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Perspective

# Perspective on the Effect of Membrane Mimetics on Dynamic Properties of Integral Membrane Proteins

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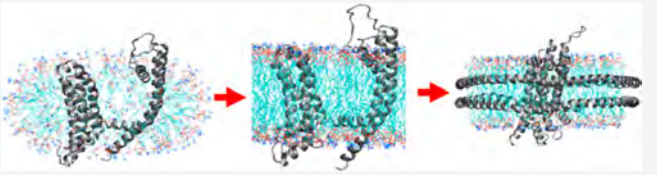
**ABSTRACT:** Integral membrane proteins are embedded into cell membranes by spanning the width of the lipid bilayer. They play an essential role in important biological functions for the survival of living organisms. Their functions include the transportation of ions and molecules across the cell membrane and initiating signaling pathways. The dynamic behavior of integral membrane proteins is very important for their function. Due to the complex behavior of integral membrane proteins in the cell membrane, studying their structural dynamics using biophysical approaches is challenging. Here, we concisely discuss challenges and recent advances in technical and methodological aspects of biophysical approaches for gleaning dynamic properties of integral membrane proteins to answer pertinent biological questions associated with these proteins.





## 1. INTRODUCTION AND SIGNIFICANCE

Integral or intrinsic membrane proteins associate permanently with biological membranes. Spanning the membrane, these proteins are surrounded by annular lipids and can be separated from the membrane only by detergents, nonpolar solvents, or denaturing agents. The most common type of integral membrane proteins that span the entire width of the biological membrane is known as transmembrane proteins. Single-pass transmembrane proteins crossing once across the membrane with their carboxyl-terminus positioned toward the cytosol can be categorized as Type 1 and amino-terminus positioned toward the cytosol can be categorized as Type 2. Type 3 contains multiple transmembrane domains in a single polypeptide. Type 4 assembles several polypeptides together in a channel through the membrane. Some examples of integral membrane proteins are bacteriorhodopsin, the prokaryotic potassium channel KcsA, phospholamban (PLB), voltage gated potassium channel (KCNQ1), KCNE1, KCNE3, *Escherichia coli* ferric citrate transporter FecA, GM2 activator protein, ABC transporter MsbA and pentameric ligand-gated ion channels (pLGICs). Integral membrane proteins contain a significant fraction of the proteins that are encoded in an organism's genome. Integral membrane proteins are mobile within the membrane lateral plane. They can form complexes by numerous molecular interactions and for various functions.[1,2] These molecular interactions lead to a highly heterogeneous organization of proteins within the membrane.[3] The interface region between the head groups and hydrophobic chains of the lipid bilayer provides the stability of certain amino acids (e.g., aromatic). The lateral pressure across the lipid chain develops the complex environment where protein folding and function occur.[4]

Integral membrane proteins are involved in several biological processes important for the survival of living organisms. They are involved in the transport of molecules across the bilayer membrane and the transduction of energy and signals. This makes integral membrane proteins functionally important and serve as drug targets.[4] Membrane-embedded enzymes are involved in catalyzing chemical reactions.[5] Misfolding and mutations of integral membrane proteins lead to several health-related disorders and diseases such as neuronal channelopathies, Charcot-Marie-Tooth disease type 1A (CMT1A), long QT syndrome, and cardiac arrhythmia.[6−9] The conformational dynamics of membrane proteins during their interaction with lipid bilayers is a critical element of their functions. Understanding the functional mechanism of integral membrane proteins requires knowledge of how these proteins are coupled to their lipid bilayer membrane environment. Despite their importance, there is an outstanding challenge in obtaining information about how the complex environment affects the structure, dynamics, and function of the membrane proteins. Dynamic properties of integral membrane proteins are essential to understand their structure−function relationship. Challenges in accessing the dynamic information on integral membrane proteins often arise due to low expression, purification yields, and lack of functional membrane mimetics under physiological

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conditions. Proper sample preparation of integral membrane proteins is needed for biophysical studies and requires extensive sample optimizations. In this Perspective, we briefly discuss challenges, current advances and effect of various membrane mimetics for studying dynamic properties of integral membrane proteins using biophysical approaches with emphasis on EPR spectroscopy.

