Development of Bio-Molecular Methods for Proteins and Aptamers/Oligonucleotide

Ankit Shrivastava[1], Dr. Pushpendra Sharma[2]

[1]Research Scholar of Sri Satya Sai University of Technology & Medical Sciences, Sehore, M.P., India.
[2]Research Supervisor of Sri Satya Sai University of Technology & Medical Sciences, Sehore, M.P., India.

Abstract

With the advent of the high-throughput technologies and exciting times for biology, the discipline of analytical methodology is experiencing a surge in the growth and the scope. Over the years, multitude of analytical techniques have evolved from a work-intensive, low sensitivity and high volume of reagent and sample consumption endeavor to automated, better selectivity, lower limit of quantification and cost-effective techniques for biological research. In this review, we give an overview of the currently available wide range of cell-based and noncell based and structural based analytical techniques, their principle and biological applications. The analytical techniques discussed in this paper includes surface plasmon resonance, electrophoresis, enzyme linked immunosorbent assay, Western blotting, flow cytometry, fluorescence activated cell sorting, mass spectrometry, nuclear magnetic resonance and x-ray crystallography.

Keywords: *Bio-Molecular Methods, Proteins, Aptamers/Oligonucleotide, Biological, Cell Electrophoresis, Structure Technique.*

1. INTRODUCTION

In the last few decades, significant progress has been in expanding the knowledge and understanding of the range of analytical technologies to analyze the biological molecules. The characterization of complex biological macromolecules such as proteins, DNA, RNA, carbohydrates and lipids using analytical techniques goes back to 19th century when scientists use simple separation methods such as acidification or crystallization following the addition of precipitation agents to extract and purify nucleic acid and proteins in order to understand their structure and determine their characteristics [1,2]. Furthermore, significant developments in the field of biology such as discovery of the DNA and protein structure more than 50 years ago has provoked scientists worldwide to develop better understanding of the complex DNA and protein interactions and other cellular processes along with the various factors affecting these processes [3]. This led to development of several advanced analytical technologies, which are automated, real-time, reliable, reproducible and cost-effective resulting in faster and more accurate monitoring and detection of biomolecules. Bioanalytical field has grown over time from being an ancillary branch of science that was used to analyze and confirm data for other disciplines and is now an area of expertise and an independent well-established scientific discipline solving complex biological problems, unearthing solutions, elucidating the functional properties and deducing the structure of biological molecules. In the past, the analytical characterization of biological molecules was complex, time-consuming, labor-intensive and limited in terms of overall throughput. Over the last few decades, major advances have been made by scientists in developing analytical tools to address challenging biological problems including the identification of potential therapeutic and diagnostic biomarkers for personalized treatment and thus bringing a revolution in human health. And it is an important stepping stone in the field of drug discovery and development. With the increasingly broad adoption of automated and forefront analytical technologies for detection of biomolecules, the sample analysis have become more efficient, accurate, rapid and cost-effective and a reduced risk of cross-contamination.

Nowadays, the researchers and scientists can leverage the discovered knowledge to make well-informed decisions, design follow-up experiments and provide compelling biological and mechanistic evidence for results. This review highlights the role of the analytical instrumentation and bioanalytical techniques utilized for assessing the biological samples. Herein, the scope of the review includes a wide array of noncell and cell based bioanalytical techniques that are used by scientists to obtain a fundamentally deeper and broader understanding of biological relationships

and approaches for structural analysis and design of biological molecules. Some of the well-established techniques that have been discussed in this paper are surface plasmon resonance (SPR), ELISA, flow cytometry (FC), x-ray crystallography, nuclear magnetic resonance (NMR) and many others that are utilized to gain better understanding of the biological molecules found on the earth surface.

2. TECHNIQUES USED TO CHARACTERIZE NUCLEIC ACID AND PROTEINS

Gel-Electrophoresis

Gel electrophoresis is a technique, used for the separation of macromolecules-especially nucleic acids, proteins and for protein-complex characterization. The relative rate at which an individual molecule move depends on several factor like net charge, molecular shape, charge to mass ratio, porosity and viscosity of the matrix through which the molecules migrates. The net charge depends on the number of positive and negative charges present in a molecule. Nucleic acids have a consistent negative charge due to the presence of phosphate backbone due to which they migrate towards the anode [3] whereas proteins can have either a net positive or net negative charge. These molecules will move towards the cathode if positively charged or towards the anode if negatively charged (Figure 1). Therefore, the different physical and chemical properties of a molecule are accountable for their movement through a gelatinous medium.

