

Advancements in Characterization Techniques of Biopolymers: Cyclic Voltammetry, Gel Electrophoresis, Circular Dichroism and Fluorescence Spectroscopy

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Abstract

Biopolymers such as DNA, RNA and proteins are the key players in the cellular processes like cell differentiation, cell growth, maintenance, repair, recombination, transcription, translation, etc. Immense efforts have been made for their isolation, purification, quantification and structural as well as functional characterization. Profound insights into the structural and functional characterization of these biopolymers could help us understand the intricate cellular machinery. A wide range of biochemical, biophysical, electrochemical and molecular biology techniques have really been beneficial in exploring the key and interdependent relationships between the structure and function of these biopolymers. Each instrumentation technique has its own advantage and disadvantage in terms of their applications, selectivity and sensitivity. There had been a lot of advancements in these techniques for achieving their desired goals, but still, lots of limitations need our attention and further improvements. For understanding, the extremely complicated biological machinery at molecular and cellular level, every small fragment of a cell has to be studied separately with a lot of intricacies. In this review, we present a brief update of the advancements made in the large repertoire of Cyclic Voltammetry (CV), Gel electrophoresis, Circular Dichroism (CD) and Fluorescence spectroscopy techniques which might be relevant for updating our knowledge of the pre-existing and well-established tools utilized by the scientists across the world.

Keywords: Biophysical and biochemical techniques; Electrochemical technique; Cyclic voltammetry; Circular dichroism; Fluorescence; Gel electrophoresis

Introduction

DNA, RNA and proteins are the most important constituent biopolymers of any cell. DNA acts as storage repository of information, which then is used by RNA for processing and giving instructions for the coding or expression of a protein. These proteins further act as the basic constructing units of the cell, as they are involved in almost each and every function of a cell. DNA and RNA binding proteins are quite specific for every biological process [1]. Proteins not only take care of the structure, function, growth and repair of cells, but it also facilitates every cellular process and its mechanisms through enzymes and hormones. Following the genomic and proteomic advances, one can easily understand the importance of the mapping of genes and their related proteins. More than 70,000 genes have now already been searched throughout the human genome, which is a quite huge number in itself [2]. Establishing the relationships between these genes and their possible functions is another quite challenging assignment. Bioinformatics had been playing a crucial role in answering the questions related with various genomes and proteomes.

Cell, being the most complicated biological machinery needs to be studied with a lot of sophistications and alertness at both molecular and cellular level. This is only possible with more advanced, selective and specific instrumentation techniques which may provide us better insights of every cellular mechanism and biological pathways. Structural diversity and polymorphism of the nucleic acids is one of the possible reasons which ultimately control the gene expression and lead to the formation of a desired or undesired protein. For controlling the formation of an undesired protein, a lot of strategies have been utilized. Utilizing pharmaceutical drugs for the same is also one of the most explored alternatives. Interestingly, DNA, RNA and proteins also being the most vulnerable targets of a large number of pharmaceutical drugs, become one of the most preferred focuses for scientists all over. For all

the above-mentioned reasons and many others, extraction, of DNA, RNA and proteins [3] along with their purification, characterization and quantification become extremely significant. Many advances in the fields of biochemistry, biophysics, electrochemistry, and molecular biology have really been based on the advanced techniques which have continuously been evolving as per the need of the research areas. In the present review, an update on some of these techniques and their applications has been discussed (Figure 1 and Table 1), with an aim of increasing the knowledge of the broad horizon of the advancements in the related fields.

Advances in Cyclic Voltammetry

Voltammetry methods are simple, sensitive, selective and time-saving in nature [4]. Analytical properties are enhanced by combining voltammetry technique with several separation methods such as capillary electrophoresis, flow injection, and HPLC. Cyclic Voltammetry (CV) technique was developed in order to study the electron-transfer in conventional redox reactions of organic as well as inorganic chemistry, multi-electron transfer processes of biochemistry. Stability of reaction products, kinetics of electron-transfer, and involvement of