## 2. CURRENT ADVANCES IN MEMBRANE MIMETIC SYSTEMS FOR STUDYING INTEGRAL MEMBRANE PROTEINS

In the past, significant efforts have been made to develop membrane mimetics that can provide a physiologically relevant membrane state for the biophysical studies of membrane proteins. However, no mimetic system is universal for studying all membrane proteins. The most widely used solvent for membrane proteins for biophysical studies is detergent micelles. It is easier to extract proteins from the plasma membrane in detergent micelles. However, the biophysical data obtained for micelle samples may not reflect the physiological state of the native lipid bilayer membrane condition due to the lack of lipid bilayer structure. A mixture of long-chain lipids and short-chain detergents has been used to solubilize the proteins to form artificial lipid bilayers known as bicelles.[10] Bicelles are smaller in size and can provide homogeneous sample conditions, but it is challenging to find a specific combination of lipids and detergents to solubilize the protein while protecting the functional integrity of the protein. A more natural mimetic condition for integral membrane proteins is solubilization in liposomes. Liposomes form a lipid bilayer membrane, but they are heterogeneous and larger in size than other mimetic systems. This limits the application of liposome systems for several biophysical studies such as NMR spectroscopy.[11] Nanodiscs or nanolipoprotein particles (NLPs) are other options for solubilization of the protein to obtain dispersed sample preparation suitable to obtain better quality biophysical measurements. This technique utilizes membrane scaffold proteins to form specific nanodisc sizes.[12−14] This can influence the optical properties of target proteins. Hence, its application is challenging for several integral membrane proteins. A recent emerging approach of using lipodisq nanoparticles or styrene maleic acid lipid nanoparticles (SMALPs) has been rapidly gaining popularity for biophysical studies of membrane proteins.[15−19] This approach utilizes a styrene and maleic acid (SMA) copolymer wrapped around protein incorporated phospholipids without the use of detergent to form lipodisq nanoparticles or SMALPs.

Lipodisq nanoparticles or SMALPs can maintain the structural and functional integrity of membrane proteins which is very difficult for traditional membrane systems.[22,23] Lipodisq nanoparticles can provide better quality experimental data for various biophysical techniques such as NMR and EPR for studying the structural dynamics of integral membrane proteins. Several forms of SMA copolymers are available in varying sizes and ratios of styrene to maleic acids to solubilize membrane proteins.[15,24,25] Various forms of reversible addition−fragmentation chain transfer (RAFT)-synthesized SMA polymers and di-isobutylene maleic acid (DIBMA) copolymers are also available to solubilize membrane proteins.[26−29] Figure 1 shows an illustrative example of KCNQ1 incorporated into different membrane environments. The dynamic motion of integral membrane proteins is higher in detergent micelles, while the dynamic motion is restricted in liposomes when compared to that in micelles and this motion is further restricted in nanodiscs or lipodisq nanoparticles.[16,30−33]

