SPIN LABELS FOR CELL SURFACES

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1. Introduction

We describe the use of spin labels that localize on the outer surfaces of membranes. We use yeast as an example of a simple eucaryotic cell system. These labels should be very useful in probing the surfaces of a variety of vesicle and cell preparations. Most spin labels designed to probe membranes penetrate throughout the membrane system resulting in an average signal of all membranes present. It is necessary to use such probes as the ones described here in order to detect many of the interactions or modifications that appear to be localized on the surfaces of cells. These probes offer the opportunity of detecting physical state changes on or near the membrane surface.

In recent years it has been well established that the cell surface is the site of many unique and important events. Virus attachments, antigen-induced antibody formation, hormone binding, lectin recognition, cell–cell recognition, and contact inhibition are all regarded as cell surface events.

Spin labels have become widely used as probes of membrane structure and function. Most studies of membranes using spin labels measure a signal that reflects an average of both halves of the bilayer and a more general average of all membranes in a given system. Spin labels may have specificity for localizing in a dielectric zone, such as near the membrane interface for some sterol [1] and aliphatic hydrocarbon labels [2], or at differing depths in bilayers depending on the nitroxide position on spin label fatty acids [3]. The spin labels we describe here localize only on the outer surface of the plasma membrane.

2. Materials and methods

Label I was synthesized by modifications of a general method [4]. Tetradecyl bromide (10 g) was mixed with two equivalents of 4-amino-2,2, 6,6-tetramethylpiperidine-*N*-oxyl in a sealed tube. After one week of standing at room temperature the precipitated bromide salt was recovered by filtration. This product was purified to homogeneity by column chromatography using 200 mesh silica gel at pH 4. This product (11 g) was treated with excess ethyliodide for 48 h in a sealed tube, the salt was collected by filtration, converted to the chloride salt, and purified by column chromatography using ether:methanol (9:1) as moving phase and 200 mesh silica gel as stationary phase. The synthesis and purification of Label II is described elsewhere [5]. Label III was synthesized by condensing the acid chloride of 3-carboxy-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl with one equivalent of 2,5-diaminobenzene sulfonic acid in dry ether containing one equivalent of triethylamine. One of the fractions crystallized from ethanol gave a positive ninhydrin test and had high spin content. This fraction was treated with myristoyl chloride in dry ether containing one equivalent of triethylamine. This product was purified to homogeneity on a column containing 200 mesh silica gel at pH 7 with ether: ethanol (1:1) as moving phase. The spin label III may have mixed products with respect to the position of the fatty acid and the pyrrolidine spin label. The product was ninhydrin negative. The synthesis and purification of IV has been dealt with elsewhere [5].

All spectra were recorded on a Varian Electron

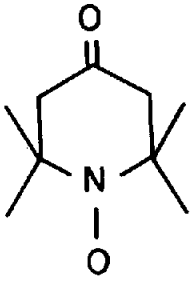
Paramagnetic Resonance Spectrometer, Model 4500. Spectra were recorded at room temperature.

Yeast cells (a haploid wild type, S288C, a strain of *Saccharomyces cerevisiae*) were grown on a synthetic medium, yeast nitrogen base, from DIFCO Laboratories (Detroit, Michigan) containing 2% dextrose. Cells were harvested in late log phase.