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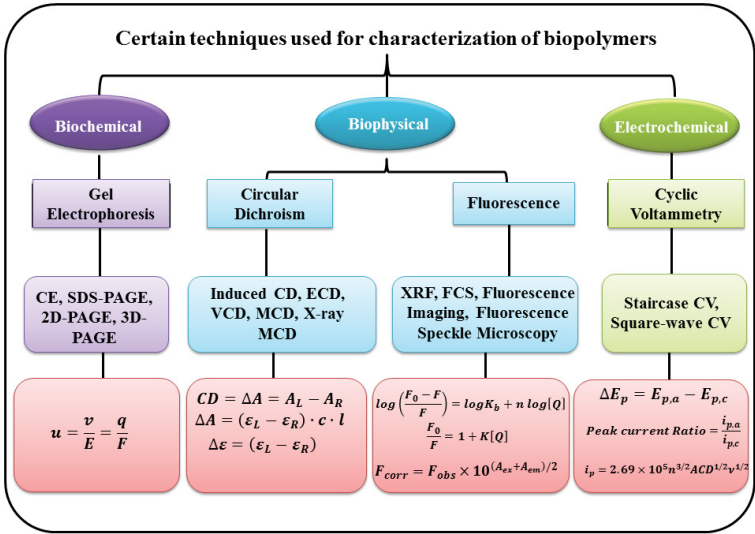


Figure 1: Classification of various techniques used for characterization of biopolymers. Equations to calculate the parameters associated with Gel electrophoresis [82], Circular Dichroism [83], Fluorescence [60,84] and Cyclic Voltammetry [85] are also shown.

S. No	Techniques	Applications
1.	Gel Electrophoresis	Tells about the molecularity of DNA secondary structures. Protein and nucleic acid molecules are separated depending on their size and net charge.
2.	Electrophoretic Mobility Shift Assay (EMSA)	It was developed to study DNA-protein interaction.
3.	Circular Dichroism	Gives information about the conformational changes in the secondary structures of biomolecules.
4.	Fluorescence Spectroscopy	Gathers information about the DNA-drug interaction, protein-protein interaction and the conformational differences in the protein structures.
5.	Fluorescence <i>in situ</i> hybridization (FISH)	Used for the study of structure and function of chromosomes
6.	UV-Visible and UV-Tm spectroscopy	Used to study DNA-ligand interactions and their thermal stability. It also measures the absorbance and purity of DNA.
7.	Cyclic Voltammetry	Gives information about the electrochemical behavior of biomolecules and pharmaceutical drugs. Used especially for the study of DNA hybridization and DNA-drug interactions

Table 1: Applications of certain techniques used for studies related to biopolymers.

intermediates in a reaction can also be very well analysed using this technique [5]. In the field of biology, it determines the concentration of compounds present in living systems [6]. Cyclic voltammetry is defined as the measurement of the current developed in an electrochemical cell, in the presence of excessive voltage (Figure 2a). In this technique, the course of the voltage scan can be reversed. The resulting current is measured, while the potential is applied to the working electrode in both forward and backward directions [4].

The implementation cost of the simple cyclic voltammetry technique can be relieved by introducing staircase ramping potential. Staircase cyclic voltammetry (SCV) was developed in order to experimentally discriminate Faradic and capacitive currents [7]. For surface redox reactions, the linear cyclic voltammetry has been replaced by staircase cyclic voltammetry experiment, which diminishes the signal to noise ratio at fast scan rate [8]. The voltammogram for SCV is a function of the scan rate, the size of the step and the point at which the current is determined at each step [7]. A further enhancement to the cyclic voltammetry technique leads to the development of square-wave voltammetry. The benefits of cyclic voltammetry and pulse voltammetric techniques together result into square-wave CV experiments (SWV). This technique is used to study the reaction mechanism and electrode kinetics of surface- and solution-phase redox reactions [9].

Cyclic voltammetry technique has immense application in the nucleic acid and pharmaceutical research. Evidence show that this

technique can be utilized for the electrochemical detection of DNA hybridization. Gold electrodes forming microchip was implemented in the cyclic voltammetry method for the detection of DNA linked to hepatitis C virus and human immunodeficiency virus. DNA structure confined to a surface experiences a conformational change induced by hybridization which results in a change in the electron-transfer, which in turn, can be measured by cyclic voltammetry [10]. In the case of molecules showing either weak absorption or electronic transition overlap, cyclic voltammetry is proved to be a suitable technique to review their interaction with the DNA. This technique specifically studies the interaction of metal-based compounds with DNA, due to involved redox processes. The diversity of various interacting compounds or numerous oxidation state of a single species can be determined via voltammetry methods. Equilibrium constants and kinetic data can be calculated with the help of potential and current measurements. Apart from these, binding strength and mode of action can also be determined via CV technique [11]. It can also be used to understand the electrochemical behavior of pharmaceutical drugs by developing modified glassy carbon-paste electrodes of several drugs [12].