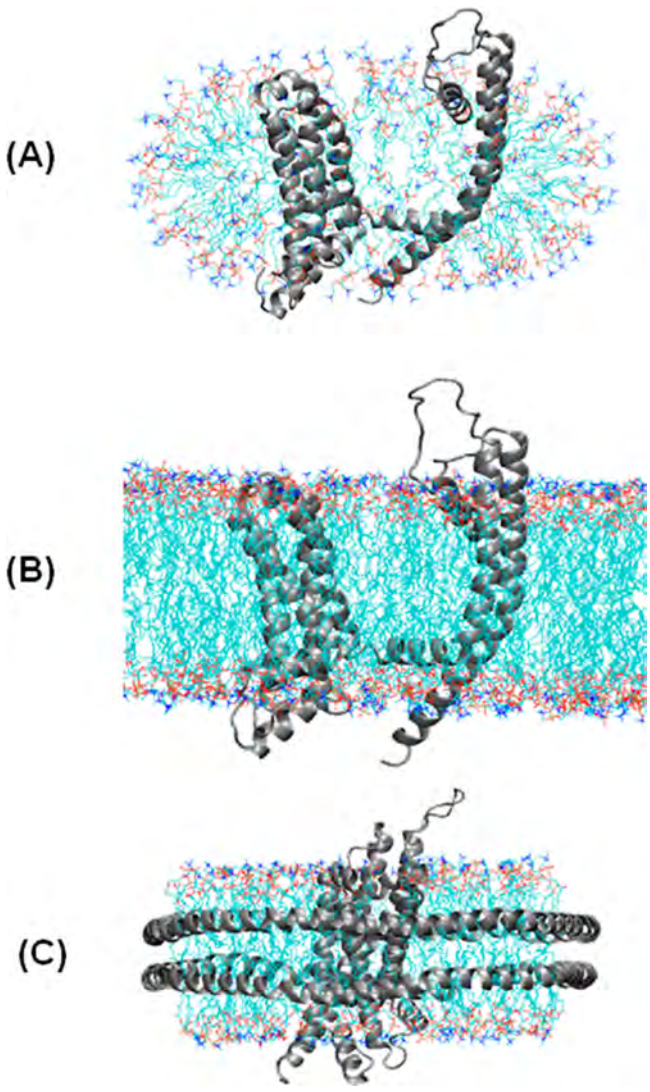


**Figure 1.** Cartoon representation of an illustrative example of a membrane protein (KCNQ1) (PDB ID: 6V00)[20] incorporated into dodecyl phosphatidylcholine (DPC) micelles (A), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) lipid bilayers (B), and nanodisc (C). The image was prepared using visual molecular dynamics (VMD)[21] and molecular modeling was performed using CHARMM-GUI (http://www.charmm-gui.org).

## 3. CHALLENGES AND PROGRESSES IN BIOPHYSICAL METHODS FOR PROBING DYNAMIC PROPERTIES OF INTEGRAL MEMBRANE PROTEINS

Great advances have been made in the technology and methodology of structural biology techniques to study structural and dynamic properties of integral membrane proteins. Challenges for applying biophysical approaches to study these proteins arise due to difficulties in obtaining sample conditions appropriate to a particular biophysical experiment to obtain a superior quality experimental data. Some modern biophysical tools that are currently widely utilized to characterize the dynamic behavior of integral membrane proteins in a lipid membrane environment are solution and solid-state nuclear magnetic resonance (NMR) spectroscopy,[34−37] Förster reso-


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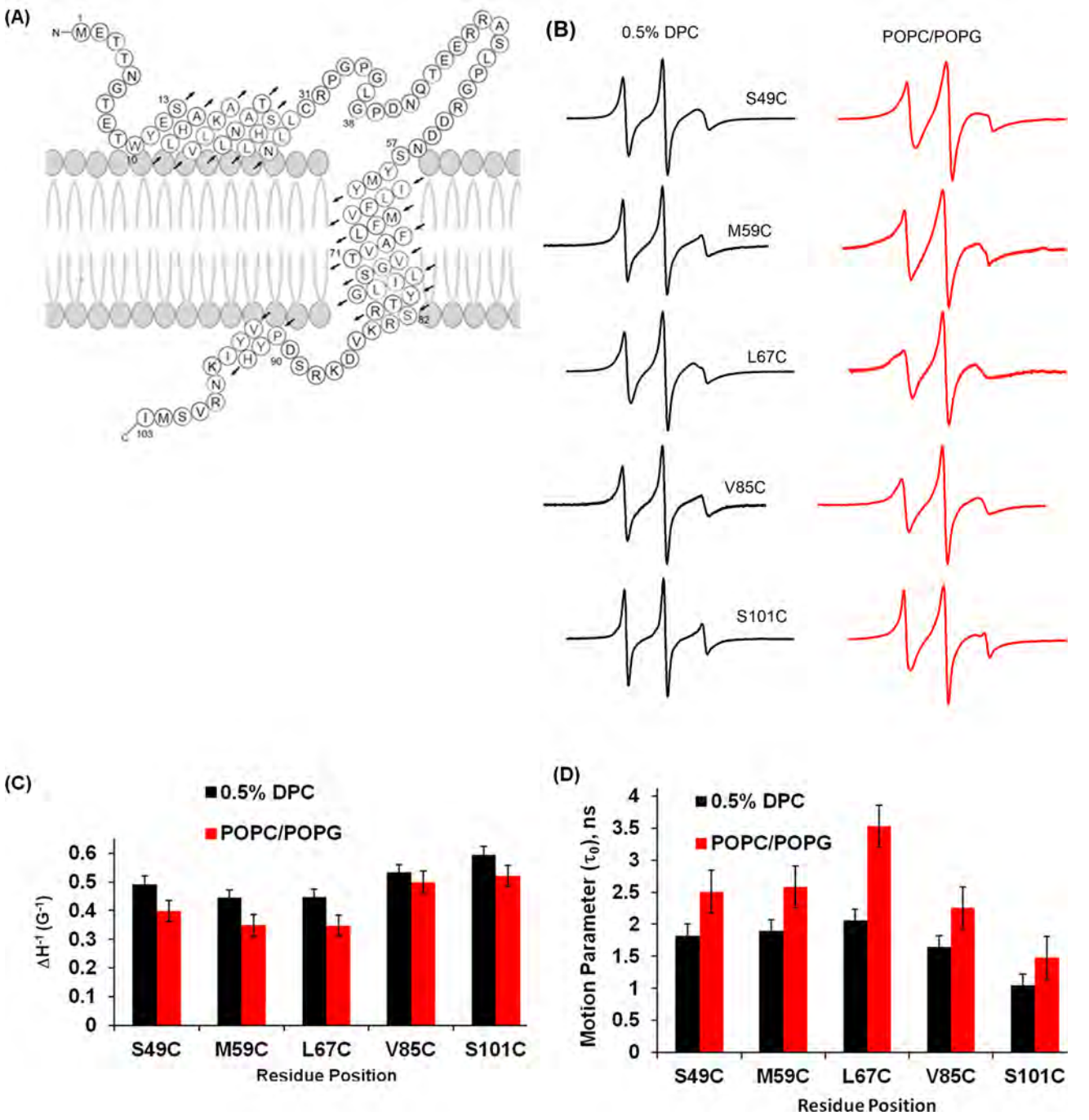


