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# Introductory Chapter: The Diversity of Biophysical Chemistry Techniques

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## 1. Introduction

Biophysical chemistry is an interdisciplinary field of study that uses concepts of chemistry and physics to understand biological systems by describing the quantitative, qualitative, energetics, structure, functions, and interactions phenomena of its physical nature. Because of the complexities of biological systems, a wide range of classical and sophisticated techniques have been employed in the field of biophysical chemistry; therefore mathematical, physical, and chemical techniques all flow into a single stream to describe biological systems. As an interesting field of research, biophysical chemistry is now growing up rapidly with major breakthroughs everywhere. In fact in 2009 Nobel Prize in chemistry have been awarded to three biophysical chemists for their work in X-ray diffraction of ribosomes [1]. The most important areas that attract biophysical chemists are molecular structure, molecular function, molecular dynamics, and kinetics, interactions, and thermodynamics of macromolecules that are located in the cell membrane or cytoplasmic constituents. In general biophysical chemistry interests in answering the following questions: how does a biological process take place, what types of molecules or particles are involved in this process and what are their structures, how long does it take for a biological process to take place and what are the energetics that accompany that change, what are the functions of biological molecules, and what are the consequences upon the cell if some biological molecules work disfunctionally?

In this introductory chapter, we would like to shed light into the importance of biophysical chemistry as a growing field of science and the broad diversity of techniques that have been used to elucidate the related phenomena. These techniques are of spectroscopic, electrochemical, thermal, and physiological origins, and we will not be able to cover all of these techniques in such an introductory chapter. Instead we will describe some selected techniques for studying the structure and functions of biological molecules. Some of these techniques adopt environmental and semi-environmental conditions as that for biological molecules in its native environment. We will focus on four types of techniques that up to this date are used in biophysical chemistry field. Each technique comes with a short description and is cited with appropriate reference for more details.

## 2. Thermal techniques

The most adequate and proper techniques that are used in this category are differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC),

which provide unique complementary information for nucleic acids, modified nucleic acids, nucleic acid-ligand interactions, and protein-ligand interaction and are very useful methods in finding thermodynamic parameters using the same simple Gibbs equation. DSC is a direct, easy-to-use, model-independent measurement tool that can be used with various physicochemical methods to obtain structural and bonding information [2–4]. Originally DSC is used to study gaining (endothermic or heat absorption) or losing (exothermic or heat generation) heat upon the biological reactions or interactions as a function of temperature and time [5].

DSC technique measures a heat change during a temperature difference, which is radiated or absorbed by the sample, in a controlled way, based on a temperature difference between the sample and the reference material [6, 7].

When conducting a DSC experiment, the sample cell, which contains the molecules of interest dissolved in appropriate solvent, and the other cell called reference cell, which contains only the same amount of solvent, are heated simultaneously, and hence temperatures of both cells are raised identically over time. Depending on the type of the energy process, whether it is endothermic or exothermic, temperature is counted. In this regard the temperature of both cells is raised simultaneously until the difference is reached. If the process is heat gaining, then more heat is needed to equilibrate the two cells, meaning more energy is required to bring the sample to the same temperature as the reference; hence, the concept of heat excess comes into the picture.

New instruments allow setting a variety of experimental parameters such as number of scans, post scan temperature, scan range and rate, as well as feedback strength, and for the goodness of the results, slower scan rates provide higher resolution, while high feedback strength will give optimal interaction or reaction sensitivity. Because of the ability of the instrument to easily measure enthalpy changes, temperature difference, and phase transitions, it is now used in many applications, for example, studying protein interaction with ligands and drugs, protein mutations [8], protein folding [9–12], lipid interaction with drugs [13], protein interaction with lipids [14], DNA duplex stability, major and minor grooves in DNA and ligand binding, helix-coil transition in DNA, thermodynamics of DNA melting, as well as DNA-based binding interactions.

Another technique is isothermal titration calorimetry. This technique is very sensitive in measuring energy released by interactions of molecule of interest and biological molecule. Mainly this technique is used for the qualitative and quantitative measurement of such interactions [15, 16]. With the aid of the Gibbs free energy equation and equation of equilibrium free energy, the interaction system can be easily understood. Two parameters can be derived directly from the ICT instrument which include the equilibrium constant for the binding process ( $K$ ) and the binding stoichiometry ( $n$ ).

### **3. Electrical techniques**

This category illustrates the most advance and versatile techniques that have been used in biological systems. The success of electrochemical methods is obvious since experiments are adopted to study biological systems *in vivo* and *in vitro*. Both types need, for instance, very tiny electrodes that are capable of entering individual biological cell without damaging it. Therefore considerable efforts have been done for developing such electrode. Recent progress shows that a new generation of ultramicroelectrodes are in use. Studying single cells are constructed by preparing working electrodes generally from 5- to 10- $\mu\text{m}$  diameter of carbon fibers. In this regard improving signal-to-noise ratio can be done considering that

the electrode size should approach the size of the detection area of interest. On the other hand a greater number of electrochemical events can be detected by larger electrode sizes [17, 18].

