Lecture 1 Introduction

This course aims to provide the detailed guidelines and procedures for carrying out various biotechnological experiments and analyzing the results obtained. The experiments include estimation of biomolecules, especially proteins and nucleic acids; separation, purification, and characterization of the biomolecules; studying biomolecular interactions using spectroscopic and immunological assays; studying the structure of cell and its components; and certain experiments related to molecular biology and genetic engineering. The course is divided into following seven modules:

Module 1: includes introduction to the course, good laboratory practices, and introduction to water and buffers

Module 2: includes the methods for estimating and characterizing the biomolecules using spectroscopic methods

Module 3: includes electrophoretic experiments for separating and characterizing the proteins and nucleic acids

Module 4: includes chromatographic experiments for separating, purifying, and characterizing biomolecules

Module 5: discusses various immonoassays

Module 6: discusses the microscopic methods for studying the cellular structure, their components, as well as the processes they are involved in

Module 7: discusses the basic tools and experiments of molecular biology.

This lecture briefly introduces the general guidelines and precautions one needs to adhere to before carrying out the experiments in a laboratory. The lecture also introduces the good laboratory practice, an internationally accepted regulation of Organisation for Economic Co-operation and Development (OECD) that ensures quality assurance of the scientific and industrial research. The lecture also briefly reviews the chemical and structural properties of proteins and nucleic acids, the classes of biomacromolecules studied in most of the experiments discussed in this course.

Guidelines/precautions for a laboratory course

Let us see what all things are important for a laboratory course, especially a course utilizing biological specimens, chemicals, and possibly harmful radiation. Let us outline the dos and don'ts for a wet-lab course:

Do's

1. Always wear the appropriate equipment of personal protection. They include an apron, a pair of gloves, safety glasses, and shoes.
2. Read thoroughly the detailed procedure before carrying out the experiment.
3. Identify the potentially hazardous materials required for performing the experiments. It is recommended to go through the Material Safety Data Sheet (please see the next section) supplied with the chemicals to understand their potential hazards and the safe usage.
4. Before starting the experiment, label your materials (tubes, vials, specimens, etc.).
5. Check the label on the container before taking out any substance.
6. Use cleaned spatula for taking out the solid chemicals.
7. Be well aware of the locations and the operating procedures of the safety equipments such as safety shower, first aid kit, fire extinguisher, eyewash, etc.
8. Use a chemical fume hood for strong acids and other fuming chemicals and solvents.
9. Use the recommended biosafety level containment while handling microorganisms.
10. Before using a biosafety cabinet, always ensure that the UV lamp is turned off.
11. Decontaminate your work bench and the equipments handled after use.
12. Decontaminate all the infectious materials before disposing them off.
13. Maintain a laboratory book and write down all the methods, procedures, and observations in detail.
14. Any spillage, accident, or injury should immediately be reported to your instructor.

Don'ts

1. Do not sit on the laboratory benches.
2. Never eat or drink anything inside the laboratory.
3. Do not handle your contact lenses or apply cosmetics in the lab.
4. Never touch any chemical or reagent with bare hands.
5. Never try to taste or smell any chemical or reagent.
6. Do not pipette any of the chemicals through your mouth.
7. If the chemicals are taken from the original container, and you happen to take out more than the amount required, never transfer back the excess amount to the original container.
8. If concentrated acids are to be diluted, never add water to the concentrated acid; concentrated acid should be added to the water drop-wise with continuous stirring.
9. Never leave your experiments unattended.
10. Never point the open end of any glassware or vial towards yourself or any other person.

Material Safety Data Sheet (MSDS)

A Material Safety Data Sheet is a document provided by the manufacturer/supplier that contains information about the potential hazard of the material. An MSDS comprises of sections on product information (product name, names and addresses of manufacturer and supplier, emergency numbers); ingredients of the material; physical properties of the material; fire or explosion hazard data; toxicological data; first aid measures in case of an accident; and information on safe handling, usage, storage, and disposal. You might have observed certain hazard warning symbols, also known as pictograms, on the chemical containers. These warning symbols allow immediate identification of the material as a hazard.

Globally Harmonized System (GHS) of Classification and Labelling of Chemicals

Globally Harmonized System of Classification and Labelling of Chemicals, introduced by United nations in 2000, is an internationally accepted system for classifying and labeling the chemical. The chemicals are classified as physical

hazards, health hazards, and environmental hazards. Some of the routinely observed symbols are shown in figure 1.1.

Flammable Oxidising Acute toxicity Corrosive

Figure 1.1 Some of the commonly observed hazard warning symbols (pictograms) on the chemicals.

Good Laboratory Practice (GLP)

What exactly the GLP is? GLP is often confused with the standards of safe laboratory practices such as wearing aprons, gloves, and safety masks. GLP goes beyond the safe laboratory practices. An internationally recognized definition of GLP goes like this: *Good Laboratory Practice (GLP) embodies a set of principles that provides a framework within which laboratory studies are planned, performed, monitored, recorded, reported, and archived*. New Zealand and Denmark were the first to introduce GLP in1972. United States was the next to introduce GLP in response to the poor scientific practices prevalent in US around that time. In early 1970s, US Food and Drug Administration realized the poor laboratory practices throughout United States. FDA also became aware of the fraudulent data produced by several toxicology laboratories; Industrial BioTest Labs being the most noted one. An international economic organization, the Organisation for Economic Co-operation and Development (OECD) published the principles of GLP in 1981 under the name 'OECD Guidelines for the Testing of Chemicals' that were internationally accepted. These guidelines are available on the OECD website: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm.
GLP makes sure that the raw data generated during the study are traceable thereby ensuring the authenticity of the published data. GLP mandates a Quality Assurance unit that is required for monitoring and auditing the studies that are underway.

Majority of the experiments discussed in this course will, directly or indirectly, utilize two major classes of biomolecules, proteins and nucleic acids. It is therefore worth reviewing the chemical nature and the structure of the proteins and nucleic acids.

