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Electron paramagnetic resonance spin label titration: a novel method to investigate random and site-speci c 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 arti cial 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-speci c 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-speci c 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 con rmed 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-speci cally immobilized versus random immobilization; (c) higher spin label incorporation in enzymes immobilized onto hydrophilic bacterial cellulose membranes versus hydrophobic modi ed poly(ether)sulfone membranes. These results are discussed with reference to analysis and utilization of biofunctional membranes.

Keywords: Electron paramagnetic resonance; Site-speci c 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 [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

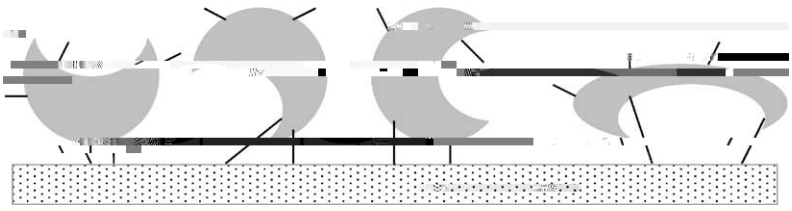

Fig. 1. Random immobilization of proteins. Indentation indicates binding/active site of the protein.

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]

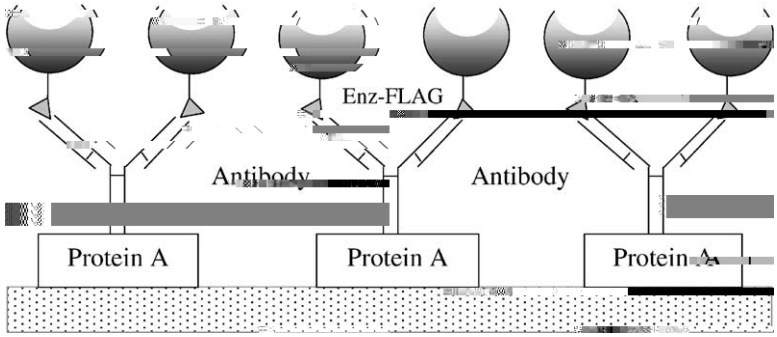

Fig. 2. Protein A and anti-FLAG monoclonal antibody mediated site-specific immobilization of FLAG-tagged proteins. Note that the active site of all enzymes faces away from the polymeric membrane surface and towards the solution.

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 1.3 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

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 SD) are given in percentage of the appropriate measure of the respective enzyme in homogenous solution. 2–4 for each measurement.

activity binding. 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.

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 ± 6% of the

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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