FROM MOLECULE TO DISCIPLINE: CONTEMPORARY AND INNOVATIVE APPROACHES IN CHEMICAL SCIENCE

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CONTENTS

CHAPTER 1

SPECTROSCOPIC APPROACHES FOR THE STRUCTURAL ELUCIDATION OF COMPLEX MOLECULES: NMR AND MASS SPECTROMETRY

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Abstract

This chapter provides a comprehensive overview of the fundamental principles, applications, and complementary roles of Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) in molecular structure elucidation. The physical basis of NMR spectroscopy is discussed through nuclear spin interactions with an external magnetic field, focusing on how chemical shift, spin–spin coupling, and relaxation times (T_1, T_2) reflect the electronic environment of molecules. The contributions of one-dimensional (1H, 13C) and two-dimensional (COSY, HSQC, HMBC, NOESY) NMR methods to structural analysis are emphasized, along with the ability of multinuclear NMR (31P, 19F, 15N) to directly reveal ligand–metal binding patterns in coordination complexes. The Magic-Angle Spinning (MAS) technique is highlighted for its role in studying crystalline heterogeneities in solid-state samples, demonstrating the broad applicability of NMR from solution to solid phases. In mass spectrometry, the choice of ionization method (ESI, MALDI, APCI) is shown to be critical for accurate structural interpretation depending on molecular properties. MS/MS fragmentation analysis enables detailed investigation of substructural units and is particularly valuable in the characterization of large biomolecules. The hybrid NMR-MS analytical approach represents one of the most powerful strategies for unambiguous molecular structure determination, where MS defines the molecular formula and NMR confirms the bonding and conformation. This synergy has proven especially effective in studying biomolecular complexes and protein–metal interactions under near-native conditions using native MS combined with high-resolution NMR spectroscopy. Furthermore, Ion Mobility Spectrometry (IMS) allows ions to be separated according to their gas-phase conformations, providing enhanced discrimination between isomeric compounds. Collectively, these analytical methods provide complementary information on stoichiometry, coordination sites, and electronic environments in coordination compounds such as Ni (II) Schiff base complexes.

1. INTRODUCTION

The accurate and detailed determination of molecular structures forms the foundation of numerous disciplines, including chemistry, biochemistry, materials science, and drug design. In this context, Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) have emerged as two complementary pillars of modern analytical chemistry. Both techniques provide high-resolution and direct information regarding a molecule's chemical composition, bonding characteristics, and spatial arrangement (Keeler, 2010; Ma et al., 2022).

NMR spectroscopy is based on transitions between energy levels of atomic nuclei possessing nuclear spin moments under an external magnetic field. This technique offers insights into the electronic environment, bond lengths, and conformational features of molecules. While 1D (1H, 13C) NMR spectra provide fundamental structural information, 2D techniques (COSY, HSQC, HMBC, NOESY) have been developed to resolve signal overlap (Claridge, 2016). In addition, multinuclear NMR applications (31P, 19F, 15N, etc.) enable the direct identification of ligand–metal binding modes in coordination complexes (Atkins & Jones, 2021).

For solid-state samples, the Magic-Angle Spinning (MAS) technique plays a critical role in characterizing structural heterogeneities within crystalline materials, allowing the differentiation of amorphous and crystalline systems (Marinova, 2024). Thus, NMR spectroscopy has become a powerful tool not only for studying molecules in solution but also for investigating solid-phase materials.

Mass spectrometry (MS), on the other hand, is based on the ionization of molecules and their separation according to mass-to-charge ratios. Various ionization methods-electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and atmospheric pressure chemical ionization (APCI)-are selected according to the chemical characteristics of the analyte, enabling highly accurate structural information (Ma et al., 2022). Particularly, MS/MS (tandem mass spectrometry) techniques are exceptionally effective in elucidating subunit structures of compounds by analyzing ion fragmentation patterns (Leitner et al., 2019).

