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Ionizable Nitroxides for Studying Local Electrostatic Properties of Lipid Bilayers and Protein Systems by EPR

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Abstract

Electrostatic interactions are known to play one of the major roles in the myriad of biochemical and biophysical processes. In this Chapter we describe biophysical methods to probe local electrostatic potentials of proteins and lipid bilayer systems that is based on an observation of reversible protonation of nitroxides by EPR. Two types of the electrostatic probes are discussed. The first one includes methanethiosulfonate derivatives of protonatable nitroxides that could be used for highly specific covalent modification of the cysteine's sulfhydryl groups. Such spin labels are very similar in magnetic parameters and chemical properties to conventional MTSL making them suitable for studying local electrostatic properties of protein-lipid interfaces. The second type of EPR probes is designed as spin-labeled phospholipids having a protonatable nitroxide tethered to the polar head group. The probes of both types report on their ionization state through changes in magnetic parameters and a degree of rotational averaging, thus, allowing one to determine the electrostatic contribution to the interfacial pK_a of the nitroxide, and, therefore, determining the local electrostatic potential. Due to their small molecular volume these probes cause a minimal perturbation to the protein or lipid system while covalent attachment secure the position of the reporter nitroxides. Experimental procedures to characterize and calibrate these probes by EPR and also the methods to analyze the EPR spectra by least-squares simulations are also outlined. The ionizable nitroxide labels and the nitroxide-labeled phospholipids described so far cover an exceptionally wide pH range from ca. 2.5 to 7.0 pH units making them suitable to study a broad range of biophysical phenomena especially at the negatively charged lipid bilayer surfaces. The rationale for selecting proper electrostatically neutral interface for calibrating such probes and example of studying surface potential of lipid bilayer is also described.

1. Introduction

Electrostatic interactions are known to play one of the major roles in the myriad of cellular and molecular biology processes ranging from protein folding to insertion of proteins, toxins, and viruses into membranes as well as other more complex events such as membrane fusion. Indeed, an essential feature of a living cell is in its ability to maintain and control an electrical potential across the lipid bilayer membrane that separates the cell from the rest of the world. Such a potential governs transport of electrically charged ions across the membrane, concentration of ions and other charged molecules at its surface, and, for charged residues of peripheral proteins, contributes to the free energy of binding to the membrane. The role of electrostatics is not limited to the membrane processes. Indeed, electrostatic forces are also directly involved in protein-protein association (1) and in structure and dynamics of DNA. (2)

Experimentally, local electrostatic interactions remain to be somewhat elusive parameters because of a relative scarcity of the spectroscopic methods capable of measuring these effects accurately and unambiguously. One of the methods for assessing local electric fields in proteins is based on the internal vibrational Stark effect of endogenous long-wavelength chromophores. (3–8) Other methods include NMR, (9–11) atomic force microscopy, (12–14) interaction force measurements, (15) second harmonic generation (SHG) charge screening measurements, (16) fluorescent spectroscopy, (17–19) and spin-probe electron-paramagnetic resonance (EPR). (20–30) All of these methods have some advantages as well as some limitations and drawbacks. For example, electrical potential of a lipid bilayer could be determined from measurements of a partition coefficient of a charged amphiphilic probe molecule between the lipid and the aqueous phases from analyzing continuous wave (CW) EPR spectra of the probe and an additional calibration. (26–28) The partition coefficient is directly related to the Gibbs free energy of transferring an amphiphilic EPR probe molecule from aqueous to a membrane phase but the location of the probe in the bilayer is largely unknown. One has also to be concerned of effects of the bilayer lipid composition on the probe's Gibbs energy as a source of additional errors.

The second group of EPR and NMR methods is based on measurements of molecular collisions between a specific site of a biomolecule such as a protein, DNA, or a lipid with charged molecules. For example, local electrostatic potentials of lipid bilayer and DNA were determined from CW electron-electron double resonance measurements of the collision frequency of a freely diffusing charged nitroxide with another nitroxide tag attached to a biomolecule in a site directed manner. (24, 25) While experimental results of the EPR collision exchange method demonstrated a remarkable agreement with the Debye-Hückel calculations for two small nitroxides, (31) in applications to larger molecules and membrane systems, this method is expected to suffer from the same uncertainty in the local diffusion constant as analogous NMR measurements of the site-specific relaxation enhancement upon collisions of exposed residues with charged paramagnetic relaxers. (9, 32) Indeed, the latter complication has been noted upon completion of a detailed NMR study of electrostatically driven molecular collisions. (33) Specifically, it was concluded that “the three different combinations of the data sets do not yield internally consistent values for the electrostatic contribution to the intermolecular free energy”. (33)

The third group of methods for evaluating local electrostatic potential is based on observing reversible ionization of specific moieties by NMR, fluorescence, or EPR. The main advantage of NMR is in not relying on any exogenous probe molecules that are typically required for EPR and fluorescence measurements. Residue-specific pK_a can be measured directly by NMR for protein and RNA samples that are labeled with ^{13}C and ^{15}N uniformly (34) or selectively. (35) Specialized NMR pulse sequences have been developed to measure the ^{13}C chemical shifts even for unfolded protein states where chemical shift overlap is the limiting resolution factor. (34) However, for unambiguous pK_a determination dynamics of proton exchange has to be slow on the NMR time scale. The latter condition limits the number of systems that could be studied.