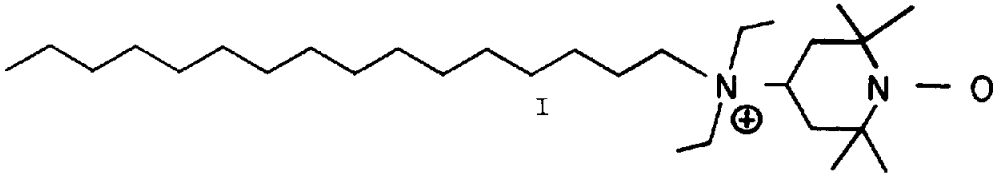
3. Results and discussion

The present study concentrates on two spin labels which are believed to localize on the surface of membranes. One of these labels has a negative charge and the other a positive charge. The hydrocarbon tails of the amphiphilic spin labels I and III should localize in the hydrocarbon zones of membranes. The charge groups should prevent the two spin labels from crossing membranous structures at a rapid rate due to charge–charge interactions. An amphiphilic phospholipid molecule spin labeled on the polar end was shown by Kornberg and McConnell to require about 6.5 h for half the phospholipids to cross the bilayer [6]. This spin label was used to measure the kinetics of the 'flip-flop' movement. We also tried similarly labeled phospholipids and found them to be much less sensitive to membrane modifications than the labels presented here. To help characterize the location of the spin labels, $NiCl_2$ and $K_3Fe(CN)_6$ are used as line broadening agents. The broadening agents effectively remove the signal originating from the population of spin label molecules located in the same environment. Since the line height of a first derivative spin label signal varies reciprocally with the square of the line width, the line height drastically decreases as the line width is increased. The broadening agents remove spin label signal by a physical interaction which requires only that the broadening agent be in the same environment as the spin label [2,7]. Both these ions are impermeable to yeast cells and therefore remove the signal outside the cells only ($NiCl_2$ permeability, see [2,7]; $Fe(CN)_6^{3-}$ permeability, see [8]). A spin label molecule having its signal quenched outside the membrane-bounded enclosure is chemically unmodified and again emits a signal upon traversing to the inside of the enclosure.

Yeast cells are used as an example of a simple cell system to illustrate the use of cell surface labels. Yeast cells grow anaerobically at near normal rates in 0.25 M $NiCl_2$ and at about half the normal rate after an initial lag period of several hours in 0.25 M $K_3Fe(CN)_6$. These cells are apparently free to drastic membrane effects induced by either ion. Thin sections and freeze-fracture replicas viewed by electron microscopy of whole yeast cells containing either 0.25 M $NiCl_2$ or 0.25 M $K_3Fe(CN)_6$ in their growth medium are indistinguishable from those of cells grown in medium containing neither. The spectrum of label I associated with intact yeast cells is shown in fig.1A. Fig.1B shows a spectrum of the same preparation after addition of 0.2 M $K_3Fe(CN)_6$. The remaining signal from I is much smaller than the 90% or greater expected of a spin label distributed randomly throughout the cellular membranes. The same experiment is repeated in fig.1C and 1D except that a relatively water-soluble label, II, was added to the yeast cells. Fig.1C shows the signal from label II dissolved in an aqueous yeast preparation. Adding 0.2 M $K_3Fe(CN)_6$ removes the signal of label II located outside the cells, leaving 15% of the signal