In recent years, hybrid analytical approaches combining NMR and MS data have made it possible to achieve highly precise molecular structure determination. While MS defines the molecular formula, NMR confirms the bonding sequence and conformation. This combination has introduced a new analytical dimension, especially in studying biomolecular complexes and protein–metal interactions under native conditions (native MS, NMR) (Hale et al., 2024).

Furthermore, ion mobility spectrometry (IMS) offers significant advantages in separating isomeric compounds by distinguishing ions according to their gas-phase conformations (Gabelica & Marklund, 2018). When used together, these techniques provide complementary information on stoichiometry, binding sites, and electronic environments of coordination compounds such as Ni (II) Schiff base complexes (Atkins & Jones, 2021).

In conclusion, this section provides an integrated overview of the theoretical principles, modern applications, and hybrid analytical approaches of NMR and MS techniques, offering a comprehensive perspective on molecular structure determination.

2. FUNDAMENTALS of NMR SPECTROSCOPY

2.1 Physical Principle of NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is based on the quantum-mechanical behavior exhibited by certain atomic nuclei when placed in a magnetic field. This technique operates on the principle that nuclei possessing a nuclear spin moment ($I \neq 0$) undergo transitions between different energy levels under the influence of a strong external magnetic field (B_0) (Keeler, 2010). Nuclei such as proton (^{1}H), carbon-13 (^{13}C), phosphorus-31 (^{31}P), and nitrogen-15 (^{15}N), which all possess spin moments, are therefore commonly used in NMR spectroscopy.

When an external magnetic field is applied, the magnetic moments of nuclei align either parallel or antiparallel to the direction of $\boldsymbol{B_0}$. A small energy difference ($\boldsymbol{\Delta E}$) arises between these two orientations, and this difference depends on the strength of the applied magnetic field and the gyromagnetic ratio ($\boldsymbol{\gamma}$) of the nucleus (Claridge, 2016). The energy gap is defined by the equation:

$$\Delta E = h\nu = \hbar\gamma B_0$$

where $\boldsymbol{\nu}$ is the resonance frequency, $\boldsymbol{\gamma}$ is the gyromagnetic ratio specific to the nucleus, $\boldsymbol{h}$ is Planck's constant, and $\boldsymbol{B_0}$ is the magnetic field strength (Keeler, 2010). Nuclei can be excited between these energy levels when exposed to radiofrequency (**RF**) energy, a process known as the resonance condition.

The amount and frequency of energy absorbed during resonance vary depending on the electronic environment surrounding the nucleus. This phenomenon is known as the chemical shift (**δ**), which is measured relative to a reference compound such as tetramethylsilane (TMS). Chemical shift values are influenced by factors such as the substituent groups attached to the atom, bond angles, electronegativity differences, and aromatic ring currents (Claridge, 2016). Consequently, the chemical shifts observed in an NMR spectrum provide direct insight into the structural characteristics of a molecule.

Additionally, magnetic interactions between nuclei of the same type give rise to spin–spin coupling (J-coupling). J-coupling occurs when the spin states of bonded nuclei influence each other, leading to the splitting of NMR signals into

multiplets (doublet, triplet, quartet, etc.) (Hore, 2015). The resulting splitting patterns provide valuable information about bonding sequences through the number of bonds between nuclei and the magnitude of the coupling constant (**J**).

Another important parameter in NMR measurements is the relaxation time. After excitation by an RF pulse, nuclei return to their equilibrium states through two distinct processes: spin–lattice (T_1) and spin - spin (T_2) relaxation (Keeler, 2010). T_1 represents the process by which nuclei lose energy and return to thermal equilibrium, while T_2 describes the loss of phase coherence. These relaxation parameters offer insights into molecular mobility, solvent viscosity, and paramagnetic effects (Claridge, 2016).