The use of the ionizable molecular probes and associated methods is well developed in fluorescence spectroscopy mainly in applications to studies of electrostatic surface potentials

of lipid bilayers. One of the most common fluorescent probes for such purposes is 4-alkyl-7-hydroxycoumarin. The alkyl tail of the probe inserts into the lipid bilayer and positions the ionizable OH-group just below the lipid phosphate moiety. (19) As was noted by Fromherz, “a local perturbation of the interface by the chromophore is unavoidable, although the coumarin is a fluorescent probe of minimal size”. (19) We also note that in lipid bilayers the ionizable OH-group of coumarin is unavoidably positioned below the dipole of the phosphatidylcholine group. Such a location is somewhat deeper than one would wish to have for studying the bilayer surface potential. Also, a chromophore positioned right at the polar head region is expected to be sensitive to any changes in the lipid packing and, thus, would require additional calibrations for temperature and other effects.

Spin-labeling EPR represents a good alternative to fluorescence labeling of protein and membrane systems primarily because of the complementary nature of the data on local structure and dynamics of biomolecules this method is capable of providing. (36) The important feature of the method is a relatively small molecular volume of nitroxide-modified side chains that are comparable to phenylalanine, (37) thus, enduring a minimal perturbation to the protein structure. (38) To this date, almost all site-directed spin labeling (SDSL) EPR studies have been carried out with nitroxide MTSL (*S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonothioate), which has methanethiosulfonate (MTS) moiety that is highly thiol-specific. While MTSL has been proven to be a very useful probe of molecular structure and dynamics, it cannot be used for local pH determination because it does not have any groups that are ionized within the useful biochemical range. In order to overcome this deficiency, we have introduced cysteine-specific labels (39, 40) and lipid-mimicking EPR probes (41, 42) that are based on derivatives of imidazoline and imidazolidine nitroxides. These nitroxides contain basic functionalities in the heterocycles making their EPR spectra sensitive to pH changes near the probe pK_a . Specifically, the protonation of such functionality will result in acquiring a localized positive charge. Then a component of the positive internal electric field directed along the N–O bond will partially stabilize the nitrogen p orbital while destabilizing the p orbital of the oxygen nitrogen, leading to a partial shift of the spin density of the unpaired electron from N to O. (43) Decrease of the spin density on N will result in a smaller isotropic nitrogen hyperfine coupling constant A_{iso} and a larger isotropic g_{iso} -factor that can be measured from the EPR spectra.

Imidazoline and imidazolidine nitroxides are considered to be the most suitable for biophysical applications because of the tunable pK_a , reversible pH effects, and high sensitivity of the EPR spectrum to pH changes. (44) We note that while some of the imidazolidine nitroxides have been employed in studies of surface potentials and polarity of phospholipid bilayers (29) and human serum albumin (29, 45) in the past, the probe attachment was not fully specific. Moreover, these earlier EPR studies were based solely on measurements of isotropic nitrogen hyperfine constant A_{iso} , which could be affected by experimental conditions other than the proton exchange reactions.

In this Chapter we describe the use of nitroxides capable of reversible ionization by protonation to probe local proton concentration and electrostatic potentials of proteins and lipid bilayer systems by EPR. Two types of the electrostatic probes will be described. The

first type of protonable nitroxides is bearing the MTS group that is highly specific for covalent modification of the cysteine's sulfhydryl groups. Such spin labels are very similar in magnetic parameters and chemical properties to conventional MTSL making them suitable for studying local electrostatic properties of protein-lipid interfaces. The second type of EPR probes was designed as spin-labeled phospholipids having a protonatable nitroxide tethered to the polar head group. The probes of both types report on their ionization state through changes in magnetic parameters and a degree of rotational averaging. We described the methods of analysis of EPR spectra for evaluating the electrostatic contribution to the interfacial pK_a of the nitroxide, and, therefore, determining the local electrostatic potential. Finally, selective applications of these methods to studies interfacial potentials are described. Potential problems and future directions are also outlined.

2. EPR Characterization of pH-Sensitive Thiol-Specific Nitroxide Labels for Mapping Local Protein Electrostatics

Figure 1 shows chemical structures of thiol-specific labels including conventional MTSL and two pH-sensitive nitroxides synthesized and characterized so far. (39, 41) IMTSL has a tertiary amino group in the structure of the heterocycle, and IKMTSL is featured with an amidino group; the pK_a 's of these functionalities determine the pK_a range these probes are capable of reporting on. Note that the strong electron withdrawing effect of the nitroxide group decreases the intrinsic pK_a 's of these groups by several units of pK_a . (46, 47) Comparison of the chemical structure of IMTSL in its basic form to that of MTSL (see Fig. 1) reveals many similarities, thus, raising expectations for physical properties, including solubility, while the same structure of the attachment group makes MTSL labeling protocols applicable for site-directed protein chain modification with IMTSL. Uncharged IMTSL is expected to have little or no effects on the protein structure the same way as MTSL does: the latter label has been shown to have small, if any, effects on the protein structure and function. (38) Similar to MTSL that is widely employed in SDSL EPR, the high specificity of covalent attachment of these nitroxide labels to thiols was achieved by incorporating methanethiosulfonate (MTS) group into the structure. Chemical properties of both probes were found to be similar to MTSL. For example, it was reported that above neutral pH IMTSL would rapidly form biradicals which became evident from characteristic five-line EPR spectra (not shown). (40) Formation of the biradicals has also been observed for MTSL under similar conditions. It was speculated that at basic pH, some of the MTS groups are hydrolyzed to thiols, which would then react with the remaining methanethiosulfonates forming disulfide biradicals. (40) Thus, for the optimal labeling it is desirable avoiding pH above the neutral values.