II



I

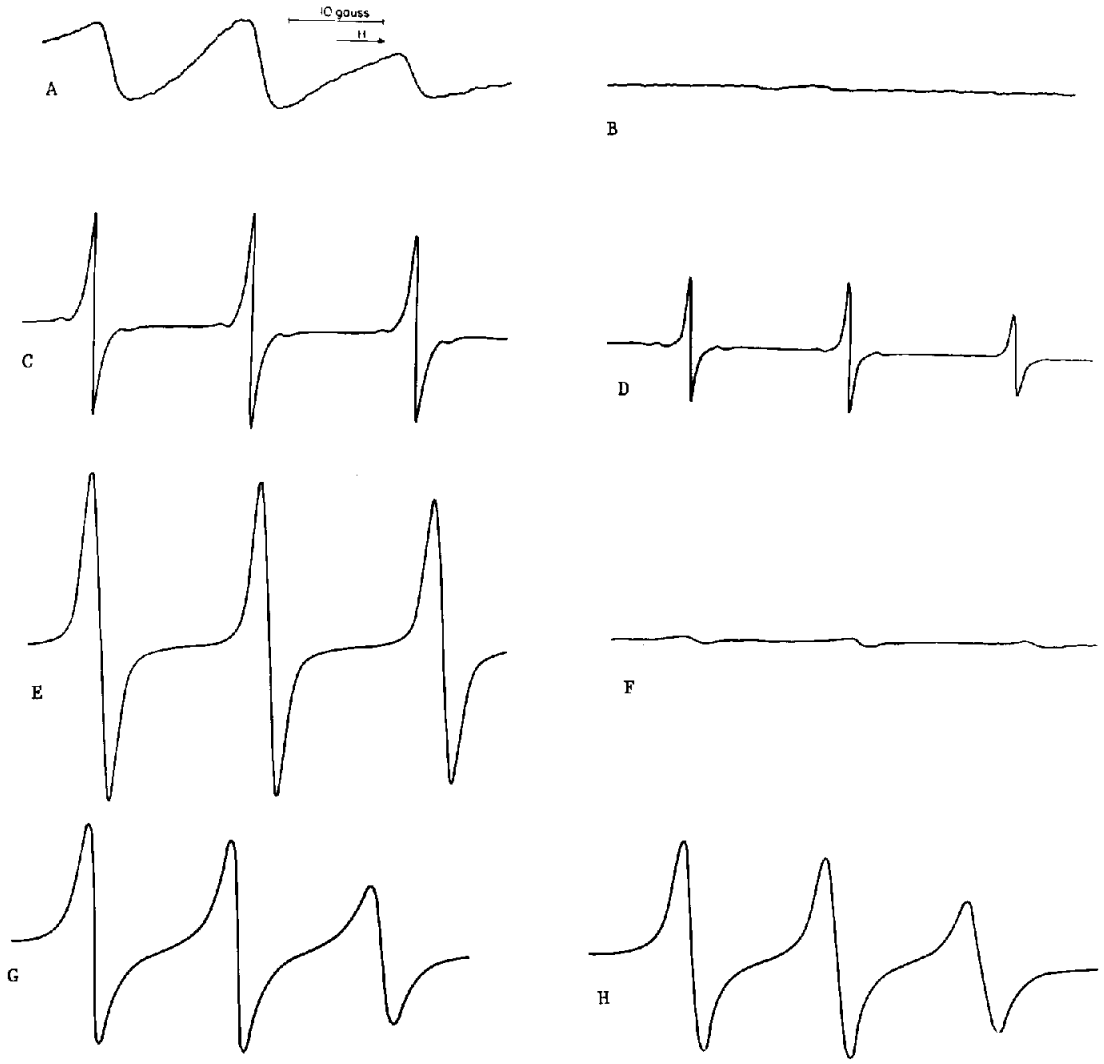
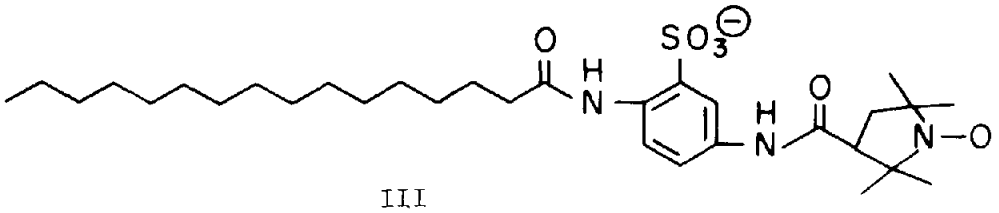


Fig.1. ESR spectra of the spin labels I, II, III and IV in yeast in the absence and presence of broadening ions. Relative instrument sensitivity is designated by S. (A) I (S=1); (B) I with 0.2 M $Fe(CN)_6^{3-}$ (S=1); (C) II (S=1); (D) II with 0.25 M Ni^{++} (S=4); (E) III (S=1); (F) III with 0.25 M Ni^{++} (S=8). (G) IV (S=1); (H) IV with 0.2 M $Fe(CN)_6^{3-}$ (S=1).

intensity. The remaining 15% corresponds closely to the expected internal cell volume (see table 1 and legend). Nearly all the signal can be removed by freezing the cells to increase their permeability to $Fe(CN)_6^{3-}$. The negatively charged surface label, III, similarly leaves only a trace of signal after treatment with 0.25 M $NiCl_2$, as is shown in fig.1E and 1F.