**Figure 2.** A) A predicted topology of KCNE3 in membrane bilayers based on previously published solution NMR studies,[70,71] (B) CW-EPR spectra of nitroxide spin-labeled KCNE3 mutants in 0.5% DPC micelles (left panel) and POPC/POPG bilayered vesicles (right panel), (C) plot of the inverse EPR central spectral line width as a function of residue position of KCNE3, and (D) plot of the motional parameter as a function of residue position of KCNE3. Reproduced from ref 31 with permission. Copyright 2022 Elsevier.

nance energy transfer (FRET)[38] and electron paramagnetic resonance (EPR)[39−42] spectroscopic techniques. X-ray crystallography[43,44] and Cryo-electron microscopy (Cryo-EM) techniques[39,45] are used to study structural aspects of membrane proteins and provide some dynamic information. These approaches have their own advantages and drawbacks.

NMR spectroscopy is used to obtain structural and dynamic properties of membrane proteins under physiological conditions. Solution NMR approaches in combination with nanodiscs can provide a high-precision dynamic information about every atom of an amino acid in a wide range of time scales from picoseconds to seconds.[37,46,47] However, this method is challenging to obtain high-quality experimental data due to the larger size of the membrane-protein complex (>∼50 kDa).[35,48−51] NMR studies of membrane proteins are also limited by the larger spectral broadening and overlapping of NMR line widths. NMR experiments also require a large amount of highly pure and folded membrane protein samples to achieve better data quality.[34,52−54] A probe-based Förster resonance energy transfer (FRET) technique is another approach to obtaining conformational dynamics of membrane proteins.[38] This technique utilizes a larger probe to obtain experimental