On the basis of the similarity of molecular sizes and geometry of the nitroxide rings of MTSL and the basic form of IMTSL and the same length and the structure of the attachment tether, one would expect very similar rotational dynamics for the two spin-labels and, therefore, similar EPR spectra. Indeed, similar to other nitroxide, EPR spectra of both IMTSL and IKMTSL either free in solution or when covalently attached to small water-soluble peptides fall into a fast motion regime resulting in a well-resolved three line EPR

spectra similar to those shown in Figure 2. The effect of pH on the EPR spectra of IMTSL and IKMTSL is associated with proton exchange between a radical $R^\bullet$ and its conjugated acid $R^\bullet H^+$:

Typically, pH-induced changes in EPR spectra of nitroxides in fast motion limit are assessed from measurements of the isotropic nitrogen hyperfine coupling constant, A_{iso} . Depending on experimental conditions, this chemical exchange could be fast or slow on the EPR time scale. Assuming that for IMTSL this reaction is diffusion-controlled with a rate constant k_1 of $\approx 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ as for other pH-sensitive nitroxides (45) and that the difference in the resonant frequencies of the high-field nitrogen hyperfine components does not exceed of a $\nu \approx 4 \times 10^6 \text{ s}^{-1}$ (*i.e.*, 1.4 G), then the EPR spectra are in a fast exchange if: (45)

$$pK_a < \log(k_1 / \Delta\nu) \approx 3.4 \quad (2)$$

Thus, according to the estimate given by eq. (2) the EPR spectra of the probes with pK_a close to the physiological range should yield resolved components corresponding to $R^\bullet$ and the $R^\bullet H^+$ nitroxide forms, thus, simplifying identification and characterization of the protonation phenomena. For probes with a low intrinsic pK_a such as IMTSL, the slow exchange conditions could be achieved by carrying out experiments at high magnetic fields (*i.e.*, by increasing ν). (39, 40)

2.1. Calibration of fast-motion EPR spectra of IMTSL- and IKMTSL-labeled thiols

Similar to fluorescent pH indicators, pH-sensitive nitroxides have to be calibrated to determine the intrinsic pK_a^i . However, pK_a of a spectroscopic probe could change upon reacting with the cysteine sulfhydryl group if the chemical modification site is close to the moiety undergoing reversible protonation. This is indeed the case for IMTSL. (39) Typically, such a calibration is carried for adducts obtained by reacting the label with small unstructured cysteine-containing peptides (39) or 2-mercaptoethanol to mimic the inductive effect of the mercaptoethyl phosphate group for labels attached to the polar head group of 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (PTE) lipid. (41)

Figure 2 shows a typical series of X-band (9.5 GHz) EPR spectra obtained upon titration of an aqueous solution of a pH-sensitive nitroxide, specifically, IKMTSL-2-mercaptoethanol adduct (IKMTSL-2me, Fig. 5). All the spectra show three well-resolved nitrogen hyperfine coupling components indicating fast rotational motion of the nitroxide on the EPR time scale (Figure 2). Similar spectra are expected for small spin-labeled peptides and unstructured protein domains. While for IKMTSL-2me no splitting of the high field nitrogen hyperfine component has been observed, the spectra recorded at pH close to the label pK_a (such as at pH=5.86 and 6.27 in Fig. 2) showed some small asymmetry of the high field nitrogen hyperfine coupling component. Such spectra were simulated using EWVoigt software (48, 49) as a superposition of two fast motion nitroxide components whereas for the rest of the

spectra simulations with a single component were sufficient. The magnitude of the isotropic nitrogen hyperfine coupling component A_{iso} was determined directly from such fits. For the two component spectra the effective A_{iso} was averaged out proportionally to the weights of the individual components. (40) The observed decrease in A_{iso} upon lowering pH (Figure 3) is associated with the protonation of the tertiary amine and, therefore, could be fitted to a modified Henderson-Hasselbalch equation:

$$A_{\text{iso}} = \frac{A_{\text{iso}}(R^{\bullet}H^+) \cdot 10^{(\text{pH}-\text{p}K_a)} + A_{\text{iso}}(R^{\bullet})}{1 + 10^{(\text{pH}-\text{p}K_a)}}, \quad (3)$$

where $A_{\text{iso}}(R^{\bullet}H^+)$ and $A_{\text{iso}}(R^{\bullet})$ are the isotropic nitrogen hyperfine coupling constants for the fully protonated and non-protonated forms of the nitroxide. These best-fit titration curves are shown as solid lines in Figure 3 for IKMTSL-2me at 17.0 and 48.0 °C. The best-fit parameters are summarized in the Table 1 together with other adducts of pH-sensitive nitroxides studied.

The Table 1 demonstrates that for both IMTSL and IKMTSL A_{iso} is a highly sensitive parameter for local pH measurements with A_{iso} ranging from *ca.* 0.87 to 1.41 G – such changes could be accurately measured from fast motion EPR spectra. For IKMTSL the changes in the $\text{p}K_a$ upon covalent attachments to sulfhydryl groups are insignificant whereas the change for IMTSL is rather large (>1.5 pH units) because the chemical modification site for IKMTSL is further away from the protonatable group. Taken together, the two probes cover an exceptionally wide pH range from *ca.* 2.5 to 7.0 pH units.