2.2 1D and 2D NMR Techniques

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most powerful analytical techniques used to determine the structural and dynamic properties of molecules. One-dimensional (1D) NMR methods - particularly those based on ^{1}H and ^{13}C nuclei - serve as fundamental approaches that provide the first insights into chemical structure. 1D, ^{1}H NMR spectra generate signals at different chemical shift (**δ**) values depending on the chemical environment of protons, while ^{13}C NMR provides information on the substituents bonded to carbon atoms and their hybridization states (Claridge, 2016). However, in complex molecules, overlapping signals and extensive spin - spin interactions can make spectrum interpretation challenging (Keeler, 2010).

In such complex systems, two-dimensional (2D) NMR techniques allow the acquisition of much more detailed structural information. 2D NMR collects data along two distinct frequency axes, visualizing correlations between signals. This enables clearer analysis of proton -proton or proton- carbon interactions across chemical shift dimensions (Claridge, 2016).

COSY (Correlation Spectroscopy) reveals proton–proton spin - spin couplings (J-couplings) and identifies pairs of directly bonded protons within the same molecule. In COSY spectra, diagonal peaks correspond to individual protons, whereas off-diagonal cross-peaks represent interacting proton pairs (Keeler, 2010). This technique is widely used for the characterization of small organic molecules and amino acid residues.

HSQC (Heteronuclear Single Quantum Coherence) spectroscopy detects direct couplings between ^{1}H and ^{13}C (or ^{15}N) nuclei. It provides information on the carbon atom to which each proton is attached, making it highly effective for determining proton–carbon correlations in complex mixtures (Claridge, 2016).

HMBC (Heteronuclear Multiple Bond Correlation), a complementary method to HSQC, identifies proton–carbon correlations across multiple bonds (typically two or three bonds) (Hore, 2015). This enables the identification of atom pairs that are not directly bonded and allows stepwise reconstruction of the carbon skeleton of the molecule.

Another important 2D technique, NOESY (Nuclear Overhauser Effect Spectroscopy), reveals spatial proximities between protons (typically < 5 Å). NOESY experiments are critical for determining spatial relationships and conformations within molecules (Claridge, 2016). Particularly in the structural elucidation of biological macromolecules, NOESY data combined with structural modeling yields high-resolution conformational information (Hore, 2015).

The combination of these techniques provides a comprehensive approach for the structural analysis of natural products, polymers, active pharmaceutical ingredients, and biomolecules. For example, even if severe signal overlap is observed in the 1D, ^{1}H spectrum of a compound, the structure can be fully elucidated by identifying proton–proton connectivity's with COSY, proton–carbon relationships with HSQC, and spatial proximities with NOESY (Keeler, 2010).

2.3 Multinuclear NMR

In the context of coordination chemistry, NMR spectroscopic analysis of different nuclei serves as a powerful tool for understanding ligand–metal interactions. Heteronuclear nuclei such as ^{31}P, ^{19}F, and ^{15}N can provide direct information regarding the nature of ligand-metal bonding, binding orientations, and geometric changes occurring upon coordination. For example, the difference between the chemical shift values of a ligand before and after coordination (**δ** _ complex – **δ** _ ligand) offers valuable insight into the binding mechanism (Pazderski, 2008).

The ^{15}N NMR nucleus exhibits pronounced changes in chemical shift when nitrogen-containing ligands coordinate to a metal center; this shift is commonly referred to as the "coordination shift" and serves as a strong indicator of binding (Pazderski, 2008). Likewise, ^{31}P NMR is frequently used to monitor interactions between metals and phosphine or phosphane-type ligands, as ^{31}P has 100% natural abundance and a wide chemical shift range, making it highly sensitive to environmental changes following coordination (Harris & Mann, 1978/2007). ^{19}F NMR, on the other hand-when fluorine is present in the ligand structure-is particularly sensitive and is widely employed to detect electronic environment changes induced by metal-ligand bonding (Xue & Cook, 2022).

This multinuclear analytical approach provides a more holistic evaluation compared to NMR studies focused on a single nucleus. For instance, simultaneous examination of both ^{31}P and ^{15}N spectra in a metal-ligand system reveals not only whether coordination has occurred but also structural reorganizations following binding (e.g., changes in bond polarization, **π**-back bonding, or redistribution of electron density from the metal to the ligand) (Vicha, 2013). Therefore, multinuclear NMR can be regarded as a "fingerprint" technique for the structural characterization of metal-organic complexes.