A Broad Range of Gel Electrophoresis

The molecularity of secondary structures of biopolymers can be determined by utilizing gel electrophoresis. Electrophoresis is defined as the technique that uses the electric current to pass the biopolymers such as DNA, RNA, and proteins for separating them through a porous

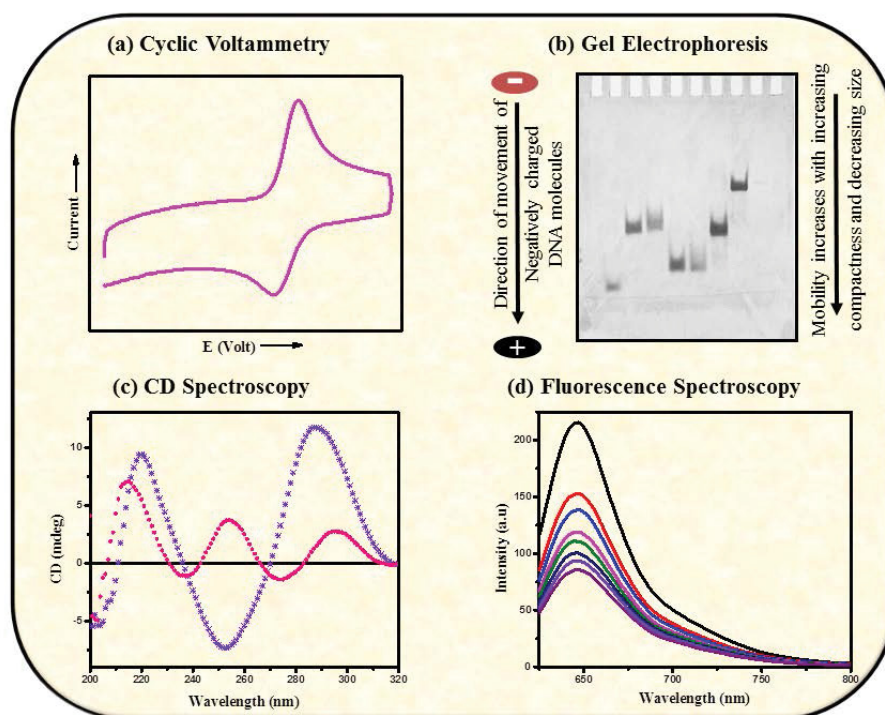


Figure 2: Representation of distinctive techniques to study the structure of DNA.

gel matrix. The gel is formed by employing molecules stacking on each other, providing a sieving medium in order to separate DNA or protein fragments according to their charge and size (Figure 2b). Gel electrophoresis can be categorized into several kinds based on the shape, net charge, size, gel-type and porosity of the matrix used [13]. Agarose and polyacrylamide gels are generally used for the biochemical analysis of macromolecules. Polymerization of β -D-galactose and 3, 6-anhydro-L-galactose results into agarose molecule forming three-dimensional matrixes. The agarose gel matrix has large pore size allowing separation of 200-20,000 bp in the size of DNA fragments [14]. This type of gel electrophoresis is generally used to separate circular DNA from different supercoiled fragments [15].

On the other hand, polyacrylamide gels are less porous and are used to separate DNA, RNA, proteins or other small molecules. Acrylamide and N, N'-bisacrylamide are polymerized in the presence of ammonium per sulfate (APS), a free radical generator and N, N', N'-tetramethylethylenediamine (TEMED), is used as a catalyst to induce polymerization. The pore size of polyacrylamide gels is smaller in comparison to agarose gels, due to which this technique can separate small DNA fragments of 5-100 bp in size and proteins of usually 5-2000 kDa in size. Polyacrylamide gels are highly cross-linked because of rapid polymerization of bisacrylamide with itself than with acrylamide. The pore size is equivalent to pores in the bisacrylamide-rich region in case of the separation of proteins, while in the case of DNA, small pores are not distinguished by these large molecules and thus, their mobility is retarded through the acrylamide-rich fibers [16]. Applied electric current would result in the migration of charged molecule either towards positive (anode) or negative (cathode) end, according to their net charge. Since phosphate backbone of nucleic acids imparts a net negative charge to them, they will migrate towards the anode whereas proteins can be positively or negatively charged depending on the amino acid present. They will migrate towards the anode, if negatively