2.2. Mapping of Local pH and Electrostatics of Peptide and Protein Systems with Methanethiosulfonate Derivatives of Ionizable Nitroxides

First and foremost the ionizable nitroxides report on local proton concentration measured as pH through changes in the EPR spectra recorded at pH close to the nitroxide $\text{p}K_a$. Thus, the initial EPR studies of peptide and protein systems with methanethiosulfonate derivatives of ionizable nitroxides were focused on measurements of local proton concentrations.

The first demonstrations of the method have been carried out using a series of IMTSL-labeled biomolecules including amino acid cysteine, short peptides (glutathione and P11 peptide (residues 925-933 of the B1 chain of the basement membrane-specific glycoprotein laminin)), (39) bacteriorhodopsin (50) and *iso*-1-cytochrome c from the yeast *Saccharomyces cerevisiae*. (40) It was shown that for IMTSL and IMTSL-labeled amino acid cysteine and a tripeptide glutathione an asymmetric charge acquired upon protonation and located chiefly on the N3 atom has a large effect on the nitroxide isotropic hyperfine coupling constant, $A_{\text{iso}} \approx 1.2\text{--}1.3$ G (Table 1), and also the isotropic g-factor,

$g_{\text{iso}} \approx 0.00026$. (40) The latter shift can be easily measured at high field/high frequency (HF) EPR experiments at 95 GHz (W-band) and above. (39, 40) The charge asymmetry with respect to the nitroxide ring provides a basis for differentiating the protonation and solvent polarity effects on magnetic parameters of IMTSL. Specifically, W-band EPR experiments with IMTSL in a series of solvents indicated that while g_{iso} correlates linearly with A_{iso} , the correlations are different for the neutral and the charged forms of the nitroxide. This was

related to the effects of solvent on the spin density at the oxygen atom of the NO group and on the excitation energy of the oxygen lone-pair orbital. (40) The observed difference in g_{iso} vs. A_{iso} correlation has been utilized in a W-band EPR study of the IMTSL-labeled P11 peptide (a fragment of the laminin B1 chain) to demonstrate that the changes in g_{iso} and A_{iso} are related to the proton-exchange reaction of the spin label but not to the local polarity changes (Fig. 4). (39) Thus, enhanced g-factor resolution of HF EPR and g_{iso} vs. A_{iso} correlations such as the one shown in Fig. 4 could be employed for mapping local polarity and proton concentration of protein surfaces.

3. pH-Sensitive Spin-Labeled Lipids for Measuring Surface Lipid Bilayers Electrostatics by EPR

Figure 5 shows chemical structures of spin-labeled phospholipids (SLP) with pH-reporting nitroxides IMTSL or IKMTSL covalently attached to the lipid's polar head group. The synthesis of these lipids has been described by Voinov *et al.* and is based on a covalent modification of a synthetic phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanol (PTE) with either IMTSL or IKMTSL. (41) Molecular probes of this type mimic the molecular structure of phospholipids as closely as possible, in both the acyl chain and the polar head regions. Due to its lipid-like nature, these probes do not partition between the lipid and aqueous phases of a lipid bilayer membrane but instead become its integral part. The chemical structure of pH-sensitive phospholipids ensures that the nitroxide moiety is positioned at the lipid bilayer interface. Perturbations to the lipid bilayer, including the phosphate group region, are expected to be minimal because of the compact volume of the nitroxide reporter group, especially for IMTSL-PTE.

3.1. Preparation of Spin-labeled Lipid Vesicles

Both IMTSL-PTE and IKMTSL-PTE are headgroup-modified phospholipids and, therefore, have very low solubility in water. For these reasons, mixing of these EPR probes with other lipids should be done as chloroform solutions although other organic solvents could be also used. Typically, doping lipid bilayers with 0.5–2.0 mol% of IMTSL-PTE or IKMTSL-PTE is sufficient for obtaining an easily detectable EPR signal without compromising properties of lipid bilayers. For the preparation of multilamellar lipid vesicles (MLVs) the readers could follow the standard procedures. (42) Homogeneously sized unilamellar or plurilamellar vesicles could be then prepared from MUVs by extrusion utilizing a filter with a desired pore size. (51)

3.2. EPR Titration Experiments with Lipid Bilayers

Similar to the EPR experiments with peptides and proteins labeled with ionizable nitroxides described in the Section 2, measurements of the surface electrostatic potential of lipid bilayers involves recording and analyzing a series of EPR spectra as a function of pH. However, one should pay attention to two additional aspects. Firstly, the phase transition temperatures of lipid bilayers are known to be pH-dependent (52) and, therefore, one should properly choose the temperature of the experiment so the lipid bilayer phase would remain the same over the entire titration range. Secondly, lipid bilayers represent a barrier to charged molecules including protons. Thus, the titration should be carried out in a way to

ensure uniform pH in the sample. The latter could be achieved by either incorporating a gramicidine A channel into lipid bilayers to provide for proton permeability (53) or by subjecting the lipid preparation to a few freeze-thaw cycles such as employed by Voinov *et al.* (42) The former approach is imperative for titration of unilamellar vesicles.