Chemical and structural features of proteins and nucleic acids

Amino acids and Proteins

Proteins constitute the cellular machinery that carry out majority of the biological reactions. They are linear polymers of L-α-amino acids. The structural of a typical L-α-amino acid is shown in figure 1.2. The R group, also known as the side-chain of an amino acid, makes a repertoire of 20 different amino acids. In proteins, amino acids are linked together through amide bonds formed between α-amino group and the α-carboxylate group as shown in figure 1.2B. As both α-amino and α-carboxylate groups are involved in making the peptide bond, the chemistry of the polypeptide is determined by the chemistry of the side-chains of the constituent amino acids. Based on the chemistry of their side-chains, amino acids have been classified as *polar* and *non-polar*. Polar amino acids have been further classified into neutral, acidic, and basic amino acids depending on the charge on the side-chain at neutral pH. Lysine side-chain, for example, has a terminal amino group that is protonated at neutral pH; lysine, therefore, is identified as a basic amino acid. Aspartic acid and glutamic acid have carboxyl group in their side chains; at neutral pH, the side chains are ionized making them the acidic amino acids.

The amino acids in a protein are linked through an amide bond, called a peptide bond (Figure 1.2B). Delocalization of the nitrogen's unshared pair of electrons over carbonyl group imparts a partial double bond character to the peptide bond. This implies that rotation about the C(O)–N(H) is not allowed, putting constraints on the conformations a polypeptide backbone can adopt. The rotations about C_α–C(O) and N–C_α, however, are allowed; and these dihedral angles are referred to as psi (ψ) and phi (φ), respectively (Figure 1.2B). The amino acid sequence of a protein, which is also termed as its primary structure, is read from N-terminal to C-terminal. The polypeptide regions within a protein can adopt local ordered structures (very similar φ and ψ values for a continuous stretch of amino acids) called secondary structures (Figure 1.2C); α-helices and β-sheets are two such secondary structures. Further assembly of the polypeptide chain leads to a compact structure, termed the tertiaty

structure (Figure 1.2D). Many proteins function as multimers *i.e.* they have more than one polypeptide chains in their functional form that interact with each other through non-covalent interactions. Such multimeric protein structures are referred to as the quaternary structures (Figure 1.2D).

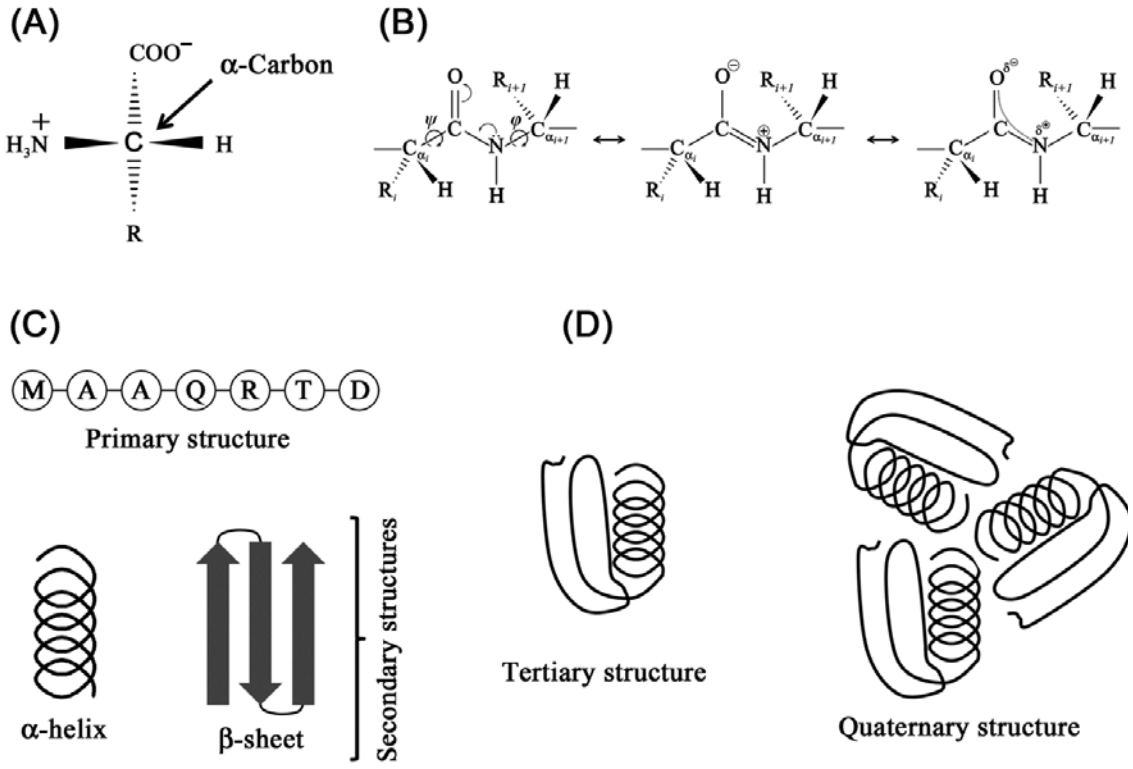

Figure 1.2 Structures of amino acids and proteins. Panel A represents the structure of a typical amino acid; the side-chain R determines the chemistry of the amino acid in a protein. Panel B shows the resonance structures and the partial double bond character of peptide bond. Panel C defines the primary and secondary structures of the proteins. Panel D shows the definitions of tertiary and quaternary structures.

Nucleotides and Nucleic Acids

A nucleotide is the monomeric unit of nucleic acids. Chemically, a nucleotide is composed of a pentose sugar attached to a heterocyclic nitrogenous base through N-glycosidic linkage and to one or more phosphate groups though phosphoester bonds (Figure 1.3A). The pentose sugar can be a ribose (the nucleotide is called a *ribonucleotide*) or a deoxyribose (the nucleotide is called a *deoxyribonucleotide*). Nucleic acids are the linear polymers of nucleotides; the polymer of ribonucleotides is called a ribonucleic acid (RNA) while that of deoxyribonucleotides is called a deoxyribonucleic acid (DNA). The nitrogenous bases present in nucleic acids are derivatives of purines and pyrimidines (Figure 1.3B).