These two spin labels associated with the membranes of intact yeast cells result in a signal indicative of considerable molecular motion. Label I is more immobilized than label III. We expect that I ionically associates with negatively charged phosphate groups on the cell surface and as a result experiences hindrance to molecular motion by components which comprise the polar zones. Label III probably ionically associates with positively charged phospholipid groups and as a result resides at a location where it would experience less hindrance to rotation. Label III also has a different



III

Table 1
The removal of spin label signal by Ni^{++} or $Fe(CN)_6^{3-}$

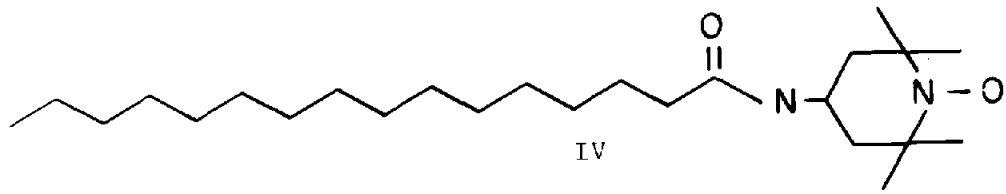
Spin label	% signal remaining in yeast after addition of 0.25 M Ni^{++}	or 0.2 M $Fe(CN)_6^{3-}$
I	0.2	$Fe(CN)_6^{3-}$
II	15	$Fe(CN)_6^{3-}$
III	0.5	Ni^{++}
IV	90	$Fe(CN)_6^{3-}$

The relative amount of spin label contributing to the signal was calculated using the relation, number of spins α W^2 h, where W and h are the first derivative line width and line height. The total internal volume of packed yeast cells was calculated and is 10% of the total vol of the sample, assuming the average diameter of each yeast cell to be 3 μm and is 16%, assuming an average diameter of 3.5 μm^3.

linkage to the ionic anchoring site which may well allow additional motional freedom.

Spin labels I and III have been employed in preliminary experiments on several cell and membrane preparations in order to obtain a general perspective in regard to potential usefulness. The spectral character undergoes marked changes in different membrane preparations. For example, spectra of these two spin labels in sarcoplasmic reticular membrane vesicles, mouse lung fibroblasts, and membrane vesicles made from bull sperm plasma membrane all indicate different degrees of immobilization. The spectra are sensitive to temperature changes, lipid composition changes, and a variety of other membrane structural modifications. The spectral changes from I are greater than those from III. These detailed studies will be presented at length elsewhere. Fig.1G and 1H show signals of label IV dissolved in the yeast cells without and with 0.2 M $K_3Fe(CN)_6$. Label IV is structurally similar to label I except that the alkyl chain is linked through an uncharged amide bond. The amide bond contributes a certain amount of polarity as does the N-oxyl group of the spin label. These two polar groups should cause the piperidine ring to orient toward the polar interface of membranes. This molecule, in fact, results in a hyperfine coupling of 15.9–16.3 gauss in yeast cells and sarcoplasmic reticular vesicles (the hyperfine coupling of IV is 17.1 gauss in water and 15.2 gauss in octane). There is only slight, if any, detectable loss of signal upon addition of 0.2 M $K_3Fe(CN)_6$ to the yeast cell preparation. The total amount of membrane interface in a yeast cell, including nuclear, tonoplast, mitochondrial, and endoplasmic reticular membranes is large compared to the outer cell or plasmalemma membrane surface; therefore, it is not surprising to see no loss of signal. This spin label has about 60% of its signal removed by $K_3Fe(CN)_6$ in a sarcoplasmic reticular membrane vesicle preparation, illustrating that label IV is distributed on both membrane surfaces and is adequately close to the membrane interface to allow quenching by $K_3Fe(CN)_6$.

These data indicate that surface labels can be used to localize the dominant part of the spin label signal on the membrane surface. The signal remaining after additon of $NiCl_2$ or $K_3Fe(CN)_6$, representing zones unavailable to the broadening agent, may arise from sites such as membrane channels, in phospholipid zones located under surface proteins, a percentage of the spin



IV

that has successfully flipped across the membrane, or perhaps other sequestered zones. We feel that the general approach of using surface spin labels has considerable promise in regard to the study of cell surface properties.

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