Finally, equilibration of lipid bilayers with electrolytes and buffers and the consequent biophysical measurements should always be carried out at the same temperature, or at least without changing the bilayer phase. Indeed, results of an EPR titration of DMPG (1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-(1-glycerol))) bilayer doped with IMTSL-PTE at 48 °C were found to be significantly different for the MLV samples that were pH-equilibrated at 40 and 20 °C. (42) A likely reason for such a discrepancy is a difference in the binding constants of counterions and/or protons to the lipid bilayers in different phase states. Thus, the bulk pH and electrolyte concentration of the aqueous phase of a sample containing a high fraction of DMPG (typically, 10–20 v%) equilibrated at 20 °C when the bilayer is in the gel phase would not necessarily remain the same after heating the same sample to 48 °C and changing the bilayer phase to fluid.

3.3. Analysis of EPR Spectra in Titration Experiments: Least-squares Decomposition of Experimental EPR Spectra Using a Two-site Slow-Exchange Model

Figure 6 shows typical results of EPR titration experiments of lipid bilayers doped with headgroup-labeled phospholipids. The left panel of Fig. 6 demonstrates nearly identical EPR spectra for DMPG MLVs doped with 1 mol % of the nonionizable MTSL-PTE reference probe from pH=2.0 to 7.0. The minor changes below pH=2.0 are likely caused by a bilayer reorganization upon protonation of the lipid phosphatidyl groups ($pK_a \approx 2-3$). (54) Excluding these minor effects, the EPR spectra of MTSL-PTE – a lipid labeled with a nonionizable nitroxide – appear to be essentially insensitive to pH-changes even in the range of protonation of the phosphatidyl moiety.

In contrast, EPR spectra of DMPG MLVs doped with 1 mol% of ionizable IMTSL-PTE display significant changes (Fig. 6, right panel) that were also found to be reversible. (42) Because the nonionizable MTSL-PTE reveals no changes in local microviscosity (Fig. 6, left), the observed changes in rotational dynamics of IMTSL-PTE must be attributable to the appearance of an ionized fraction of the nitroxide and electrostatic interactions of the protonated nitroxide with the negatively charged bilayer interface. (42) This ionized fraction is responsible for the appearance of a low field shoulder at pH<5.0 that is characteristic of a slower and a more restricted nitroxide tumbling. We note here that while the magnetic parameters of the nitroxide are also affected by protonation, it is the effect of electrostatic interaction on the nitroxide tumbling rate that causes the largest and easily detectable changes in the EPR spectra shown in Fig. 6 (right panel).

For the purpose of EPR titration experiments we are only interested in determining a fraction of the protonated species, f_{RH^+} , rather than extracting the whole set of nitroxide motion parameters from the experimental spectra. Therefore, in order to reduce the number of adjustable parameters and to increase the accuracy of f_{RH^+} measurement, the following simplified slow chemical exchange model was found to be useful. Specifically, an

experimental EPR spectrum at an intermediate pH, $I(B)$, is assumed to be a superposition of the spectra from the neutral and ionized species, $F_R(B)$ and $F_{RH^+}(B)$, respectively:

$$I(B) = a \cdot F_R(B) + b \cdot F_{RH^+}(B) \quad (4)$$

For the purpose of our decomposition procedure $F_R(B)$ and $F_{RH^+}(B)$ spectra could be measured experimentally and then used for a least-squares decomposition to derive the coefficients a and b . The fraction $f = D_R / (D_R + D_{RH^+})$ of the nonprotonated form of the nitroxide is then calculated from the double integrals D_R and D_{RH^+} of the corresponding $F_R(B)$ and $F_{RH^+}(B)$ spectra. However, we note that some of the experimental EPR spectra may contain an admixture of an out-of-phase EPR signal and also be shifted in magnetic field due to a shift in the resonator frequency. To account for these experimental factors we have employed a convolution filtering that allows for correcting of the phase distortion by digital convolution with a Lorentzian function of the desired phase. (55) Specifically, the phase shift φ and φ_+ , magnetic field B and B_+ parameters as well as the intensity coefficients a and b were adjusted during the least-squares Levenberg–Marquardt optimization to minimize:

$$I(B) \otimes L(0, 0, \Delta B_{pp}) - \left\{ a \cdot f_R(B) \otimes L(\Delta B_R, \Delta \varphi_R, \Delta B_{pp}) + b \cdot f_{RH^+}(B) \otimes L(\Delta B_{RH^+}, \Delta \varphi_{RH^+}, \Delta B_{pp}) \right\} \rightarrow 0 \quad (5)$$

where the operator $\otimes$ stands for the convolution integral and $L(B_i, \varphi_i, B_{pp})$ are amplitude-normalized Lorentzian functions with peak-to-peak width of B_{pp} , phase shift from the pure absorption of φ_i and magnetic field shift B_i .

This form of fitting allows for adjusting the phase misalignment and the field positions by varying parameters of the corresponding $L(B_i, \varphi_i, B_{pp})$ functions that are calculated analytically while $F_R(B)$ and $F_{RH^+}(B)$ are measured experimentally. Typically, the parameter B_{pp} (which should be the same for all the three Lorentzian functions in the expression (5)) was set to be equal to three digitizing intervals of an EPR spectrum.

Note that this decomposition method requires experimental reference spectra for the protonated and non-protonated nitroxide forms. Because pH corresponding to such spectra is not known *a priori*, the EPR spectra are usually measured at increments of about 0.5 pH units and then compared. If no measurable changes are observed in the two consecutive spectra, either could be taken as a reference.

Figure 7 illustrates a least-squares decomposition of an experimental two-component X-band EPR spectrum from 1 mol% IMTSL-PTE in 65 nm unilamellar DMPG vesicles equilibrated at 17 °C with 50 mM buffer at pH=4.88 and measured at 17 °C. The least-squares decomposition into non-protonated and protonated components is shown as B and C, respectively. The residual of the fit – the difference between the experimental and two

simulated spectra (Fig. 7E) – demonstrates that the two-site slow exchange model and the decomposition procedure through convolution filtering work exceptionally well.