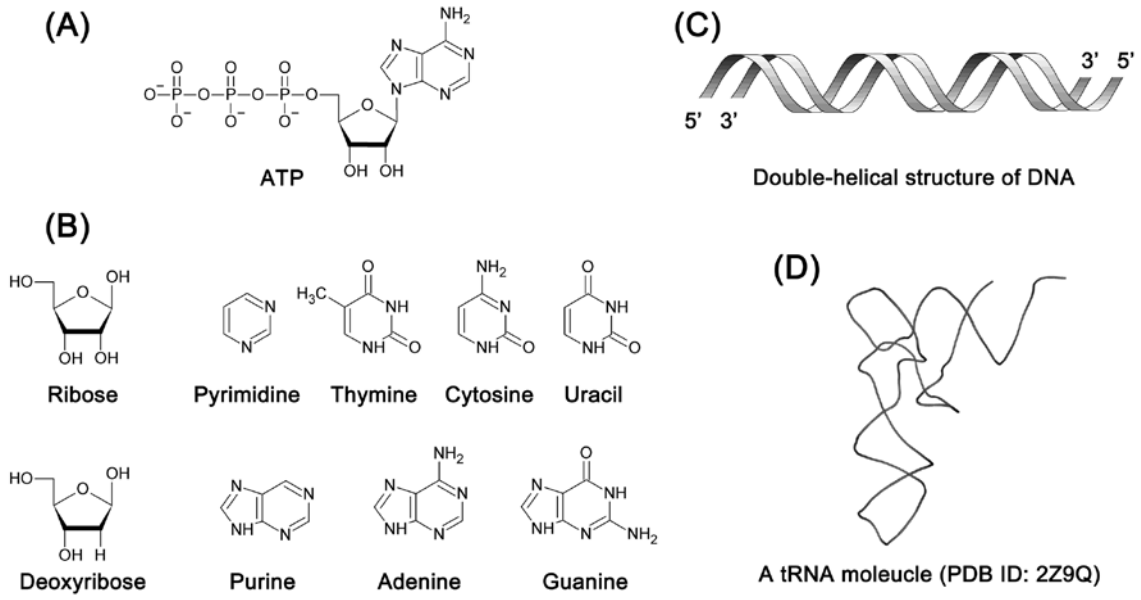

Figure 1.3 Structures of nucleotides and nucleic acids. Panel A represents the structure of Adenosine triphosphate (ATP). Panel B shows the structures of the sugars and the bases present in the nucleic acids. Panel C shows the double-helical structure of DNA; notice the directionality of the two complementary strands. Panel D shows the structure of a transfer RNA (tRNA) molecule (PDB ID: 2Z9Q).

The bases present in DNA are *adenine* (A), *thymine* (T), *cytosine* (C), and *guanine* (G). In RNA molecules, *thymine* is replaced by *uracil* (U). DNA is usually made up of two antiparallel chains coiled around each other in a double helical structure (double-stranded DNA or dsDNA). Adenine-thymine, adinine-uracil, and guanine-cytosine are said to constitute the complementary base pairs in nucleic acids. The two strands of the DNA molecules are antiparallel and complementary to each other *i.e.* thymines and cytosines on one strand are paired with adenines and guanines, respectively on the

other one (Figure 1.3C). RNA molecules are typically single stranded but can be double-stranded as well *e.g.* dsRNA viruses. RNA molecules can have intramolecular complementary sequences causing them fold into defined three-dimensional structures *e.g.* transfer RNA molecules (Figure 1.3D).

Nucleotides such as adenosine triphosphate (ATP), guanosine triphosphate (GTP), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and coenzyme A (CoA) play critical roles in metabolism and intracellular signaling.

Lecture 2 Water and aqueous solutions

Water is the most abundant chemical component present in living systems. It constitutes around 60% or more of the total mass of most of the organisms. The first organisms are believed to have originated in the aqueous environment. Most of the biological reactions are carried out in water; many of them directly involving water as one of the reactants or products. It will not be incorrect if we call water as the biological solvent. To understand the chemistry of the biological processes and the behavior of biological molecules in water, we need to understand the physical and chemical properties of water. Biochemical experiments are mostly carried out in aqueous solutions having a defined chemical composition and pH. In this lecture, we shall be discussing the physical and chemical properties of water, concepts related to solutions, and pH.

Hydrogen bonding and physical properties of water

Water (H_2O) is a molecule wherein the oxygen atom is sp^3 hybridized; two of these hybridized orbitals make σ bonds with hydrogen atoms while the other two are occupied by the unshared pairs of electrons. Water, therefore, is a bent molecule with an H–O–H angle of 104.5º (Figure 2.1A). The large difference in the electronegativities of oxygen and hydrogen atoms make O–H a highly polar bond. Owing to the angular geometry of the molecule, the dipole moments of the two O–H bonds do not cancel each other out, imparting it a high dipole moment of 1.85 Debye. The oxygen atom is partially negatively charged while the hydrogen atoms are partially positively charged. This leads to an electrostatic attraction between the oxygen atom of one water molecule and the hydrogen atoms of the other. This interaction is referred to as the hydrogen bond (Figure 1.2B). It is due to these hydrogen bonds, that water molecules are held together making it a liquid at room temperature. Hydrogen bonds (H-bonds) between the water molecules impart it high melting point, boiling point, and heat of vaporization. Low molecular weight and the ability to form H-bonds also impart water a very high specific heat; it takes 1 calorie to increase the temperature of 1 gram of water by 1 ºC at standard temperature and pressure. Water, therefore, is as an excellent temperature buffer. Hydrogen bonds are not restricted to water; they are readily formed between an electronegative atom (called a hydrogen acceptor; usually fluorine, nitrogen, and oxygen) and a hydrogen

attached to another electronegative atom (called a hydrogen donor). Hydrogen bonds are weak but play very crucial roles in the structure and function of the biomolecules. Common hydrogen bonds observed in biological systems are shown in figure 2.1C.

