine residues on the enzyme and a functional group (–CHO) on the surface of the MPS (Gelman Sciences, Ann Arbor, MI, USA) or bacterial cellulose (BC) membranes (Minnetonka, MN, USA). For random immobilization, a known amount of enzyme in PBS buffer (150 mM NaCl, 5 mM phosphate buffer, pH 7.4) was allowed to circulate though a membrane convective flow cell for 2 h at a flow rate of 2 ml/min. The enzyme solution was then analyzed using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) to determine the final concentration of enzyme. The amount of enzyme that has been loaded (immobilized) onto the membrane surface was determined from the difference between the amount of enzyme before and after introduction to the membrane surface. The spin label for the active site of subtilisin, 4-(ethoxyfluorophosphinyloxy)-TEMPO, was purchased from Sigma.

The method used to gain site-directed immobilization in this report is a fusion protein approach. Molecular biology was used to fuse an affinity tag, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG), to the C-terminus of the enzymes OPH [4] and subtilisin BPN′ [19]. Specifically, the coding sequence for the affinity tag was incorporated into the reverse primer for the polymerase chain reaction (PCR). The amplified gene was then introduced into *Escherichia coli* cells and expressed. Purification of the expressed fusion protein was performed as described in [4,12].

For site-directed immobilization, 1 mg of protein A (Sigma), which specifically binds to the F_c region of the antibody, in 20 ml of PBS buffer was circulated through a convective flow cell containing either the MPS or BC membrane for 2 h at a flow rate of 2 ml/min. The membrane was then extensively washed using a 1 M NaCl solution and then with PBS buffer in order to remove any unbound protein A. After that, 100 μg of anti-FLAG monoclonal antibody (Sigma) was added to 20 ml of PBS buffer and allowed to circulate through the membrane flow cell for 1 h. The membrane was again extensively washed using 1 M NaCl and PBS buffer. Dimethyl pimelimidate (DMP, 1 mg in 200 μl of 0.20 M triethanolamine, pH 8.2), was then allowed to circulate through the membrane flow cell for 1 h at a flow rate of 2 ml/min. DMP serves as a cross-linker that binds the antibody even tighter to protein A to make the complex resistant to harsh pH changes. The membrane was again extensively washed using the NaCl and PBS solutions. Enzyme-FLAG of known concentration in 10 ml of PBS was introduced to the membrane at the same flow rate for 2 h, forming a complex in which the enzyme active site faces the solution and away from the membrane surface [8] (Fig. 2). After 2 h, the flow cell was washed with the NaCl and PBS solutions. The amount of enzyme-FLAG that has been site specifically immobilized onto the membrane surface was determined by BCA assay as in random immobilization.

2.1. Spin label

To use EPR to study an enzyme following immobilization, a paramagnetic species (spin label) must be introduced to the active site of the enzyme. The spin label must be specific for the active site of the enzyme in question in order to correlate spectral intensity differences, before and after reaction, with active site availability, and, hence, activity. The spin label used to study the serine protease subtilisin was

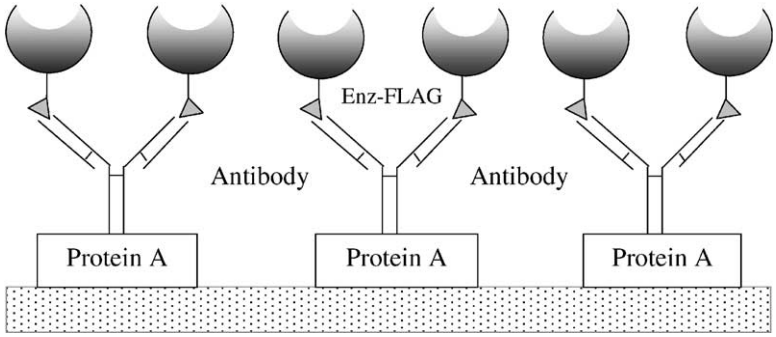

Fig. 2. Protein A and anti-FLAG monoclonal antibody mediated site-specific immobilization of FLAG-tagged proteins. Note that the active site of all enzymes faces away from the polymeric membrane surface and towards the solution.

4-(ethoxyfluorophosphinyloxy)-TEMPO (Sigma), which binds to the nucleophilic serine residue in the active site of the enzyme. The active site of the enzyme OPH was specifically spin labeled with 4-[(*p*-sulfonamido)benzoyloxy]-2,2,6,6-tetramethylpiperidine-1-oxyl (Fig. 3), which complexes with the Co^{2+} ions in the active site. The spin label was prepared and characterized as described previously [22].