3.4. Interfacial pK_a and the Choice of an Electrically Neutral Reference Interface

For an ionizable group of a molecule located at a charged interface, such as pH-sensitive nitroxides tethered to the lipid head groups, the observed interfacial pK_a^i contains contributions arising from changes in the Gibbs free energy, G_{pol} , upon transferring the probe from the bulk water onto the interface with a different local electric permittivity, ϵ_1 , and the term G_{el} attributed to the local electric potential, Ψ , that affects the equilibrium between charged and uncharged species. Then the observed interfacial pK_a^i is given by:

$$pK_a^i = pK_a^0 + \Delta pK_a^{el} + \Delta pK_a^{pol}, \quad (6)$$

where pK_a^0 is an intrinsic pK_a of the probe observed in a pure water, and ΔpK_a^{pol} and ΔpK_a^{el} are the electrostatic and the polarity contributions, respectively. (17, 19) The surface electrostatic potential Ψ is related to ΔpK_a^{el} as:

$$\Delta pK_a^{el} = -e\Psi / \ln(10)kT, \quad (7)$$

where e is the elementary charge, k is the Boltzmann constant, and T is the absolute temperature.

The electrostatic pK_a shift, ΔpK_a^{el} , can be derived from equation (6) if other contributions to pK_a^i are otherwise determined. While pK_a^0 can be obtained from the experimental EPR titration of nitroxides in water or model compounds (*i.e.*, IMTSL-2-mercaptoethanol adduct) when the latter are practically insoluble in water (such as IMTSL-PTE), (41) the value of ΔpK_a^{pol} could be affected by the position of the spectroscopic reporter group with respect to the interface, as well as by specific chemical moieties in the immediate vicinity of the probe. Experimentally, this problem could be resolved by measuring pK_a^i for a probe incorporated into a model system with a chemically similar but uncharged interface, so that $\Psi=0$ and $\Delta pK_a^{el}=0$. Early studies of fluorescent pH indicators employed micelles composed of the nonionic detergent polyoxyethylene isooctyl phenyl ether (Triton X-100), as a neutral (uncharged) reference interface.^{17,19} More recently, slightly more polar non-ionic amphiphilic alkylglucoside surfactants have also been suggested and tested. (56) One disadvantage of the alkylglucoside micelles is that the measured ΔpK_a^{pol} value could be affected by specific interactions of the hydroxyl groups of the surfactant with the molecular probe and the surrounding water molecules. (57) Other authors employed zwitterionic DMPC vesicles as an “uncharged” reference for deriving the electrostatic surface potential of charged lipid bilayers. (29, 58, 59) We note here that while the vesicles formed from zwitterionic DMPC exhibit zero net charge, there is a charge separation within the lipid polar head group. The latter leads to an electrical potential across the polar head region of

the lipid (or detergent) monolayer and may produce a proton (ion) concentration gradient between the interface and the bulk water. Moreover, in addition to formal charges localized at the polar head groups of the lipid molecules, a significant density of molecular dipoles is thought to exist between the head group and the hydrophobic region of the membrane. (60) These combined dipole moments may noticeably affect the local surface potential especially for bilayers formed from zwitterionic lipids even though these molecules formally have the net zero charge. (61, 62) For these and other reasons, the use of zwitterionic DPPC and DSPC references for ΔpK_a^{pol} was noted to be inappropriate. (63) Furthermore, experimental pK_a^i of IMTSL-PTE in DMPC MLVs reported by Voinov *et al.* (42) provided another argument that DMPC vesicles may be unsuitable as electrically neutral references for measurement of bilayer electrostatic potential. To conclude, despite numerous literature suggestions for better reference systems, Triton X-100 micelles are thought to be the most suitable choice for the neutral (uncharged) reference interface as first described more than 30 years ago.^{17,19}

Thus, we have chosen the Triton X-100 micelles as the reference interface. (41, 42) The pK_a^i of IMTSL-PTE in Triton X-100 micelles was shown to have only a slight temperature dependence yielding very similar values of 2.52 ± 0.01 and 2.39 ± 0.03 units of pH at 23.0 and 48.0 °C, respectively (see also Table 1). (41) Using the intrinsic $pK_a^0 = 3.33 \pm 0.03$ of IMTSL-PTE, the ΔpK_a^{pol} values were determined to be -0.81 ± 0.03 (23.0 °C) and -0.94 ± 0.04 (48.0 °C). These values of ΔpK_a^{pol} demonstrate that the pH-sensitive nitroxide of IMTSL-PTE experiences a significantly less polar environment at the interface of the Triton X-100 micelles *vs.* that of pure water. Voinov *et al.* also reported a series of EPR titrations of IMTSL-2-mercaptoethanol adduct in mixed water/isopropyl alcohol solution that allowed for calibrating the polarity-induced pK_a shifts, ΔpK_a^{pol} , *vs.* bulk solvent dielectric permittivity, ϵ . (41) These calibration data allowed for estimating the local dielectric constant, ϵ_{eff} , experienced by the reporter nitroxide of the IMTSL-PTE lipid incorporated into the nonionic Triton X-100 micelles as 60 ± 5 and 57 ± 5 at 23 and 48 °C, respectively. (41)

To conclude, once the reference interface is chosen and ΔpK_a^{pol} is determined, then ΔpK_a^{el} and the corresponding local electrical potential Ψ can be determined from eqs. (6) and (7). For the lipid bilayer interface, one can use experimental data for IMTSL-PTE in Triton X-100 micelles summarized in the Table 1 to determine ΔpK_a^{pol} . Other ionizable EPR probes can be calibrated the same way.