To further narrow this application, a single biological molecule can be investigated using patch-clamp technique. This technique is capable of directly recording ionic current that could flow from a single ion channel. Moreover conductance and conformational changes between nonconducting and conducting conformations can be detected as well [19]. Since this technique could measure the conductance of a single channel, and as the conductance is the movement of ion through a specific area, this process could interfere with ion concentration. Therefore special considerations should be taken to avoid such interference.

The current recording from the instrument that is produced from a single or few channels can be used to derive two important phenomena: one is the conductance of a single channel, and the other is the time required for opening and closing the channel or what we call gating kinetics. Initially this can be achieved by inserting gramicidin A as model pores into planar lipid bilayer membranes. Using this step we look for sufficient amounts of ion channels to be inserted in the bilayer, and then both patch-clamp and voltage-clamp of expressed channels can be recorded, and background noise can be significantly reduced by applying gigaseal resistance, which could be achieved by forming such high resistance between recording electrode and the membrane patch. Patch-clamp methodology is reviewed extensively in the following references [20, 21].

Three techniques are nowadays applied to record single-channel conductance: the inside-out patch, the outside-out patch, and whole-cell patch. More details can be found in [22].

The keystone in the area of electrochemical techniques that are applied in biological systems is the appropriate electrode. The electrode that is actively used in biological analysis is now called bioelectrodes. By the name it should be micro-electrodes or less since all biological operations taking place in the diameter of that of living cells and more precisely its electrode whose tip diameter is less than 10  $\mu\text{m}$  while ultramicroelectrodes whose tip diameter is less than 1  $\mu\text{m}$ . There are so many applications of such electrodes in biological systems. Among them are neurochemical analysis, mutagenicity and toxicity detection, analysis of blood ions and gases, blood flow analysis, and analysis of small molecules, nucleic acids, and proteins.

Some secondary techniques can be used in combination with patch-clamp recording to study specific proteins. For example, rapid perturbation techniques can be used to investigate pre-steady-state kinetics of membrane transporters. In this regard the current produced across the membrane by a transporter can be measured, and hence the mechanism behind can be elucidated. One problem with this is the feature of transporter itself since ion fluxes and consequently current produced by transporters are much lower than that produced by channels. Therefore whole-cell or inside-out patch method is used.

Another interesting technique that is actively used is voltage jump fluorometry combined with site-directed fluorescence labeling. This technique is mainly used to detect local protein motion in real time under native environmental conditions. Cysteine as amino acid is used for this purpose and could be inserted in specific location within a protein structure. The more useful way to do that is site-directed mutagenesis. Through this some amino acids can be cut and replaced by the desired ones, which eventually results in cysteine being in a desired location. New structure of protein can be expressed in *Xenopus* oocytes and allow for fluorescently active dyes to bind cysteine, for example, tetramethyl rhodamine-6-maleimide. Expressed protein can be stimulated by, for example, voltage pulse which leads eventually to recording of fluorescence signals and current responses in an electrophysiological



experiment which allows structural changes to be correlated with specific ion transport steps, and hence detailed mechanism can be illustrated. More details can be found in the following references [23-26].

#### **4. Spectroscopic techniques**

Under this section lie so many advance techniques that successfully discover many features of biological systems. Few of them will be selected and discussed. The most important technique is X-ray crystallography [27], which is considered to be the primary tool for determining the macromolecule biological structure. This could be done by studying the X-ray scattering pattern that is produced from macromolecule prepared structure. Here we should note that not all biological macromolecules can form crystals. Some of them could form very nice crystals which have their molecules arranged in regular array called and this is used as a scattering surface of the crystal, biological molecules that aren't capable to form crystal could their structure be determined using solution NMR method, determination of the structure is subject to mathematical formulas of X-ray diffraction and scattering applied in the field, this process is very complicated and could take very long time to determine one macromolecular structure. Another application of X-ray is its use as a tool for spectroscopy. X-ray absorption spectroscopy (XAS) [28] is quite the same as the regular absorption methods, but this uses X-ray radiation for excitation of electrons. This technique is sensitive to the element that is involved in the absorption, and because of such sensitivity, this is become a growing technique. Two methods have been developed from XAS. The first one is to use XAS as X-ray absorption near edge structure (XANES), and hence element order, geometry, and oxidation state can be elucidated. The second one is to use XRA as extended X-ray absorption fine structure (EXAFS). In this case the active site by which metal ion can bind to macromolecule can be defined. The resolutions of both methods are in the range of Angstrom. One of the advantages of this methods is their ability to analyze any type of sample including perfect crystals, torsional crystals, and noncrystalline samples or amorphous samples, and the main disadvantage of this technique is its less sensitivity to the amount of material of interest that is less than mg/g.