charged or cathode, if positively charged [17]. In order to gather the information about the secondary structure of the DNA molecule, native Polyacrylamide gel Electrophoresis (Native-PAGE) is used, while to check its purity and the size (length) denaturing PAGE is used, where urea and formamide are commonly used denaturing agents.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the method for differentiating proteins, in which SDS is utilized to denature the secondary, tertiary or quaternary structures of proteins. The SDS-polypeptide molecules will have a negative charge that is attracted towards the anode. The proteins having a size less than 30 kDa can be rectified using Tricine-SDS-PAGE, in which low concentrations of acrylamide is used which is essential for hydrophobic proteins [18].

A more convenient way to separate proteins is via capillary gel electrophoresis, as SDS-PAGE is a time-consuming technique. Polyacrylamide gels are prepared inside the capillary column, which eliminates the problems of shrinkage of gel and bubble formation [19]. For proteins, other sieving matrices such as poly (N, N-dimethyl acrylamide) [20] and poly (vinyl pyrrolidone) are used to improve the resolution of proteins and to prevent their absorption [21]. For suppressing protein absorption, quaternized cellulose which is hydrophobic in nature is used as a novel coating for capillary electrophoresis [22].

To analyze several proteins simultaneously, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) came into the picture in mid-70's. The 2D-PAGE resolves proteins according to two distinct principles, based on their net charges obtained by isoelectric point and their molecular masses. At first, proteins get separated by electrophoresis in the first dimension (isoelectric focusing), followed by the second dimension in the existence of sodium dodecyl sulfate. Resolution of 2D-PAGE is much greater than 1D-PAGE, which is not

lost after the dimension separation. Capillary gels with pH gradient are used in the foremost separation by isoelectric focusing. SDS-PAGE used for further separation is much easier to stain in comparison to isoelectric focusing in the first step [23]. 2D-PAGE cannot be used on a large scale separation since the second step of SDS-PAGE is carried out in slab gels. It becomes labour-intensive and time-consuming, as it also involves mass spectrometry after the second procedure.

A new method involving three-dimensional gel electrophoresis (3D-gel) was introduced to overcome the problems of 2D-PAGE. This method employs agarose gel, polyacrylamide gel and SDS-polyacrylamide gel electrophoresis for the separation of proteins and DNA [24]. 3D-gels are resolved by using photo detection of laser-induced fluorescence online, after carrying out electrophoresis. It increases the automatization degree to a larger extent and also enhances the efficiency of protein analysis [25].

Till now, toxic staining dyes including EtBr, bromophenol blue, coomassie blue and stains-all solution were used for gels but recently, a green staining method has been developed that uses fluorescent copper nanoclusters. These nanoclusters are non-toxic and thus enhance the stability and sensitivity of the staining method [26].

Electrophoretic mobility shift assay (EMSA) was developed by Fried and Crothers in 1981, to study the DNA-protein interactions. This method follows the fundamental principle which states that a protein bound DNA has a retarded mobility through polyacrylamide gel in comparison to unbound DNA [27,28]. EMSA is considered the most sensitive method for DNA binding proteins, as it employs radioisotope-labeled DNA. Binding affinity and parameters of a protein can be determined using EMSA technique. Higher-order complexes

involving more than one protein can also be analyzed by this method [29]. Variations such as assay using chemiluminescence, fluorescence, and immunohistochemical detection can be used, in case high-sensitive analysis is not required. DNA with varying structures or lengths is well suited for EMSA technique [28]. One of the foremost applications of EMSA is the analysis of binding of transcription factors and other proteins which are DNA sequence-specific. This technique can also be used to detect the specificity of protein-DNA complex [30]. As DNA labeling is a hazardous process and involves radioisotope, Jing et al., developed a method which eliminated the use of radioisotope-labeled DNA. In this protocol, nucleic acids are identified by SYBR Green EMSA dye, whereas proteins are identified by SYPRO Ruby EMSA dye [31]. However, EMSA is itself a resourceful and informative technique, but in the combination of other techniques such as immunoblotting, immunodepletion and shift-western blotting, it becomes even more powerful [32].