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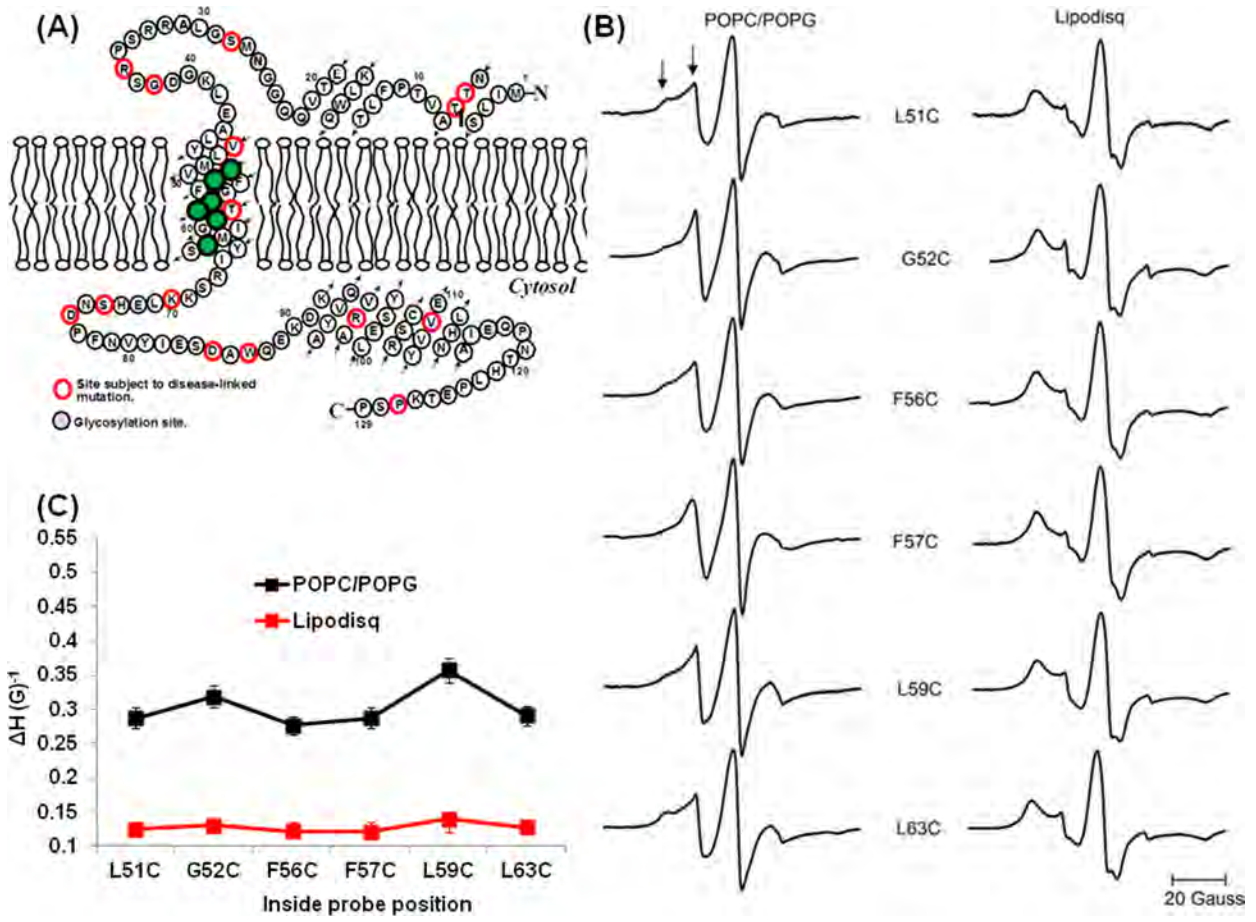


**Figure 3.** (A) Predicted membrane topology of KCNE1 based on previous solution NMR studies[72] with spin-labeling sites represented by green filled circles, (B) CW-EPR spectra of different mutants of KCNE1 in POPC/POPG vesicles and POPC/POPG lipodisq nanoparticles, and (C) plot of the inverse EPR central spectral line width as a function of residue position of KCNE1. Reproduced from ref 30 with permission. Copyright 2017 American Chemical Society.

data that may perturb the structure of the membrane protein. It is also difficult to incorporate the site-specific FRET probe on the membrane protein sequence.[42] A recent example of using NMR spectroscopy is the study of Leukotriene B4 receptor 2 (BLT2) in lipid nanodiscs.[47] BLT2 receptors are G protein-coupled receptors (GPCRs). GPCRs represent a large family of eukaryotic integral membrane proteins. They are involved in several essential biological processes. Pozza et al. utilized two-dimensional (2D) $^{1}H,^{13}C$ SOFAST-HMQC NMR spectroscopy through three transmembrane $^{13}CH_3$ reporters, in residues $M105^{3.35}$, $M19^{75.54}$, and $I229^{6.40}$ to study the conformational ensemble of BLT2 GPCR in POPC/POPG and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) lipid discs.[47] The results suggested that these three methyl NMR reporters are sensitive to the conformational plasticity of the receptor. The NMR data analysis of BLT2 in nanodiscs of various compositions and in the presence of agonists showed that BLT2 represents a complex conformational dynamic landscape in eukaryotic membranes enriched in POPC/POPG or in more rigid DPPC membrane.