3.5. Surface Charge and Potential Calculation Using the Gouy-Chapman Theory

The experimental measurements of the bilayer surface potential described in the preceding section could be compared with the widely accepted the Gouy-Chapman (GC) theory. According to the GC theory the surface potential, Ψ_{GC} , of a lipid bilayer is given by: (64)

$$\Psi_{GC} = \frac{2k_B T}{e_0} \operatorname{asinh} \left(\frac{\lambda_D e_0 \sigma}{2\epsilon_0 \epsilon k_B T} \right), \quad (8)$$

where σ is the lipid surface charge density, λ_D is the Debye screening length, k_B is the Boltzmann constant, e_0 is the elementary electric charge, ε_0 is the permittivity of vacuum, ε is the dielectric constant of the medium ($\varepsilon=78$ for water), and T is the absolute temperature.

The Debye screening length is given by:

$$\lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon k_B T}{2000 e_0^2 N_A C_{el}}}, \quad (9)$$

where N_A is Avogadro's number and C_{el} is the bulk molar electrolyte concentration.

The surface charge density can be estimated as:

$$\sigma = -\frac{e_0 \alpha}{A_L}, \quad (10)$$

where A_L is the surface area per ionizable group of a lipid and α is the degree of dissociation of the phosphatidyl group of a lipid. If the pK_a of this group is lower than the range of the pH values used in the experiments, then $\alpha \approx 1$.

Experimentally determined surface electrostatic potentials can also be used to estimate the surface charge density, σ , of lipid bilayers using the Gouy-Chapman theory. For a monovalent electrolyte with bulk concentration C_{el} the surface density σ is given by:

$$\sigma = \sqrt{8000 k_B T \varepsilon \varepsilon_0 C_{el} N_A} \sinh\left(\frac{e_0 \Psi}{2 k_B T}\right), \quad (11)$$

4. Conclusions and Outlook

In this Chapter we described the use of nitroxides capable of reversible protonation to probe local proton concentration and electrostatic potentials of proteins and lipid bilayer systems by EPR. These probes report on their ionization state through changes in magnetic parameters and a degree of rotational averaging. We have also outlined experimental procedures to characterize and calibrate these probes by EPR and also the methods to analyze the EPR spectra by least-squares simulations. We have summarized EPR and ionization parameters for IMTSL and IKMTSL and their derivatives including phospholipids. Together, these two probes cover an exceptionally wide pH range from ca. 2.5 to 7.0 pH units making them suitable to study a broad range of proton and charge transfer-related phenomena especially at the negatively charged lipid bilayer surfaces. We have also described the rationale for selecting proper electrostatically neutral interface for calibrating such probes and example of studying surface potential of lipid bilayer. The methods are general and can be applied to characterize other ionizable nitroxides synthesized in the future.

The authors believe that the use of ionizable nitroxides in biophysical EPR remains to be largely unexplored. The method is expected to shed new light on the role of electrostatic interactions in the protein function - from the operation of ion channels to assembly of membrane proteins and to the charge separation in membranes of photoactive proteins. The method is also uniquely capable of mapping local dielectric constant along membrane protein-lipid bilayer interface as a function of the basic thermodynamic parameters such as temperature and ion concentration. Thus, the fundamental questions regarding effects of protein sequence and lipid composition on the dielectric properties of the interface could be answered by this method. Another promising direction lays in studies of local electrostatics of oligonucleotides and oligonucleotide-protein complexes.

However, several problems remain. Firstly, at the moment of this writing none of the ionizable nitroxide labels or head-group-labeled lipids described in this Chapter are available commercially. This could be changed rapidly as the synthetic methods are described in the literature and are not protected by the US Patents, to the best of the authors' knowledge. Secondly, new probes with higher intrinsic pK_a would be beneficial for probing local dielectric environment inside lipid bilayers, where the local proton concentration is a few orders of magnitude lower vs. the bulk solution. One may also wish to develop ionizable nitroxides in a form of unnatural amino acids and/or less bulky than IKMTSL. Finally, but not lastly, additional method development is needed to evaluate polarity contribution ΔpK_a^{pol} at different protein sites. In this respect, High Field/High Frequency (HF) EPR experiments at low temperatures could be very useful because magnetic parameters of the nitroxides (both g -matrix and A -tensor) determined from the rigid limit HF EPR with a high accuracy are known to report on local polarity. (65, 66) However, one should proceed with a caution because the effective polarity of frozen solvent glasses in the vicinity of dipolar solutes could be altered by a partial ordering of the solute molecules. (67)

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Figure 1.

Chemical structures of thiol-specific nitroxide labels including conventional MTSL (*S*-(1-oxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonylthioate) that is insensitive to pH changes and pH-sensitive IMTSL (*S*-(1-oxy-2,2,3,5,5-pentamethylimidazolidin-4-yl)methyl methanesulfonylthioate) and IKMTSL (*S*-4-(4-(dimethylamino)-2-ethyl-5,5-dimethyl-1-oxy-2,5-dihydro-1*H*-imidazol-2-yl)benzyl methane-sulfonylthioate).