Variety in Circular Dichroism Spectroscopy

Circular Dichroism (CD) is a convenient and accurate spectroscopic technique to get information of conformational changes in the secondary structure of nucleic acids or proteins. CD spectra are given by asymmetric molecule, as they can absorb the dissimilar amount of left and right-handed polarized light. The conformational changes in DNA structure upon ligand binding can also be accessed using CD spectroscopy. It is commonly used to discriminate between structural and conformational polymorphs of DNA (A-, B-, Z- etc.), which have different CD characteristics [33]. Structural polymorphs of DNA give different CD signatures. (Figure 2c). The B-form of DNA is characterized by a positive peak at 275 nm, which corresponds to base stacking and a negative peak at 245 nm correspond to right-handed

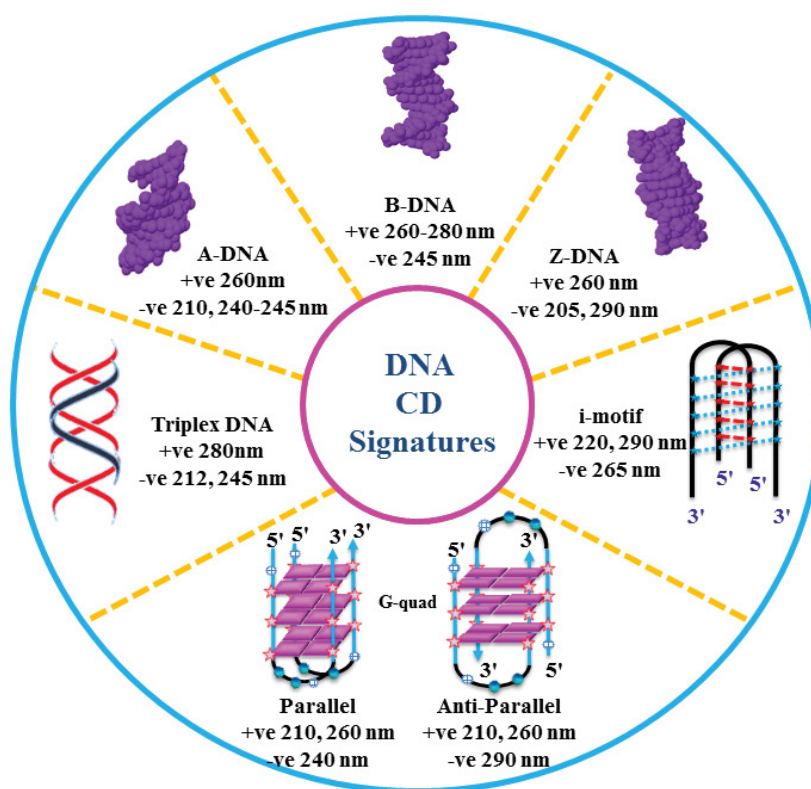


Figure 3: Generalized CD signatures of various structural polymorphs of DNA.

helicity [34-36]. All the major CD signals of various conformations of DNA have been summarized in Figure 3. CD spectra can easily detect the percentage of an alpha helix or beta sheet conformation in proteins [37-39]. Various conformations of G-quadruplex (parallel, antiparallel or mixed) can be differentiated because of their characteristic CD signatures [40-42] and had been quite extensively utilized.

Vibrational Circular Dichroism (VCD) is the advanced version of CD spectroscopy to study infrared and near-infrared ranges [13]. VCD can provide us a vital piece of information of the electronic ground state vibrations of the molecule. The absolute configuration of natural products or synthesized compounds can also be obtained with the help of VCD [43]. The characteristic C=O, C=N and C=C vibrations of nucleobases are examined in the spectral region 1800-1300 cm^{-1} and therefore VCD can be used to monitor this spectral region [44]. Recently, the absolute configuration of a variety of metal complexes has also been resolved by VCD [45-48]. This technique was used to explore the effect of the various compositions of mixed solvents and temperature on the conformation and aggregation of the two synthesized polypeptides (PBLA, PBLG) [49]. In a recent report, a new method in VCD is used which is called as Exciton chirality method. It can quite easily get the absolute configuration of the molecule, which used be very difficult by other spectroscopic techniques [50].