EPR spectroscopy is a powerful biophysical technique to overcome the limitations associated with conventional methods and provide important structural and dynamic information on integral membrane proteins incorporated into various membrane environments.[40,42,55−59] When EPR is combined with site-directed spin-labeling (SDSL), it can provide structural dynamics of nitroxide side chain, protein topology, solvent polarity, and intra- or intermolecular distances between two nitroxide spin-labels on membrane proteins.[40,41,56,58,60,61] In SDSL, the natural cysteine on the protein sequence is replaced by alanine or serine, and the site-specific amino acid is replaced by cysteine. The mutated form of the protein containing cysteine at the specific site is then reacted with MTSL to generate a stable nitroxide spin-label having an unpaired electron that is EPR active. The dynamic behavior of the nitroxide spin-label makes it easier to introduce them at any desired position of the protein sequence. This method has no protein size restriction. Recent improvements in technical and methodological aspects of SDSL EPR spectroscopy have made it very popular for studying structural dynamic properties of integral membrane proteins. EPR spectroscopy combined with SDSL has been widely used to study dynamic properties of important integral membrane protein systems.[40,41,56,58] A pulse EPR approach of double electron−electron resonance (DEER) spectroscopy can be also used to obtain structural dynamics of integral membrane proteins by measuring distances and corresponding distance distributions between two nitroxide spin-labels attached to these proteins.[40,62,63] Details of the application of EPR spectroscopy to study membrane proteins have been reviewed in the literature.[40,41,56] Some of the biologically important integral membrane protein systems recently investigated using EPR spectroscopy include transient receptor potential vanilloid 1 (TRPV1) channel, bacteriophage pinholin, KCNE1, KCNE3,


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KCNQ1 voltage-sensing domain (VSD), sensory rhodopsin II of *Natronomonas pharaonis* (NpSRII).[16,31,64−68]

A recent example of probing dynamic properties of integral membrane protein is a study of transient receptor potential vanilloid 1 (TRPV1) channel.[67,69] TRPV1 consists of six transmembrane domains in which the fifth and sixth transmembrane domains are involved in pore formation. TRPV1 is expressed by primary afferent sensory neurons and contributes to the pain hypersensitivity mechanisms. It is an essential component of the cellular mechanism where noxious stimuli give rise to pain. Velissety et al. performed site-directed spin-labeling continuous wave (CW)-EPR spectroscopic experiments to probe the dynamics of residues located at the pore domain (e.g., extracellular vestibule, hydrophobic plug, and TRP domain) of TRPV1.[67] Authors measured mobility parameters calculated as the inverse central line width of the first derivative absorption spectra ($\Delta H_0^{-1}$) obtained for several spin-labeled eTRPV1 mutants in DDM detergent micelles as well as in asolectin liposomes. The data revealed higher mobility values for the spin-label residues exposed to the aqueous environment when compared to the residues in a proteinaceous environment at the intracellular gate and linker domain. The results suggested that these mobility parameters could potentially monitor changes in dynamics during activation gating.[67]

Another recent illustrative example of the dynamic properties of an integral membrane protein is the study of KCNE3 using SDSL EPR spectroscopy.[31] KCNE3 is a single membrane-spanning potassium channel accessory protein that modulates the function and trafficking of voltage-gated potassium channels such as KCNQ1. Voltage-gated potassium channels are very important in several biological functions including cardiac, nervous, and auditory systems. They are targets of several modern medical drugs. KCNE3 interacts with KCNQ1 forming KCNQ1/KCNE3 channels important for the transportation of potassium ions through epithelial cells during salt homeostasis. The dysfunction and mutations in KCNE3 have been linked to several disorders including long QT syndrome (LQTS), cardiac arrhythmia, cystic fibrosis and secretory diarrhea, periodic paralysis, tinnitus, and Ménière's disease. Campbell et al. performed nitroxide-based SDSL CW-EPR spectroscopic experiments on KCNE3 in both DPC detergent micelles and POPC/POPG (3:1) lipid bilayered vesicles to obtain the side chain mobility and motion parameter of the KCNE3.[31] The spectral line shape analysis suggested that the dynamic motion of the nitroxide spin-labels is lower in the lipid bilayer membrane environment when compared to that in detergent micelles. Figure 2 shows the predicted topology of KCNE3 in lipid bilayers based on previous solution NMR studies,[70] CW-EPR spectra, inverse central spectral line width, and empirical motional parameter as a function of residue position. The motional parameter, also known as rotational correlation time, describes the time required for the spin-label to rotate an angle of one radian.