2.2. Spin label titration

A spin label solution with a concentration of 3 μM was prepared in 10.5 ml of PBS buffer. After a known amount of enzyme was immobilized onto a membrane, the spin label solution was allowed to circulate through the flow cell containing the membrane with the immobilized enzyme for 2 h at a flow rate of 2 ml/min. The flow cell was then allowed to drain completely, and the solution was kept in an amber glass bottle. The membrane complex was then extensively washed using 10 ml of 1 M NaCl followed by several washes with 10 ml of PBS. All of the washes were collected in amber glass bottles to prevent UV destruction of the spin label. EPR analysis of each sample was then performed.

EPR can discern between differing spin label concentrations as low as 5×10^{-7} M. As the concentration becomes more dilute, the spectrum's peak heights become much smaller in size. To avoid a potential problem in which differing peak heights depend on the *Q*-value of the resonant cavity due to placement of the quartz aqueous sample cell, a reference standard

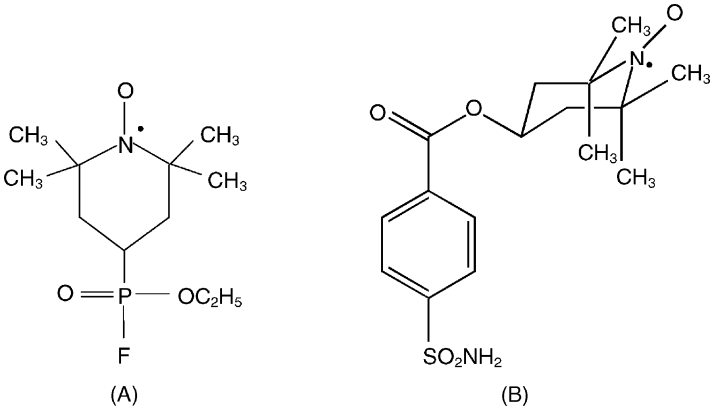

Fig. 3. Spin labels used for (A) labeling the active site of subtilisin, 4-(ethoxyfluorophosphinyloxy)-TEMPO and (B) labeling the Co^{2+} in the active site of organophosphorus hydrolase (OPH), 4-[(*p*-sulfonamido)benzoyloxy]-2,2,6,6-tetramethylpiperidine-1-oxyl.

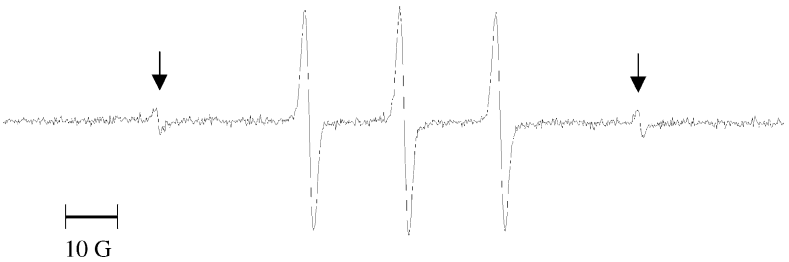

Fig. 4. EPR spectrum of the spin label used for the subtilisin studies along with the standard mixture of 1 part of calcium oxide and 20 parts of silica powder. The spin label concentration is 6 μM. Arrows indicate the reference standard signals of manganese oxide in the solid standard mixture that is attached to the quartz aqueous sample cell.

of defined spin density was included in each sample. Consequently, a ratio of the sample peak height to the reference peak height (which occurs at a different resonant field) eliminates this potential problem. The reference standard used for the EPR analysis was a silica powder and calcium oxide mixture. The calcium oxide is not a paramagnetic species, but it has an impurity, manganese oxide, that is paramagnetic and results in two peaks on the outside of the three peaks given by the spin label, which can be used as the reference signal (Fig. 4). The amount of calcium oxide that is added is about 1 part for every 15–20 parts of silica powder. The resulting powder is mixed well and then placed in a capillary tube and sealed. The capillary tube is then attached, using parafilm, to the EPR quartz cell that holds the sample so that both can be analyzed at the same time and the reference signal stays constant for all samples [23].

In order to correlate peak height to concentration, a set of spin label samples with a known concentration must be analyzed using EPR and compared with the reference signal. The peak height of the midfield line of the spectrum of known concentration samples is then divided by the peak height of the reference signal. A calibration plot is derived by plotting the different concentrations against their respective peak height ratio. The samples of unknown spin label concentration are then analyzed by EPR, and their peak height ratio can determine their concentration using this calibration plot. The concentrations are converted to moles, and the amount of spin label that was bound to the immobilized enzyme is determined. The amount of spin label bound is converted to a percentage of the total spin label moles present. The percentages are then compared to the percentages of immobilized enzyme activity. The following instrumental parameters were used for EPR experiments: sweep width, 150 G; center field, 3480 G; modulation amplitude, 0.3 G; modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 20 mW; time constant, 0.64 s.