Electronic circular dichroism (ECD) spectroscopy is very convenient, fast and sensitive in determining the electronic structure of the molecule. ECD measures the impact of the excited state vibration during the absorption process. This technique requires very less (<1 mg) sample, and the information provided by this technique is restricted to chromophores only. The absolute configuration can be assigned by using semi-empirical or empirical rules which are supported by quantum mechanical calculations [51]. The ECD is advantageous over VCD in terms of smaller sample and time requirement for the experiment. Both the techniques (ECD and VCD) are used complementarily, to detect the absolute configuration of the molecule. It is also suggested that more than one chiroptical techniques should always be used, to increase the authenticity of the absolute configuration of any compound [52].

Magnetic circular dichroism (MCD) is almost similar to conventional CD. The only difference is that, in MCD, the sample is placed between magnetic field, which is aligned parallel to the direction of the propagation of light. This technique is advantageous for the samples which are too weak to be detected in circular dichroism. In MCD, the CD signals are induced in the sample by using a magnetic field, as in the presence of magnetic field, left and right circularly polarized light do not interact evenly to the absorption medium [53]. MCD signal for a particular compound can only be acquired at a wavelength where it has optical absorption. MCD can be used to explore the information of both ground and excited state of the molecule. It has its prime application to investigate the electronic as well as the geometric structures of the metal center in metalloproteins [54]. MCD is found to be perfect in determining spin as well as the oxidation state of the metal, in the case of ferric heme protein [55]. Various applications of MCD for porphyrins and phthalocyanines are reviewed by Nagao and co-workers [56]. MCD is also used for the quantitative analysis of proteins, for the amount of tryptophan present in that particular protein; in fact, among natural amino acids, tryptophan is the only amino acid which gives MCD signals [57].

Extensions of Fluorescence Spectroscopy

Fluorescence spectroscopy is commonly used to decipher the DNA-drug interactions. Its selectivity and sensitivity make it

an advantageous technique over other spectroscopic techniques. Generally, compounds consist of aromatic functional groups having low $\pi \rightarrow \pi^*$ transition levels show intense fluorescence spectra [11]. The fluorescence intensity of any fluorescent molecule is quenched after the binding of an external molecule to it (Figure 2d). Several kinds of molecular interactions are responsible for the quenching such as energy transfer, complex formation in the ground state, excited state reactions, molecular rearrangements, and collisional quenching [58]. Two types of quenching mechanisms are known: static and dynamic. In static quenching, the complex formation is observed between the fluorophore and the quencher; while such kind of complex formation is absent in the case of dynamic quenching. The type of quenching can be monitored by tracking the Ksv values, the Stern-Volmer constant. With an increase in temperature, Ksv value decreases in case of static quenching, while an increase is noticed in the case of dynamic quenching [59]. The intrinsic binding constant (K_b), and the number of binding sites (n) can also be calculated with the help of the following equation [60].

Foster resonance energy transfer or fluorescence resonance energy transfer (FRET) is the transfer of non-radiative energy from one molecule (excited state) to another molecule (ground state). It is highly sensitive technique used in the field of biology and chemistry. It is used to explore the information of DNA-drug interactions, protein-protein interactions, and the conformational variation in the structure of the proteins [61]. The distance between two chromophores and their relative orientation are very crucial for the efficiency of the energy transfer [62]. To check the formation of the complex, one molecule is labeled as a donor and the other as acceptor. For this purpose, generally cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are used in combination; where CFP acts as a donor, while YFP is an acceptor [63]. The inorganic fluorophores (quantum dots) are used as an alternative due to their distinct photophysical property and nanoscale size. Daniel and Niko have reviewed the modern expansion of the use of quantum dots as a fluorophore [64]. Recently, graphene and their derivatives have reported being a good fluorophore due to its biocompatibility, strong photoluminescence emission, and adjustable band gap [65]. FRET-based sensors have also been developed which can be effectively used in the live cell monitoring [66].