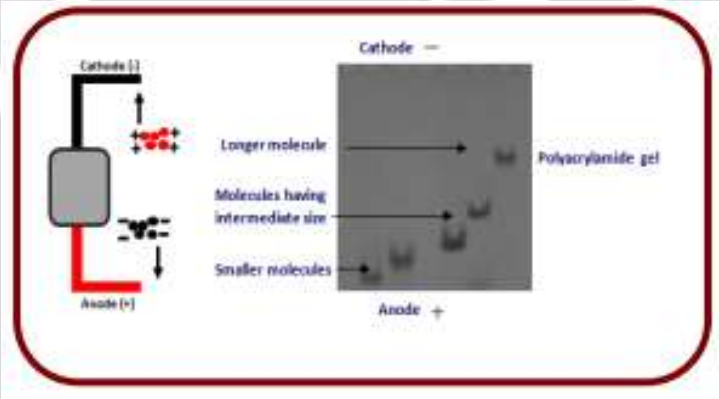

Figure 1: Migration of charged molecules in an electric field on the basis of (a) charge and (b) size

The basic principle involved in the process of electrophoresis is similar, but the major difference lies on the type of support medium like cellulose or thin gels. Polyacrylamide and agarose gels are used as support medium for larger molecules. For low molecular weight biomolecules such as amino acids and carbohydrates, cellulose is used. However, denaturing gels are also performed to check the purity of the biomolecules.

Denaturing Gels

Denaturing gels have been used for the separation and purification of single stranded fragment of DNA and RNA. Denaturation is the destruction of the molecular conformation of the native protein and nucleic acids, and results in the loss of their biological activity. Denaturation can be carried by the addition of denaturants, strong acid or base (i.e. changing the pH of the solution), an organic solvent (e.g., alcohol or chloroform), or heat. Under nondenaturing conditions, such samples (nucleic acid or proteins) migrate in a manner determined largely by their intrinsic electrical charge.

(i) Urea-Polyacrylamide Gel Electrophoresis (Urea –PAGE)

Denaturing polyacrylamide gels are used to allow the separation of molecules by their relative size by keeping the molecules denatured. Formamide and urea is often used to denature DNA and RNA and allow their separation on the basis of size by keeping the molecules single stranded. Samples can also be heat treated for denaturation. The secondary structure of nucleic acid is stabilized by hydrogen bonding between the base pairs. Similar hydrogen bonds are involved in stabilization of secondary, tertiary and quaternary structures of proteins. High concentration of

urea (7-8 M) interrupt the hydrogen bonding between two functional groups, leading to complete disruption of secondary, tertiary and quaternary structures.

(ii) Sodium Dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE is the technique used for the analysis of protein mixture qualitatively. It is mostly used for the purification of protein and their molecular mass determination. SDS (CH3-(CH2)10-CH2OSO3 - Na+) is used to distort the secondary, tertiary and quaternary structure of proteins to produce linear polypeptide chains. Each protein in the mixture gets fully denatured by this technique. Consequently, SDSpolypeptide complexes will have net negative charge and migrate towards anode at rate based solely on size and molecular weight of the polypeptides. After electrophoresis and dye staining, mobilities are measured and molecular weight can be determined graphically.

2. Native Gels

(i) Polyacrylamide Gel

Polyacrylamide gel electrophoresis (PAGE) is a dynamic technique, used for the physical-chemical characterization of proteins and nucleic acid on the basis of size, conformation, and net charge. These gels are formed by the free radical polymerization of acrylamide (monomer) into long chains and the cross linking agent is- N-N'-methylene-bisacrylamide. The initiation process is carried out by the addition of ammonium per sulfate and the base N, N, N', N'- tetramethylene diamine (TEMED). An alternative method that can be used to polymerize acrylamide gels is photopolymerisation. Photopolymerization can be instigated by riboflavin, which is used in place of ammonium persulfate and TEMED. The gel is kept in front of a bright light for 2-3 hours after being poured, and polymerization is initiated by the free radical generated by the photodecomposition of riboflavin. There are several advantages of using polyacrylamide gels such as, it is chemically inert, provides high resolution and gels can be prepared with wide range of pore sizes.

(ii) Agarose Gel

PAGE is used for analyzing small fragments of nucleic acids (500bp) in molecular size; however, large nucleic acid fragments cannot be adequately analyzed due to the small pore sizes. These gels are used for both nucleic acid and proteins. These gels have a larger pore size in comparison to polyacrylamide gels and therefore can be used to analyze large biomolecules. Agarose is a polysaccharide made up of the agarobiose, which comprises alternating units of D-galactose and 3, 6- anhydro-L-galactose. The mobility of nucleic acids in agarose gels depends on the conformation of the nucleic acid, the molecular size and agarose concentration. For separation of nucleic acids, gels having 0.3 to 2.0 % agarose concentration are most effective. DNA molecules of the same size but with different conformations migrate at different rates. For example, the small, compact, supercoiled forms usually have the higher mobility, in comparison to the linear double stranded DNA. The extended, circular DNA migrates more slowly. However, agarose concentration and ionic strength are also considering factors while determining migration pattern of the various forms of DNA.