The chemical shift changes observed upon coordination can serve as indicators of bond strength and the surrounding electronic structure. For example, a significant downfield shift in the ^{15}N chemical shift after coordination may indicate strong **d-π** or **π-d** interactions between the ligand and the metal center (Pazderski, 2008). Additionally, the presence-or absence - of a P-M (^{31}P- metal) coupling signal in the ^{31}P spectrum can offer clues regarding the geometry and symmetry of the metal-ligand bond. In this context, simultaneous analysis of multiple nuclei such as ^{1}H, ^{13}C, ^{19}F, and ^{31}P is a common strategy in solution-phase studies of transition-metal complexes (Xue & Cook, 2022).

2.4 Solid-State NMR

Solid-state nuclear magnetic resonance (NMR) spectroscopy is applied in environments where molecules are not free to tumble, in contrast to solution-state NMR. Consequently, it is inherently limited by line broadening and reduced resolution, primarily due to the persistence of chemical shift anisotropy (CSA) and dipolar interactions. One of the most important techniques developed to mitigate these interactions and enhance resolution in solid samples is Magic-Angle Spinning (MAS). MAS is based on spinning the sample at high speed with the rotor axis set at an angle of 54.74° relative to the magnetic field (Schurko, 2010). This special angle corresponds to the zero point of the second-order Legendre polynomial, allowing the time-averaging and effective suppression of dipolar interactions and chemical shift anisotropy (Deng & Hou, 2019).

As a result of MAS, orientation-dependent magnetic interactions in solid samples become isotropic, leading to significant line narrowing and the acquisition of spectra with resolution approaching that of solution-state NMR (Marinova, 2024). This capability is particularly advantageous for identifying molecular heterogeneity, crystal-phase differences, and local structural disorder in crystalline or partially amorphous systems. Therefore, solid-state NMR can differentiate between various phases in polycrystalline or composite materials and enables atomic-level analysis of the material (Recent Progress, 2024).

The MAS technique is commonly combined with cross-polarization / magic-angle spinning (CP/MAS). This combined method enhances the sensitivity of low-γ nuclei such as ^{13}C or ^{15}N by enabling magnetization transfer from more abundant nuclei such as ^{1}H (Deng & Hou, 2019). In this way, both sensitivity is increased and the detectability of nuclei that strongly interact with protons in the solid state is improved. CP/MAS is widely used in the investigation of heterogeneous systems such as polymers, biomaterials, and porous solids (Recent Progress, 2024).

The spinning speed under MAS directly affects spectral quality. As the spinning speed increases, broadening interactions are averaged more effectively. Ultrafast MAS (≥ 60 kHz) can suppress proton–proton dipolar interactions, enabling high-resolution proton detection (Ultrafast MAS, 2021). These technological advances now allow proton-detected solid-state NMR experiments to be performed at high resolution for organic and biological solid samples (Schurko, 2010).

However, there are certain limitations to using MAS-based signals directly for quantitative analysis. In CP/MAS experiments, signal intensity is influenced by factors such as dipolar coupling strength, relaxation times, and the efficiency of magnetization transfer (Deng & Hou, 2019). Therefore, when quantitatively evaluating crystal-structure heterogeneity, care must be taken, and appropriate reference materials and calibration methods should be employed.

3. FUNDAMENTALS of MASS SPECTROMETRY

3.1 Ionization Methods

In mass spectrometry (MS), the ionization of molecules is the most critical step of the analytical process, as ionization efficiency directly influences spectrum quality and the accuracy of structural interpretation (Gross, 2017). The selection of an appropriate ionization method depends on factors such as the volatility, polarity, molecular weight, and thermal stability of the sample. Among the most widely used ionization techniques in modern MS systems are Electrospray Ionization (ESI), Matrix-Assisted Laser Desorption/Ionization (MALDI), and Atmospheric Pressure Chemical Ionization (APCI) (Ma et al., 2022).