Fluorescence correlation spectroscopy (FCS) is a powerful tool in biological and analytical chemistry. FCS is widely used to determine the kinetic chemical reaction rate and diffusion coefficients by observing the inconstancy in the fluorescence intensity of the system. It is advantageous over many biophysical and other fluorescence techniques due to its high sensitivity. It can read from nanomolar to picomolar concentrations in a small volume (1 μm^3) [67]. Change in the concentration of fluorescent molecule in a defined volume is the main factor which is responsible for the fluctuation in the fluorescence intensity. It is used in the context of confocal microscopy to make it easier, to work with fluorescent proteins in live cells and to characterize the bimolecular interactions at physiological pH [68]. It is apparent that FCS can work accurately, only with smaller concentrations, when it has to detect very few molecules simultaneously [69]. FCS technique is also applied to get the possible mechanism of the molecular lateral diffusion of the plasma membrane of the living cells [70]. Kai et al. have explained the mechanism of PEGylation of silica nanoparticle with the help of FCS [71]. It is also used in the study of aggregation and oligomerization of the proteins *in vitro* as well *in vivo* [72].

Fluorescence *in situ* hybridization (FISH) is a fast growing technique which is used to study the structure and the function of the cell, particularly the chromosomes. It uses special types of

fluorescence probes which hybridize to the specific part of the chromosome with a prominent sequence complementarity. After hybridization of the target DNA sequences, fluorescence microscopy is used to envision the hybridized probe on the target material [73]. It permits the examination of the genetic variation and tissue framework and monitors the presence or absence of particular DNA sequences. It is used in a number of areas including disease diagnosis, therapeutic targeting, gene mapping, and karyotype analysis [74]. It is advantageous over other similar techniques, as it can distinguish many targets at one time by using multiple fluorochromes. The tumor repetition and progression can also be monitored using FISH technique [75]. Recently, this technique was used to detect the renal primitive neuroectodermal tumor in the human body [76]. FISH can not only be used for the identification and visualization but it also gives the localization of the microorganism in the tissue [77]. It is also used for the detection of genomic alterations and disturbances in the patients with chronic lymphocytic leukemia and dermatofibrosarcoma protuberans [78]. FISH can also be utilized to compare the genome of two different biological species to get information on evolutionary relationships. On the basis of thermodynamic models, Silvia et al. have reviewed various aspects for the maintenance of melting temperature during FISH experiment [79].

Recently, multistranded guanine-rich DNA structures known as G-quadruplexes have been visualized *in vivo* by employing fluorescent imaging with structure-specific BG4 antibody which binds with the telomere G-quadruplex with high selectivity [80]. Detection of RNA in the whole organism is utmost important for resolving the tissue expression pattern of a single transcript. Since the 1980s, *in situ* hybridization was the only technique to visualize RNA in cells, but recently, various methods for RNA imaging have been explored. For example, RNA-binding proteins, usually via fluorescence *in situ* hybridization (FISH), through binding of fluorophore and hybridization results into fluorescence of RNA aptamer. But still, research in the same field is required to develop a method which eliminates the challenges of low abundance of target RNA and permeability of dyes into cells [81].

Outlook and Future Directions

In spite of continued research in the field of characterization techniques of biopolymers, there are still lacunae left in the knowledge related to biological entities. Our knowledge in terms of isolation, identification, purification and structural as well as functional characterization of a large number of DNA, RNA and proteins is still limited. Even though there are advanced techniques to characterize a wide range of biopolymers which are designed or extracted from different sources like plants, animals, viruses, bacteria's, etc., traditional techniques cannot be precluded. In fact, up-gradations in the traditional techniques facilitate the data gathered from the advanced techniques. New structures and conformations of DNA, RNA and proteins have continuously been deciphered and many of them have already shown their *in vivo* existence. Collecting data from different techniques, interpreting and analyzing the same for reaching to some conclusion really needs quite meticulous technical data. Generation of these datasets for giving us new directions for interdependent relationships between structure and function of a cell and their constituents is essentially crucial for mining the information related to molecular pathways and mechanisms. This review bolsters the advancements in biochemical, biophysical, electrochemical, thermodynamic and molecular biology related techniques which are of utmost importance for exploring and understanding the intricate machinery of the cell.

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