3. UV-THERMAL DENATURATION

Spectroscopy is a branch of science which deals with the study of interaction of electromagnetic radiation with matter and the measurements which are based on light and other forms of electromagnetic radiation are called spectrochemical methods/techniques. These techniques are widely used for the elucidation of molecular structure as well as the qualitative identification and quantitative estimations of both organic and inorganic compounds. The stability of the secondary and tertiary structure of biomolecules is important for many biological processes. The stability of nucleic acids and proteins can be evaluated by different spectroscopic techniques and one of them is UV-Thermal Denaturation. Nucleic acids absorb strongly in the UV region (around 260 nm) whereas proteins absorbs at 280 nm. As the temperature of the solution (containing DNA/protein sample) increases, the absorbance increases. This increase in absorbance (hyperchromicity) results from the unwinding of the two strands of DNA/ dissociation of the secondary/tertiary structures to the primary structure. When a solution containing DNA is heated, a change in absorbance properties occurs, which reflect conformational changes in the molecule [4, 5]. The temperature at which 50% of the DNA sample is melted is called melting temperature (Tm) and determines the thermal stability of the duplex or structure of interest. It not only provides the stability of any biomolecule of interest but is one of the possible methods to predict the stabilization or destabilization of a nucleic acid/ protein-structure by a ligand.

4. CIRCULAR DICHROISM (CD)

Circular Dichroism (CD) is powerful spectroscopic technique widely used in biochemistry to detect conformational alterations of nucleic acids, in particular DNA [6], proteins and DNA-protein interaction [7]. CD is a form of optical activity which arises from the differential absorption between the components of left- and right circularly polarized light by a solution of chiral molecules. Optical activity is a characteristic of many organic and almost all biological molecules and, each optically active compound has a characteristic specific rotation. A very large fraction of biological molecules contain optically active centers and when applied to molecules such as proteins, nucleic acids and carbohydrates, CD can provide their detailed structural conformations. Non-chiral molecules exhibit no CD signal in solution, however a CD signal can be obtained when any achiral ligand binds to a chiral host [8]. The structures of proteins and DNA are examined in the wavelength range from 160 to 400 nm because in these regions electronic transitions occurs in the purine and pyrimidine bases, peptide backbone and side chains in proteins. Biopolymers such as nucleic acids and proteins are folded into secondary, tertiary and quaternary structures by ionic interactions, stacking interactions, hydrogen bonding and hydration. CD spectroscopy is quite informative in elucidating the conformations adopted by various nucleic acid and protein secondary structures, conformational changes due to interactions with ligands and environmental effects [9]. DNA exhibits structural polymorphism and can adopt either A-, Band Z- conformation depending on the oligonucleotide sequence and solution conditions. The native conformation of DNA in solution under physiological condition is B- form. A typical B- form of DNA shows positive peak at 280 nm and a negative peak at 245 nm. Runs of homopurine homopyrimidine DNA sequence [poly(dG)•poly(dC)], forms an A- like helix and give positive peak at 260 nm and negative peak at 240 nm. Z- DNA can form in alternating purine•pyrimidine tracts (dGCGCGCGC). The Zform exhibits a negative peak at 290 and a positive peak at 260 nm.

5. CONCLUSION

The ever-evolving and exponentially growing field of analytical method development and validation demand effective and high sensitive detection of minute traces of complex biological molecules. In order to so, there is an increasing demand for new analytical technologies and constant improvements in the sensitivity and specificity of the current analytical methodologies. Within next 5–10 years, the development of new, innovative and reliable analytical tools, techniques and technologies is anticipated particularly the early drug development stage which can be extensively employed for detection of vast array of biological molecules such as monoclonal antibodies, oligonucleotides, aptamers and peptides. In today's era with stringent regulations in place, the development of sound analytical method is of paramount importance in the field of drug discovery and drug development process and the obtained data during this process must meet the scientific and regulatory standards. Considering the recent trends and future scenario, this review paper discusses some of the vital analytical techniques both noncell based and immunological assays and structural methods that are utilized by the galaxy of scientists worldwide for the quantitative detection and evaluation of biological molecules, which is critical for the successful drug development studies. We believe a good fundamental understanding of these everyday analytical techniques along with their pros and cons and their broad applications in biology could spur a greater interest in the scientific community particularly analysts to employ them to gain better insights about the intricate biological molecules at minuscule level.

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