One example of studying dynamic properties of an integral membrane protein is a study of potassium channel accessory protein KCNE1 using EPR spectroscopy. KCNE1 is a single-pass transmembrane protein containing 129 amino acids. It is involved in modulating the function of several voltage-gated potassium channels including KCNQ1.[72−74] KCNE1 forms a complex with KCNQ1 in the human heart and generates the slow delayed rectifier current. This is represented by its slow activation and deactivation kinetics.[75] This is very important for the cardiac action potential repolarization phase. Sahu et al. performed CW-EPR spectral line shape analysis on several spin-label sites of the transmembrane domain of KCNE1 in POPC/POPG lipid bilayers and POPC/POPG lipodisq nanoparticles.[30] The results showed a significant increase in EPR spectral line broadening and corresponding inverse central line width of spin-labeled KCNE1 residues for lipodisq nanoparticles samples decreases when compared to lipid vesicle samples. This suggested the spin-label side chain motion of KCNE1 is restricted in POPC/POPG lipodisq nanoparticles when compared to that in POPC/POPG liposomes. Figure 3 shows a cartoon representation of the membrane topology of KCNE1 with spin-labeling sites and CW-EPR spectra and inverse central line width data of KCNE3 in difference membrane mimetics.[30,72] The arrows on the spectra represent two spectral components with slower/rigid components (left arrows) and faster/higher motional components (right arrows).

Another recent example of the investigation of dynamic properties of an integral membrane protein is a study of a biologically significant integral membrane protein antipinholin ($S^{21}68_{IRS}$) in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) proteoliposomes.[64] Antipinholin has 71 amino acids containing two transmembrane domains (TMDs) embedded into the inner cytoplasmic membrane. This makes the N- and C-termini stay in the cytoplasm. Antipinholin is responsible for delaying the formation of the active dimer required for the formation of the pinholes. Ahmmad et al. performed nitroxide-based SDSL CW-EPR experiments on 35 different nitroxide spin-label (R1) side chains of antipinholin ($S^{21}68_{IRS}$) in DMPC liposomes.[64] CW-EPR spectral line shape analysis was carried out to obtain relative mobility and the rotational correlational times of R1 side chains in DMPC liposomes. The results suggested a restricted mobility of both TMDs when compared to the N- and C-termini.

Another recent example of conformational dynamics study of an integral membrane protein is the study of Sensory rhodopsin II in nanolipoprotein and styrene−maleic acid lipid particles using EPR spectroscopy.[68] The NpSRII is a membrane-embedded photoreceptor that moderates the photorepellent response to potentially harmful blue light. The NpSRII forms a transmembrane complex with its conjugate transducer NpHtrII. This complex plays a key role in negative phototaxis. It can be used as a unique model system for studying the light-induced transfer of a conformational signal between two integral membrane proteins. Mosslehy et al. utilized site-directed spin-labeling CW-EPR spectroscopic data to compare the conformational dynamics of NpSRII2/NpHtrII2 complex reconstituted into SMALPs with that reconstituted into nanolipoprotein particles (NLPs) and liposomes.[68] The researchers recorded CW-EPR spectra for R1 spin-labeled NpSRII-L159R1 with and without the transducer $NpHtrII_{157}$ in liposomes, NLPs and SMALPs. The spectra of L159R1 were almost identical in all three environments showing a spectral powder pattern (immobile) with a resolved hyperfine line in the high field region.[68] These data revealed that the nitroxide side chain dynamics in the interior of NpSRII were not affected by different lipid environments, suggesting that the tertiary structure of the protein close to position 159 was not influenced. Additionally, the CW-EPR spectra of $NpSRII/NpHtrII_{157}$-A94R1 in liposomes, NLPs, and SMALPs showed a composite spectral shape, indicating the presence of at least two components related to mobile and immobile fractions of the spin-label side chain. The dynamics of the NpHtrII157-A94R1 side chain with the protein reconstituted into NLPs was similar to that of the