2.3. Measurement of enzyme activity

An excess amount (2 mM) of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPF-*p*NA) was permeated convectively through the membrane with immobilized subtilisin in the flow cell at 2 ml/min in 50 mM phosphate buffer, pH 8.7. The enzyme activity was determined at 24 °C by monitoring the increase in absorbance over time at 410 nm using a Baush & Lomb Spectronic 1001UV–VIS spectrophotometer as *p*-nitroaniline is being formed. For immobilized OPH, the substrate used was 2 mM paraoxon in 50 mM CHES buffer (Sigma), pH 9.6; the enzyme activity was determined by monitoring the increase in absorbance at 400 nm over time.

3. Results and discussion

Subtilisin activity studies showed that the mean activity for this serine protease randomly immobilized onto a hydrophobic MPS membrane was 10.6% that of the homogenous enzyme (Table 1). If the EPR titration method introduced in this paper is a valid method, the percentage of accessible active sites should be similar. Using EPR to estimate the amount of immobilized enzyme with accessible active sites revealed a value of 11.7 ± 2.7% (Table 1), in close agreement with the

Table 1
Comparison of spin label titration (SLT) and the activity method for determining active immobilized enzyme (%) on MPS and bacterial cellulose membranes[a]

Immobilization technique	Membrane	Method	Subtilisin	Subtilisin-FLAG	OPH	OPH-FLAG
Random	MPS	SLT	11.7 ± 2.7		9.4 ± 1.9	
		Activity	10.6 ± 4.3		8.0 ± 5.2	
Random	BC	SLT	31.5 ± 4.0		34.9 ± 1.5	
		Activity	27.4 ± 5.5		37.0 ± 4.8	
Site-specific	MPS	SLT		28.5 ± 1.7		51.0 ± 1.6
		Activity		28.1 ± 6.8		49.0 ± 7.2
Site-specific	BC	SLT		82.5 ± 2.6		84.3 ± 1.2
		Activity		80.6 ± 9.1		89.0 ± 9.2

[a] The results (mean ± S.D.) are given in percentage of the appropriate measure of the respective enzyme in homogenous solution. $N = 2$–4 for each measurement.

activity finding. The low percentage of active enzyme upon random immobilization is due to three factors, the membrane surface, the type of immobilization, and the possibility of multiple-point attachment of the enzyme. The MPS membrane is a hydrophobic membrane. The lack of polar groups on the membrane surface causes the hydrophobic portions of the enzyme to interact with and spread across the surface of the membrane. The effect of this spreading of some of the enzymes across the surface would be to alter the active site conformation, resulting in lower spin label binding and in a much lower percentage of active enzymes on the surface of MPS. Another factor affecting the low percentage of active enzyme after random immobilization is the random immobilization itself. Since the point of immobilization onto the surface of the membrane is anywhere on the enzyme backbone that has a lysine group, the enzyme can orient itself in random fashion on the membrane surface (Fig. 1). The third factor is the possibility of multi-point attachment of the enzyme through more than one lysine group. This could have the effect of making the enzyme rigid and inflexible. Only a small percentage of the immobilized enzyme would be attached to the MPS membrane in a way that would allow its active site to face away from the membrane surface and, consequently, be accessible to spin label binding.

The percentages of active enzyme site-specifically immobilized onto a MPS membrane determined though the spin label titration and activity methods are 28.5 and 28.1%, respectively (Table 1). These percentages are higher than those for random immobilization. These results are consistent with the notion that the spin label titration experiment is a valid method to determine the amount of active enzyme on a membrane surface. The increase of active subtilisin immobilized on MPS membranes in a site-specific fashion relative to randomly-immobilized enzyme is likely due to two factors, the site-specific immobilization and the space between the immobilization surface and the active site structure. Using site-specific immobilization, the enzymes are oriented in the same fashion with the active sites facing away from the membrane surface. Also, with the protein A, antibody, and affinity tag acting as a spacer between the membrane and the enzyme, there is sufficient space between the two that some of the hydrophobic interactions between the membrane surface and the protein are minimized.

Using a different, more polar, membrane, the percentage of active subtilisin randomly immobilized on the surface of the BC [12] membrane increased when compared to random immobilization onto the MPS membrane. The spin label titration method yielded the percentage of active enzyme as 31.5 ± 4.0%, while that determined using the activity measurements was 27.4% (Table 1). The membrane used in this experiment is a better choice for enzyme immobilization due to the minimal interaction of the enzyme with the cellulosic membrane surface. These results again suggest that the spin label titration method is a valid method for determining the amount of active immobilized enzyme. A cellulosic membrane is hydrophilic and has many polar groups in its polymeric backbone. These polar groups on the surface of the membrane

minimize enzyme–surface interactions. Therefore, the membrane has a smaller effect on the membrane surface and the only effect on the enzyme is where it is attached to the membrane surface. To increase the percentage of active immobilized enzyme even further, the use of site-specific immobilization was employed.