Figure 2.

Representative X-band (9.5 GHz) EPR spectra of IKMTSL-2-mercaptoethanol adduct (IKMTSL-2me) measured at 17.0 °C in a series of 50 mM buffer solutions of various pH indicated next to the spectra. Vertical dashed lines mark approximate positions of the maximum of the high field nitrogen hyperfine coupling components corresponding to the protonated $R^{\bullet}H^{\ddagger}$ and the non-protonated $R^{\bullet}$ forms of IKMTSL-2me and are given as guides for an eye. Approximate magnitude of the isotropic nitrogen hyperfine coupling constant, A_{iso} , is shown by an arrow.

Figure 3.

Experimental X-band (9.5 GHz) EPR titration data for IKMTSL-2-mercaptoethanol adduct (IKMTSL-2me) measured at 17.0 (●) and 48.0 °C (○) in 50 mM buffer solutions. Least-squares fits to Henderson-Hasselbalch equation (3) are shown as solid lines with the parameters listed in the Table 1.

Figure 4.

Isotropic magnetic parameters g_{iso} vs. A_{iso} obtained from room temperature solution W-band (95 GHz) EPR spectra. Open squares: IMTSL in a series of protic and aprotic solvents and their mixtures: 1 - toluene, 2 - acetonitrile, 3 - acetone, 4 - iso-propanol, 5 - ethanol, 6 - water/ethanol mixture (3:7, v/v), 7 - water/ethanol mixture (7:3, v/v), 8 - water (buffered to pH 6.0). Titration parameters for: IMTSL (open circles) and IMTSL-P11 (filled squares). Linear regressions are shown as solid lines and discussed in the text. Reproduced with permission from Voinov *et al.* (40)

Figure 5.

Chemical structures of phospholipid-based nitroxide electrostatic EPR probes IMTSL-PTE ((*S*)-2,3-bis(palmitoyloxy)propyl 2-(((1-oxyl-2,2,3,5,5-pentamethylimidazolidin-4-yl)methyl)disulfanyl)ethyl phosphate) and IKMTSL-PTE ((*S*)-2,3-bis(palmitoyloxy)propyl 2-((4-(4-(dimethylamino)-2-ethyl-1-oxyl-5,5-dimethyl-2,5-dihydro-1*H*-imidazol-2-yl)benzyl)disulfanyl)ethyl phosphate) are compared with non-ionizable analogue of IMTSL-PTE – MTSL-PTE ((*S*)-2,3-bis(palmitoyloxy)propyl 2-(((1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl)disulfanyl)ethyl phosphate), and of the adduct of IKMTSL with 2-mercaptoethanol (IKMTSL-2me).

Figure 6. Representative X-band (9.5 GHz) EPR spectra from pH titration experiments of DMPG MLVs doped with 1 mol % of either pH-sensitive IMTSL-PTE (right) or control MTSL-PTE (left). Spectra were acquired at 17 °C when the bilayer is in a gel phase. Reproduced with permission from Voinov *et al.* (42)

Figure 7.

(A) Experimental X-band (9.5 GHz) EPR spectrum of 65 nm unilamellar IMTSL-PTE-doped POPG vesicles (1:100 mol%) equilibrated at 17 °C with 50 mM buffer at pH=4.88 and measured at 17 °C shown after a digital convolution with a Lorentzian function with peak-to-peak linewidth of just $B_{pp}=0.146$ G (or 3 digitizing intervals Least-squares decomposition of the spectrum (A) into component corresponding to nonprotonated (B) and protonated (C) forms of the nitroxide. (D): Simulated composite spectrum. (E): Residual of the fit, *i.e.*, the difference between the experimental and the sum of the simulated spectra. Panels B and C are the actual reference spectra convolved with the same $B_{pp}=0.146$ G and scaled by amplitude.

Table 1

Experimental pK_a and isotropic nitrogen hyperfine coupling constants A_{iso} for the fully protonated (R^+H^+) and non-protonated (R^+) forms of the nitroxides titrated in aqueous solutions buffered at 50 mM and IMTSL-PTE incorporated into Triton X-100 micelles at 0.5 mol%. Full chemical names of IMTSL and IKMTSL adducts are given in the text.

Nitroxide	T, °C	$A_{iso}(R^+H^+)$, G	$A_{iso}(R^+)$, G	pK_a
IMTSL	19.0	14.34±0.04	15.75±0.03	1.58±0.03
IMTSL	37.0	14.36±0.06	15.75±0.04	1.54±0.08
IMTSL-cys	19.0	14.52±0.04	15.79±0.04	3.29±0.08
IMTSL-glu	19.0	14.51±0.01	15.82±0.02	3.17±0.03
IMTSL-2me	19.0	14.58±0.01	15.86±0.01	3.33±0.03
IKMTSL	17.0	14.56±0.04	15.33±0.04	5.68±0.01
IKMTSL-cys	17.0	14.58±0.02	15.48±0.02	5.92±0.04
IKMTSL-glu	17.0	14.62±0.01	15.46±0.02	6.15±0.03
IKMTSL-2me	17.0	14.64±0.01	15.53±0.01	6.16±0.03
IKMTSL-2me	48.0	14.65±0.01	15.52±0.01	5.70±0.02
IMTSL-PTE, Triton X-100	23.0	14.19±0.01	15.19±0.01	2.52±0.01
IMTSL-PTE, Triton X-100	48.0	14.25±0.01	15.13±0.01	2.39±0.03