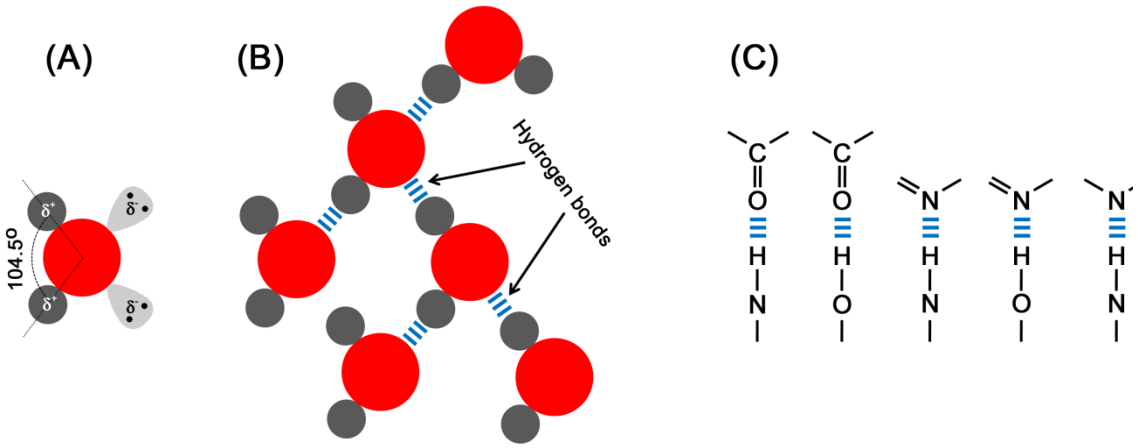

Figure 2.1: Structure of water and hydrogen bonding. Panel A shows the bent structure of water molecule; the big red sphere represents the oxygen atom while small spheres represent the hydrogen atoms. Panel B shows the hydrogen bonding between water molecules. Panel C shows the types of hydrogen bonds frequently found in biomolecules.

Polar solutes can form hydrogen bonds with the polar water molecules thereby dissolving in water. Non-polar solutes, on the other hand, do not make favorable interactions with the water molecules and clump together. Water can easily dissolve the charged solutes by hydrating and stabilizing the solute ions. Sodium chloride, for example, readily dissolves in water; the Na^+ and Cl^- ions aquire greater freedom after leaving the salt crystal lattice. This increase in entropy makes the overall free energy change negative thereby favoring dissolution. The compounds that readily dissolve in water are termed hydrophilic while those that do not dissolve in water are termed hydrophobic. Certain molecules have spatially separated hydrophilic and hydrophobic regions; such molecules are termed as amphipathic compounds.

Chemical properties of water

Pure water undergoes weak ionization to yield H^+ and OH^- ions.

$$H_2O \rightleftharpoons H^+ + OH^- \qquad \text{---------- (2.1)}$$

Free H^+ ions do not exist in water; they exist as hydrated ions called hydronium ion (H_3O^+). Equation 2.1 can therefore be written as:

$$H_2O + H_2O \rightleftharpoons H_3O^+ + OH^- \quad \text{---------- (2.2)}$$

The dissociation of water molecule can be expressed in terms of the equilibrium constant, K_{eq}.

$$K_{eq} = \frac{[H^+]\,[OH^-]}{[H_2O]} \quad \text{---------- (2.3)}$$

Dissociation of water is so weak that the concentration of undissociated water can be assumed to be constant *i.e.* 55.5 M. Equation 2.3 can therefore be written as follows:

$$(55.5\ M)(K_{eq}) = [H^+]\,[OH^-] = K_w \quad \text{---------- (2.4)}$$

where, K_w is called the ion product of water. The concentrations of H^+ and OH^- at 25 °C for pure water are 1×10^{-7} M each. Therefore, the ion product of water is 1×10^{-14} M^2. When the concentration of H^+ and OH^- are same, the water or any aqueous solution is said to be at neutral pH.

The pH scale

The concept of pH originates from the ion product of water.

$$[H^+]\,[OH^-] = 10^{-14} \quad \text{---------- (2.5)}$$

Taking logarithms:

$$\log[H^+] + \log[OH^-] = -14 \quad \text{---------- (2.6)}$$

Multiplying by –1:

$$-\log[H^+] - \log[OH^-] = 14 \quad \text{---------- (2.7)}$$

$-\log[H^+]$ and $-\log[OH^-]$ are defined as pH and pOH, respectively where "p" denotes the negative logarithm. Equation 2.7 can therefore be written as:

$$\text{pH} + \text{pOH} = 14 \quad \text{---------- (2.8)}$$

The above discussed definition of pH does not pose any limits on the values it can take. It is however convenient to define a pH scale ranging from a concentration of 1

M H^+ to 1 M OH^-. The pH scale thus obtained ranges from 0 – 14. It should be clear by now that pH is a scale for defining the acidity or basicity of aqueous solutions. A solution having a pH<7 is acidic while that having a pH>7 is basic.

pH is an important concept for living organisms. pH defines the ionization state and therefore the function of the biomolecules; most biomolecules function within a narrow pH range.

Expressing concentrations

As most biological processes are carried out in the aqueous environment, it is important to know how the concentrations of the solutions are expressed:

Concentrations based on volume: concentrations represented as the amount of substance per unit volume of solution are the most widely used in a biochemical laboratory.

Molarity (M): Molarity is the number of moles of solute per litre of solution. One mole is defined as the amount of substance that contains as many atoms, molecules, or other elementary units as the number of atoms present in 12 g of Carbon 12. One mole is the mass in grams numerically equal to the formula weight of the substance.

$$\text{Number of moles} = \frac{mass\ of\ the\ substance\ in\ grams}{molecular\ weight\ of\ the\ substance}$$

Normality (N): Normality is the number of equivalents of solute per litre of solution.