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complex into liposomes, suggesting no effect on the equilibrium between the mobile and immobile components. However, the motion of the transducer R1 side chain in SMALPs was more restricted indicated by the more pronounced immobile component, suggesting the shift in the equilibrium between the mobile and immobile toward the immobile component. These results suggested less flexibility of the protein in SMALPs.[68]

## 4. CONCLUSION AND FUTURE PERSPECTIVE

Integral membrane proteins are crucial for several biological functions needed for the survival of living beings. Due to the complex behavior of integral membrane proteins in cell membranes, a suitable solvent condition is required for extracting the protein from the cell membrane to obtain dynamic properties. Large improvements have been achieved in developing membrane mimetic environments appropriate for biophysical approaches for studying dynamic properties of integral membrane proteins. Despite recent improvements in methodology and biophysical approaches, the major challenge in this field is to develop sample preparation conditions for the improvement of the expression yield and stability of the integral membrane protein in physiological lipid bilayer membrane environments. It is also very challenging to find the integral membrane protein solubilization condition that can maintain physiological conditions while obtaining superior quality experimental data from biophysical measurements to answer structural dynamics-related questions. There are still open questions for researchers in structural biology fields such as, is there a universal membrane mimetic system that can be suitable for all integral membrane protein systems for biophysical studies? The EPR studies have suggested a reduced spin-label side chain motion of the protein when moving from detergent micelles to lipid bilayer membranes and further to the SMALPs.[30,74] The degree of the reduction in the spin-label side chain motion varies depending upon the protein−lipid systems studied. The incorporation of the protein−lipid complex into SMALPs increases the viscosity of the system causing a reduction of the global tumbling motion.[30] This can help to understand the local spin-label side chain motion of the protein such as protein backbone dynamics. However, there are not enough biophysical studies available in the field to generalize this behavior. Additional future studies will help understand dynamic behavior of the protein with respect to various membrane mimetic systems. The ongoing progress in developing new derivatives of SMA copolymers for solubilizing membrane proteins will open the path for obtaining reliable structural dynamic information on integral membrane proteins using biophysical methods. The In-cell EPR approaches will also provide avenues for obtaining reliable structural dynamic information on these proteins.[76]

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### Notes

The authors declare no competing financial interest.

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Indra D. Sahu received B.Sc. and M.Sc. Degrees from Tribhuvan University, Nepal in 2002, and M.S. and Ph.D. degrees from State University of New York (SUNY), University at Albany, NY, in 2009 in Biomedical Physics. He worked as a Postdoctoral fellow (2010−2013) and Research scientist (2013−2019) at Miami University. He is working as an Assistant Professor of Physics (2019 to present) at Campbellsville University. He is also an Adjunct Assistant professor at Miami University (2019 to present). His research interest includes structural biology and biophysics of membrane proteins.

Gary A. Lorigan received a BS (1990) in Chemistry at California State University, Sacramento, CA, and a Ph.D. in Chemistry (1996) from the University of California at Davis, CA. He was a NIH postdoctoral researcher fellow (1996−1998) at the University of Pennsylvania, Philadelphia, PA, in the area of biophysical chemistry. Currently, he is Chair and Distinguished Professor of Chemistry at Miami University in Oxford, OH. His research focuses on studying the structural and dynamic properties of membrane proteins using EPR and solid-state NMR spectroscopic techniques.

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## ■ ABBREVIATIONS

SDSL, site-directed spin labeling; TMD, transmembrane domain; MTSL, 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate; PDB, Protein Data Bank; SMALPs, styrene maleic acid lipid particles; SMA, styrene maleic acid; NMR, nuclear magnetic resonance; FRET, Förster resonance energy transfer; Cryo-EM, cryogenic electron microscopy; EPR, electron paramagnetic resonance; CW-EPR, continuous-wave electron paramagnetic resonance; DPC, dodecyl phosphatidyl-choline; DDM, *n*-dodecyl-*β*-maltoside; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt); DMPC, (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine

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