For site-directed immobilized subtilisin, the percentage of active immobilized enzyme increased dramatically compared to the other enzyme immobilization techniques. The activity study showed that this site-specific immobilization method yielded 80.6% of the immobilized enzyme active, while the spin label titration method determined that $82.5 \pm 2.6\%$ of the immobilized enzyme is active (Table 1). The much higher activity is due to the hydrophilic nature of the membrane surface. With the use of the BC membrane, subtilisin–membrane interactions are minimized. Consistent with these findings using this novel EPR technique, site-specific immobilization of enzymes using the fusion protein method has been shown to be a promising means to immobilize an enzyme to a membrane while keeping most of its activity [8].

The spin label titration method showed a good correlation with the enzymatic activity for subtilisin. However, in order to be able to use the spin label titration method in future studies, this method must be applicable to more than one type of enzyme with different masses and different active sites that require the use of different spin labels. In order to test the generality of this new EPR active site titration method, the immobilization of OPH was studied. Just as with the random immobilization of subtilisin on the hydrophobic MPS membrane, the percentage of active randomly-immobilized OPH is low when compared to its homogenous state. The spin label titration experiment showed that $9.4 \pm 1.9\%$ of the immobilized enzyme was active, and the activity study showed that 8% of the immobilized enzyme was active (Table 1). The reasons for the low active percentage of this randomly-immobilized enzyme to a hydrophobic membrane were noted above for subtilisin.

By immobilizing OPH-FLAG site specifically onto a MPS membrane, the percentage of active enzyme increased substantially as was seen with subtilisin-FLAG. The percentage of active immobilized enzyme was 49%, while the spin label titration method showed that $51.0 \pm 1.6\%$ of the enzyme remained active upon immobilization (Table 1). The spin label titration experiment again shows a good correlation with the activity method.

The reason that the percentage of active site-specifically immobilized enzyme is higher for OPH-FLAG than the subtilisin-FLAG is likely that the OPH-FLAG is almost three times larger than subtilisin and is a dimeric protein. Thus, it is more likely that one of the subunits is accessible to the substrate.

Based on the results of subtilisin on the BC membrane, the percentage of active OPH enzyme that remains upon random immobilization onto a BC membrane is predicted to increase dramatically when compared to random immobilization onto a MPS membrane. The experimental results confirm this prediction. The percentage of active immobilized OPH using the spin label titration experiment was found to be $34.9 \pm 1.5\%$, and the percentage using the activity experiment was found to be 37% (Table 1). These results are in agreement when compared with each other. The reason for the increase in the amount of active OPH relative to random immobilization is due to the effects of a hydrophilic membrane as noted above.

The use of site-directed immobilization and a hydrophilic membrane increases the amount of active immobilized enzyme substantially. The activity results demonstrate that the percentage of active immobilized enzyme is 89% (Table 1), while the spin label titration method yielded a percentage of active immobilized OPH of $84.3 \pm 1.2\%$. These results are in close agreement with each other and the percentage of active immobilized enzyme is one of the highest reported using a cellulose membrane. The reason for the high percentage of active enzyme is that both the fusion protein complex and the hydrophilic membrane play important roles.

4. Conclusions

This paper reports the development of a new method to determine the amount of active enzyme immobilized on membranes. The method reported here utilizes EPR to detect the amount of spin label bound to the enzyme active site by difference in intensities of spin label before and after the enzyme was immobilized on biofunctional membranes. The amount of spin label bound to the active site residue correlates to the activity of the enzyme. Compared to random

immobilization, significantly higher enzymatic activity is retained when enzymes are site-specifically immobilized in such a way that their active sites are pointed away from the immobilization surfaces. We also demonstrated that hydrophilic membranes used as immobilization supports invariably gave catalytic biofunctional membranes with higher enzymatic activity than did those using hydrophobic membranes.

It is possible to measure indirectly an enzyme activity by enzyme active site spin label titration using EPR. This is particularly advantageous when light scattering prohibits the use of traditional spectroscopy measurements once an opaque sample, such as biofunctional membranes with immobilized biomolecules, is used. The spin label titration assay for the amount of active immobilized enzyme was validated using the accepted method of comparing activities. Due to the sensitivity of EPR, the spin label titration method coupled with active site-specific spin labels can be used to detect changes in the amount of spin label bound to enzymes. The spin label titration method gave results that appear to be generalizable over two different types of enzymes, two different types of spin labels used, and two different types of functionalized membranes. This novel EPR method should find great utility in the study of biofunctional membranes.

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