One equivalent (or equivalent weight) of an acid or base is the mass that contains one mole of replaceable hydrogen or hydroxyl.

One equivalent (or equivalent weight) of a compound involved in oxidation-reduction reaction is the mass that donates or accepts one faraday of electrons (1 mole of electrons).

$$\text{Number of equivalents} = \frac{mass\ of\ the\ substance\ in\ grams}{Equivalent\ weight}$$

Weight/volume percent (%w/v): The weight of solute in grams per 100 ml of solution.

Os*molarity*: Osmolarity is the molarity of the particles in a solution. Osmolarity of 1 M solution of a dissociable molecule is *n* Osmolar, where *n* is the number of ions produced per molecule.

Ionic strength $\left(\frac{I}{2}\right)$: Ionic strength is a measure of all the charges present in a solution:

Ionic strength $\left(\frac{I}{2}\right) = \frac{1}{2}\sum M_i Z_i^2$, where M_i is the molarity of the i^{th} ion and Z_i is the net charge of the ion (sign does not matter).

<u>*Concentrations based on weight*</u>:

Weight/weight percent (%w/w): The weight of the solute in grams per 100 grams of solution

Molality (m): the number of moles of solute per 1000 grams of solvent

Mole fraction (X_i): Mole fraction of a compound in a solution is the fraction of the total number of moles represented by the compound in question. If a solution contains n_1 moles of compound 1, n_2 moles of compound 2, n_3 moles of compound 3, and n_4 moles of compound 4; the mole fraction of compound 3 is given by:

$$X_3 = \frac{n_3}{n_1+n_2+n_3+n_4} \qquad \text{---------- (2.9)}$$

We shall be discussing about the buffers and preparation of buffers in the next lecture.

Lecture 3 Buffers

In this lecture, we shall be discussing about the ionization of weak acids and preparation of buffers using weak acids and bases. By the end of this lecture, you should be able to prepare a buffer of any strength (concentration) and any pH.

Ionization of weak acids and weak bases

In biochemistry, it is convenient to use the Brønsted concept of acid and base; a substance that donates protons is an acid while a substance that accepts the protons is a base. When an acid loses a proton, a base is produced. The acid and the corresponding base are said to constitute a conjugate acid-base pair.

$$HA + B^- \rightleftharpoons A^- + HB \qquad \text{---------- (3.1)}$$

In the above ionization reaction, *HA* and A^- make one conjugate acid-base pair while B^- and *HB* make another conjugate acid-base pair.

Strong acids like HCl, H_2SO_4, and HNO_3 and strong bases like NaOH and KOH completely dissociate in dilute aqueous solutions. The weak acids and bases are those that do not dissociate completely when dissolved in water. The dissociation of a weak acid in water can be written as a reversible reaction:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^- \qquad \text{---------- (3.2)}$$

The ionization can be represented by the equilibrium constant, K_{eq}:

$$K_{eq} = \frac{[H_3O^+][A^-]}{[H_2O][HA]} \qquad \text{---------- (3.3)}$$

Again, the concentration of the undissociated water can be assumed to be constant (55.5 *M*). Equation 3.3 can therefore be written as

$$[55.5\ M]K_{eq} = \frac{[H_3O^+][A^-]}{[HA]} \qquad \text{---------- (3.4)}$$

[55.5 M] K_{eq} is another constant; represented as K_a for the dissociation of acids. The equation 3.4 can therefore be rewritten as:

$$K_a = \frac{[H_3O^+][A^-]}{[HA]} \text{ or simply } \frac{[H^+][A^-]}{[HA]} \quad \text{---------- (3.5)}$$

Taking logarithm of equation 3.5

$$\log K_a = \log[H_3O^+] + \log[A^-] - log[HA] \quad \text{---------- (3.6)}$$

Rearranging equation 3.6:

$$-\log[H_3O^+] = -\log K_a + \log[A^-] - log[HA] \text{---------- (3.7)}$$

$$pH = pK_a + log\left[\frac{A^-}{HA}\right] \quad \text{---------- (3.8)}$$

where, p is a shorthand for negative logarithm (*–log*). Equation 3.8 is known as the Henderson-Hasselbalch equation.From Henderson-Hasselbalch equation, pK_a can be defined as the pH at which an acid is 50% ionized.

Titration of a weak acid

Titration of a weak acid (acetic acid) with a strong base (NaOH) is shown in figure 3.1. Addition of NaOH neutralizes the H^+ ions present in the solution; thereby increasing its pH. Consumption of H^+ ions drives the dissociation of the CH_3COOH into CH_3COO^- and H^+(Le Chatlier's principle). As more and more of NaOH is added, the pH of the solution increases. This increase in pH is small when the solution reaches the pH close to the pK_a of acetic acid. A weak acid, therefore, provides a resistance to change in pH near its pK_a value. This resistance to change in pH by an acid near its pK_a is the concept of a buffer.