Another interesting spectroscopic technique is stopped-flow fluorometry [24-26] in conjugation with voltage-sensitive fluorescence membrane probes. The stopped-flow technique allows the kinetics of ion pumps to be followed in the millisecond time range. Partial reactions of pumps which involve charge movement within the membrane produce local changes in electric field strength, which shift the fluorescence excitation spectrum of the probes and thus produce a fluorescence response.

Nuclear magnetic resonance (NMR) allows measurement of steady-state fluxes across cell membranes [29], which can be achieved due to the different chemical environment inside and outside cells. Signals coming from NMR active nuclei on both sides of cell membranes can be distinguished. Time-resolved changes in the intensity of these signals, thus, allow the steady-state kinetics of channels, pumps, and transporters to be detected and analyzed.

Time-resolved infrared spectroscopy recently became very frequent in use as a growing spectroscopic technique [30], since, with the help of pulsed lasers, it is possible to study processes that occur on timescales as short as  $10^{-9}$  seconds or lower. Many transporters or pumps can be activated by the photochemical release of caged compounds. During ion transport processes, changes in the infrared absorbance spectrum of the pump or transporter under investigation can occur. Apart

from following the kinetics of the ion transport reactions, in contrast to fluorescence spectroscopy, infrared spectroscopy allows more detailed structural information concerning the involvement of individual amino acid residues to be deduced.

Fluorescence correlation spectroscopy (FCS) is a technique with single-molecule sensitivity that correlates the fluorescence fluctuations due to the diffusion of individual fluorophores through the focal volume of the microscope [31, 32]. It can be used to quantify protein dynamics and concentrations, as well as protein–protein interactions in the dual-color version of the method. This technique is similar to single-particle tracking, which is a complementary technique that also provides information about protein dynamics and the oligomeric state of the proteins analyzed at the single-molecule level.

Another technique worth to be discussed is single-molecule fluorescence imaging [33]. In this case there is a wide range of single-molecule methods capable of examining transporter and channel and pump function in vivo and in vitro. With probing membrane protein molecules, conformational changes and measurements of ionic flux across the bilayer can be defined.

Flash photolysis is a very useful technique for studying the kinetics and mechanism of light-activated ion pumps and channels [34], which undergo changes in their UV/visible absorbance spectrum as part of their reaction cycle. One interesting example of kinetics and mechanism that have been resolved by this technique is that of the light-activated proton pump bacteriorhodopsin and the newly discovered light-activated channelrhodopsins.

Electron spin resonance as a powerful technique for structural elucidation of protein structure is extensively used [35, 36]. For this site-directed spin-labeling electron spin resonance spectroscopy (SDSL-EPR) is used as a highly sensitive technique for determining the structure and dynamics of, for example, membrane proteins within their native membrane environment. Site-directed mutagenesis methods are used to introduce a unique cysteine at a desired location to which a paramagnetic nitroxide spin label is then attached. The EPR spectrum yields information about side-chain mobility, solvent accessibility, the polarity of its immediate environment, and inter-nitroxide distances. Using these measurements it is possible to determine secondary structure, orientation of elements, and protein topography which allow questions concerning membrane protein structure, conformational changes, and membrane translocation phenomena to be investigated. The time-scale of sensitivity of the EPR technique is also well matched to the motions of lipids in membranes and conformational changes of functional relevance (picoseconds to seconds).

The last technique I would like to shed light on is atomic absorption spectrophotometry (AAS) [37]. It can be used to quantify the activity of cation transporting P-type ATPases by measuring cation uptake into individual cells by atomic absorption spectrophotometry using heterologous expression in *Xenopus* oocytes. The method is a sensitive and safe alternative to radioisotope flux experiments and is explicitly suited to the assessment of transport activity of electroneutrally operating transporters, which cannot easily be measured by electrophysiology. The method exhibits a low background of unspecific cation uptake and facilitates complex kinetic studies. Furthermore, since cation uptake can be determined on single cells, the flux experiments can be carried out in combination with two-electrode voltage clamping (TEVC) to achieve accurate control of the membrane potential and current.

## 5. Miscellaneous techniques

Long timescale molecular simulations [38–40]—In the past, the size and timescale of molecular simulations of macromolecules have been limited by the available

computational power. As a consequence it has been difficult to include the full complexity of the natural environment of proteins and to simulate for long enough to capture biological processes taking longer than tens of nanoseconds. However, recently the steady increase in computer power has allowed for much longer simulations to be conducted, with a few cutting-edge studies reporting simulations of millisecond duration. Thus, it is now possible to directly investigate global conformational changes of proteins that are responsible for protein function as well as to quantify the energetic of physiological processes.

Radioactivity-based analysis technique [41]—Using this technique flux (influx and efflux) measurements of ions and substances across biological membranes using radioisotopes can be determined. Radioactive-based analysis of membrane transport is the method of choice if the transporters are operating electroneutrally (in this case the patch-clamp method cannot be applied) or if sensitive fluorescent dyes are not available. The latter is especially true for Na<sup>+</sup> and K<sup>+</sup> transport.

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