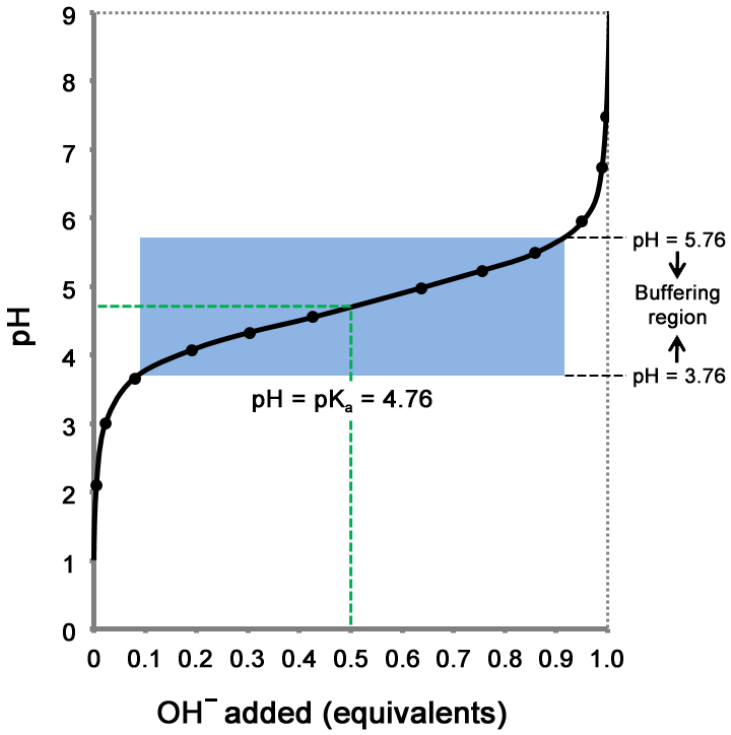

Figure 3.1: Titration curve of acetic acid. Acetic acid is titrated with NaOH and pH is measured after every addition. The pH is plotted against the amount of NaOH expressed as a fraction of the total amount of NaOH required to ionize all the acetic acid. Addition of NaOH drives the dissociation of CH_3COOH into CH_3COO^- and H^+. Addition of NaOH causes increase in pH; notice the buffering region (blue) wherein addition of NaOH causes little increase in pH.

Common ion effect

Common ion effect is the change in the ionization equilibrium due to the addition of a solute that provides an ion taking part in the equilibrium. Let us take an example with acetic acid.

Example 3.1: You are given a 0.1 *M* solution of acetic acid.

(a) Calculate the concentrations of all the chemical species present at equilibrium (Given K_a of acetic acid is $1.7 \times 10^{-5} M$).

(b) Calculate the concentrations of all the species when 0.05 *M* of sodium acetate is added to the solutions.

Solution (a): $CH_3COOH + H_2O \rightleftharpoons CH_3COO^- + H_3O^+$

Concentration			
Starting:	*0.1 M*	*0*	*0*
Change:	*–x M*	*+x*	*+x*
Equilibrium:	*(0.1–x) M*	*x*	*x*

$$K_a = \frac{[CH_3COO^-][H_3O^+]}{[CH_3COOH]} \quad \text{---------- (3.9)}$$

$$1.7 \times 10^{-5} = \frac{[x][x]}{[0.1-x]} \quad \text{---------- (3.10)}$$

If the acid is <10% ionized *i.e.* if $K_a < 10^{-3}$, *0.1–x* can be approximate to 0.1. Equation 3.10, therefore, reduces to:

$$x^2 = 1.7 \times 10^{-6} \quad \text{---------- (3.11)}$$

$$x = 1.3 \times 10^{-3} M \quad \text{---------- (3.12)}$$

Therefore, the equilibrium concentrations of CH_3COOH, CH_3COO^-, and H_3O^+ are 98.7 *mM*, 1.3 *mM*, and 1.3 *mM*, respectively.

Solution (b): Sodium acetate dissociates completely in aqueous solutions; 0.05 *M* sodium acetate therefore provides 0.05 *M* of acetate ions.

$$CH_3COOH + H_2O \quad \rightleftharpoons \quad CH_3COO^- + H_3O^+$$

Concentration			
Starting:	*0.1 M*	*0.05*	*0*
Change:	*–x M*	*+x*	*+x*
Equilibrium:	*(0.1–x) M*	*0.05+x*	*x*

$$K_a = \frac{[CH_3COO^-][H_3O^+]}{[CH_3COOH]} \quad \text{---------- (3.13)}$$

$$1.7 \times 10^{-5} = \frac{[0.05+x][x]}{[0.1-x]} \quad \text{---------- (3.14)}$$

Again, *0.1 –x* can be approximated to *0.1* and *0.05+x* can be approximated to *0.05*. Equation 3.12, therefore, reduces to:

$$0.05x = 1.7 \times 10^{-6} \quad \text{---------- (3.15)}$$

$$x = 3.4 \times 10^{-5}\ M \quad \text{---------- (3.16)}$$

Therefore, the equilibrium concentrations of CH_3COOH, CH_3COO^-, and H_3O^+in the presence of 0.5 *M*sodium acetateare ~99.966*mM*, 0.084*mM*, and 0.034*mM*, respectively.

In case, *x* is not small compared to the concentration of the acid and the common ion provided by the solute, the quadratic equation needs to be solved. Let us take equation 3.14:

$$x^2 + \{0.05 + (1.7 \times 10^{-5})\}x = 1.7 \times 10^{-6} \text{ ---------- (3.17)}$$

Here we see that $0.05 + (1.7 \times 10^{-5}) \approx 0.05$; equation 3.17 therefore reduces to:

$$x^2 + 0.05x = 1.7 \times 10^{-6} \text{ ---------- (3.18)}$$

$$x = \frac{-0.05 \pm \sqrt{(0.05)^2 - (6.8 \times 10^{-6})}}{2}$$

$$x = \frac{-0.05 \pm \sqrt{(0.0025) - (6.8 \times 10^{-6})}}{2}$$

$$x = \frac{-0.05 \pm \sqrt{0.0024932}}{2}$$

$$x = \frac{-0.05 \pm 0.049932}{2}$$

$$x = -0.05 \; and \; 0.000034$$

As concentration cannot be negative, $x = 3.4 \times 10^{-5}$. The result is same as obtained in equation 3.16. This shows that the approximations we made earlier were correct.

Preparation of buffers

Let us now see how we make buffers. Suppose you are asked to prepare a buffer of pH 7.4, the pH of human blood. The first thing you need to do is to select appropriate conjugate acid-base pair. The selection is based on the pK_a of the acid; an acid can be used to prepare a buffer within a pH range of $pK_a \pm 1$. This means that you cannot prepare an acetate buffer of pH 7.4 because acetic acid-acetate conjugate base pair is a good buffer only for the pH range of 3.76 – 5.76 (see figure 3.1). Let us see the dissociation of phosphoric acid:

$K_{a_1} = 7.6 \times 10^{-3}$ $K_{a_2} = 6.16 \times 10^{-8}$ $K_{a_3} = 4.79 \times 10^{-13}$

$H_3PO_4 \rightleftharpoons H_2PO_4^- \rightleftharpoons HPO_4^{2-} \quad \rightleftharpoons \quad PO_4^{3-}$ ---------- (3.19)

For phosphoric acid, $pK_{a_1} = 2.12$, $pK_{a_2} = 7.21$, and $pK_{a_3} = 12.32$

We can therefore see that the pK_{a_2} of phosphoric acid is suitable for preparing the buffer of pH 7.4. Let us see how to prepare a phosphate buffer of pH 7.4.

Example 3.2: Preparation of 500 *ml* of 200 *mM* phosphate buffer of pH 7.4

Methodology: As is evident from equation 3.19, phosphate buffer of the required pH can be prepared starting with any of the four chemical species. Suppose we have following four chemicals in the laboratory:

H_3PO_4	(Molecular weight: 98Da)
NaH_2PO_4	(Molecular weight: 119.98 Da)
$Na_2HPO_4{\cdot}7H_2O$	(Molecular weight: 268.07 Da)
Na_3PO4	(Molecular weight: 163.94 Da)

We can prepare phosphate buffer of pH 7.4 in the following ways:

(a) Take the required amount of H_3PO_4 or NaH_2PO_4 and dissolve it in 400 *ml* water. The pH of the solution will be less than 7.4. Titrate the solution with NaOH until a pH of 7.4 is reached; adjust the final volume to 500 *ml* with water.

(b) We can take NaH_2PO_4 and $Na_2HPO_4{\cdot}7H_2O$ in the amounts that would give a pH of 7.4 when dissolved in water so as to obtain a solution of 500 *ml* volume. The pH obtained this way, however, is seldom correct.

Okay then. Let's go and prepare it (let us go stepwise):

Calibration of pH meter: pH meters need to be calibrated every time a buffer is to be prepared. A pH meter is calibrated with standard buffers of pH 4.0, 7.0, and 9.2. The pH meter is calibrated with pH 7.0 buffer; the second standard buffer to be used is that of pH 4.0 if the buffer that needs to be prepared is acidic and that of pH 9.2 if the buffer to be prepared is basic.

1. Switch "ON" the pH meter and allow it to stabilize for 20 *min*.

2. Take out your standard buffers and allow them to come to room temperature (standard buffers are usually stored at 4 °C).
3. The electrode of the pH meter is kept in 3 *M*HCl.
4. Take out the electrode and wash it thoroughly with distilled water.
5. Measure the pH of "pH 7.0 standard buffer", set this as pH 7.0 in the pH meter.
6. Take the electrode out of the standard buffer and wash it thoroughly with water.
7. Measure the pH of "pH 9.2 standard buffer", set this as pH 9.2 in the pH meter.

Method 1: Preparing buffer with H_3PO_4

1. Prepare 100 *ml* of 1 *N*NaOH solution
2. Take 400 *ml*distilled water in a 1 *litre* conical flask.
3. Add 11.53*ml*of H_3PO_4dropwise in the 400 *ml* distilled water
    a. The amount of H_3PO_4 required

$$= \frac{Mol.wt. \times Molarity \times Volume\ in\ ml}{1000}\, grams$$

$$= \frac{\left(98\frac{g}{mol}\right) \times (0.2M) \times (500ml)}{1000} =$$

$9.8\ grams$

    b. Concentrated H_3PO_4 is 85 % (wt/v) with a density of 1.685*g/ml*
    c. Therefore, the volume of concentrated H_3PO_4 required:

$$= \frac{amount\ required\ in\ grams}{Concentration\ (\%\ wt/v)} \times 100\ ml$$

$$= \frac{9.8}{85} \times 100\ grams = 11.53\ ml$$

4. Wash the pH electrode thoroughly with distilled water and place it in the H_3PO_4 solution.
5. Add 1 *N*NaOHdropwise; shake well and measure the pH.
6. When the pH of the solution nears 7.4, be careful in adding NaOH so that pH does not overshoot.
7. Transfer the contents of the flask in a 500 *ml* volumetric flask.

8. Add water to make the final volume of the solution to 500 *ml*.
9. The required buffer is ready.

Note: The same method can be used for preparing the buffer starting with NaH_2PO_4. The amount of NaH_2PO_4 required:

$$= \frac{Mol.wt\ of\ NaH_2PO_4 \times Molarity\ of\ buffer \times Volume\ in\ ml}{1000}$$

$$= \frac{119.98 \frac{grams}{mol} \times 0.2 \frac{moles}{litre} \times 500ml}{1000} \approx 12\ grams$$

12 g of NaH_2PO_4 is dissolved in 400 *ml* water in a 1 *liter* conical flask and then steps 5-9 are followed.

Method 2: Preparing buffer with NaH_2PO_4 and Na_2HPO_4

NaH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$ completely dissociate in water to provide conjugate acid ($H_2PO_4^-$) and conjugate base (HPO_4^{2-}). As is evident from the Henderson-Hasselbalch equation (equation 3.8), the pH of the solution is determined by the ratio of conjugate base to the conjugate acid. Let us calculate this ratio:

$$pH = pK_a + log\left[\frac{A^-}{AH}\right] \quad \text{---------- (3.8)}$$

For the given pair of conjugate acid and base, pK_{a_2} of phosphoric acid is to be used:

$$7.4 = 7.21 + log\left[\frac{HPO_4^{2-}}{H_2PO_4^-}\right] \quad \text{---------- (3.20)}$$

$$log\left[\frac{HPO_4^{2-}}{H_2PO_4^-}\right] = 0.19 \quad \text{---------- (3.21)}$$

$$\left[\frac{HPO_4^{2-}}{H_2PO_4^-}\right] = 10^{0.19} = \frac{1.5488}{1} \quad \text{---------- (3.22)}$$

Therefore, $Na_2HPO_4 \cdot 7H_2O$:NaH_2PO_4 ratio for preparing the required buffer is 1.5488:1. For preparing one liter of buffer, the required number of moles of phosphate species = 0.2 *i.e.* 200 *mmoles*.

The required number of moles for a *V ml* of buffer is given by $= \frac{Molarity\ of\ buffer}{1000} \times V\ (ml)$

Therefore, the number of moles required for the 500 *ml* of 200 *mM* buffer $= \frac{0.2}{1000} \times 500 =$ **0.1**

Therefore, moles of conjugate acid (NaH_2PO_4) required

$= X_{acid} \times total\ number\ of\ moles$

$= \frac{1}{1.5488+1} \times 0.1 = \mathbf{0.039}$

Moles of conjugate base ($Na_2HPO_4 \cdot 7H_2O$) required = $X_{base} \times total\ number\ of\ moles$

$$= \frac{1.5488}{1.5488 + 1} \times 0.1 = \mathbf{0.061}$$

Therefore, the amount of NaH_2PO_4 required:

$= number\ of\ moles\ of\ \ NaH_2PO_4\ \times molecular\ weight$

$= 0.039\ moles \times 119.98 \frac{grams}{mole} = 4.68\ grams$

The amount of $Na_2HPO_4 \cdot 7H_2O$ required:

$= number\ of\ moles\ of\ Na_2HPO_4 \cdot 7H_2O \times molecular\ weight$

$= 0.061\ moles \times 268.07 \frac{grams}{mole} = 16.35\ grams$

Let us now see how to prepare the buffer:

1. Weigh accurately 4.68 *g* of NaH_2PO_4 and 16.35 *g* of $Na_2HPO_4 \cdot 7H_2O$.
2. Transfer the salts into a 1 *liter* conical flask and add 400 *ml* distilled water.
3. Shake the flask well to achieve complete dissolution of the salts
4. Wash the electrode of the pH meter thoroughly with distilled water.
5. Place the pH electrode into the phosphate solution and measure its pH.
    a. Although the amounts of the conjugate acid and conjugate base are weighed so as to achieve a pH of 7.4, the pH of the solution is seldom 7.4.
6. If the pH of the solution is <7.4, add 1 *N*NaOHdropwise, shake the flask well and measure the pH. Keep doing this until a pH of 7.4 is achieved.

7. If the pH of the solution is >7.4, add 1 *N* H_3PO_4dropwise, shake the flask well and measure the pH. Keep doing this until a pH of 7.4 is achieved.
    a. Addition of H_3PO_4 increases the concentration of a phosphate species in the solution; you will therefore end up with a buffer that will have a pH of 7.4 but the concentration of the buffer will be slightly more than 200 *mM*.

Which method of buffer preparation one should choose?

Which method do you think is better for preparing a buffer.Method 1 might appear better than method 2 because we are required to titrate with NaOH; this implies that we are not going to add any phosphate species that could change the strength of buffer. There is, however, a possibility of overshooting the required pH while adding NaOH. What do we do if we actually overshoot the required pH? We are left with two options:

i. Discard all the contents and prepare fresh buffer.
ii. Add H_3PO_4 to bring the pH down to 7.4; this would, however, increase the strength of the buffer.

Increase in buffer strength, however, is not that big a problem. The prepared can be diluted with water so as to obtain the buffer of desired strength. But, is there a way out that we don't change the buffer strength and still achieve the correct pH no matter how casually we perform the titrations? The answer is "yes". This is how we go about it:

We know that $Na_2HPO_4{\cdot}7H_2O$ and NaH_2PO_4 are required in 1.5488: 1 molar ratio. We can prepare the 200 *mM* solutions of both $Na_2HPO_4{\cdot}7H_2O$ and NaH_2PO_4 in a 1.5488:1 volume ratio. As we need to prepare 500 *ml* buffer, we would perform the calculation for a slightly higher volume, say 550 *ml*.

1. Prepare $\frac{1}{(1.5488+1)} \times 550\ ml = 215.8\ ml$ of NaH_2PO_4 solution in water. Let us prepare 220 ml of the NaH_2PO_4 solution (volume rounded off to the above 'multiple of 10' integer):

   a. Weigh $\frac{Mol.wt \times Molarity \times Volume\ in\ ml}{1000} = \frac{119.98 \times 0.2 \times 220}{1000} grams = 5.28\ grams$ of NaH_2PO_4, dissolve it in 200 *ml* distilled water and add distilled water to make a final volume of 220 *ml*.

2. Prepare $\frac{1.5488}{(1.5488+1)} \times 550\ ml = 334.2\ ml$ of $Na_2HPO_4 \cdot 7H_2O$ solution in water. Let us prepare 340 ml of the $Na_2HPO_4 \cdot 7H_2O$ solution (volume rounded off to the above 'multiple of 10' integer):
   a. Weigh $\frac{Mol.wt \times Molarity \times Volume\ in\ ml}{1000} = \frac{268.07 \times 0.2 \times 340}{1000} grams = 18.23\ grams$ of $Na_2HPO_4 \cdot 7H_2O$, dissolve it in 300 *ml* distilled water and add distilled water to make a final volume of 340 *ml*.
3. Mix $\frac{1}{(1.5488+1)} \times 500\ ml = 196.17\ ml$ of NaH_2PO_4 with $\frac{1.5488}{(1.5488+1)} \times 500\ ml = 303.83\ ml$ of $Na_2HPO_4 \cdot 7H_2O$. This gives you the 500 *ml* of 200 *mM* phosphate buffer.
4. Measure the pH of the solution.
5. If the pH of the solution is less than pH 7.4, titrate with 200 *mM* $Na_2HPO_4 \cdot 7H_2O$ until the pH reaches 7.4; if the pH is greater than 7.4, titrate it with 200 *mM*NaH_2PO_4 until the pH reaches 7.4.
6. The 200 *mM* phosphate buffer with pH 7.4 is ready.

Although we have discussed the preparation of phosphate buffer, the concept and the protocol for making the buffer are general.