Electrospray Ionization (ESI) is particularly preferred for polar and thermally labile compounds such as biomolecules, peptides, and proteins. In this technique, the sample in solution is ionized via fine spraying under a high voltage. The resulting charged droplets gradually evaporate, yielding gas-phase ions that retain their charge (Cole, 2010). One of the most significant advantages of ESI is that it

generates multiply charged ions, which enables high-molecular-weight species-such as proteins-to be analysed within a lower m/z range (Gross, 2017).

The MALDI (Matrix-Assisted Laser Desorption/Ionization) technique is commonly employed for the analysis of large biomolecules and polymers. The sample is mixed with a UV-absorbing matrix compound and irradiated with a laser beam; the matrix absorbs the energy and ionizes the analyte while transferring it into the gas phase (Dass, 2007). Because MALDI preserves the integrity of the analyte during ionization, it is classified as a "soft ionization" technique. This method has gained extensive application in proteomics and biotechnology research (Ma et al., 2022).

APCI (Atmospheric Pressure Chemical Ionization), like ESI, is compatible with liquid chromatography (LC-MS) systems and is effective for analysing organic compounds of moderate polarity. In APCI, the sample is vaporized and ionized within a corona discharge plasma. This method generally outperforms ESI in ionizing analytes with lower polarity (Gross, 2017).

3.2 MS/MS Fragmentation Analysis

Tandem mass spectrometry (MS/MS) is a powerful analytical technique that enables the elucidation of molecular structural features by examining the fragmentation behaviour of ions generated after the ionization of a compound (Leitner et al., 2019). This method is indispensable for the identification of high-molecular-weight compounds-such as peptides, proteins, metabolites, and pharmaceutical derivatives-particularly within complex biological matrices (de Hoffmann & Stroobant, 2014).

The fundamental principle of MS/MS analysis is the selection of precursor ions, followed by the introduction of energy before the second mass analyser, allowing these ions to dissociate into product ions. This process is typically carried out using techniques such as collision-induced dissociation (CID) or electron transfer dissociation (ETD) (Gross, 2017). The resulting fragmentation patterns provide direct information regarding atomic connectivity, bond types, and functional groups within the molecule.

For example, in peptide and protein analyses, MS/MS is used to determine amino acid sequences. Under CID conditions, peptides characteristically produce b and y ion series; by examining the m/z values of these ions, the peptide sequence can be reconstructed step by step (Leitner et al., 2019). ETD, on the other hand, offers advantages for the analysis of post-translational modifications (such as phosphorylation or acetylation), as it preserves the peptide backbone

while providing clearer information on side-chain modifications (Coon & Syka, 2015).

Tandem MS is not only used for peptide sequencing but is also widely applied in metabolomics and lipidomics. Fragmentation patterns of small molecules serve as powerful tools for differentiating structural isomers and identifying unknown compounds. Additionally, when combined with high-resolution mass spectrometers (HRMS), MS/MS allows for accurate determination of elemental composition through isotopic pattern analysis (Gross, 2017).

With technological advancements, the time required for data acquisition has decreased to the millisecond scale, making LC-MS/MS systems routine tools in fields such as clinical biochemistry, pharmaceutical analysis, and environmental chemistry (de Hoffmann & Stroobant, 2014). In proteomics, MS/MS fragmentation analysis-integrated with data-dependent acquisition (DDA) and data-independent acquisition (DIA) strategies-enables the simultaneous qualitative and quantitative analysis of thousands of proteins (Leitner et al., 2019).

3.3 Ion Mobility Spectrometry

Ion mobility spectrometry (IMS) is an advanced analytical technique that enables the separation of ions in the gas phase based on their drift velocities and, consequently, their three-dimensional conformations. IMS significantly enhances the structural selectivity of mass spectrometry by providing high-resolution separation, particularly for isomeric and conformeric compounds (Hale et al., 2024). In this technique, the drift time of ions through a carrier gas (typically nitrogen or helium) under an applied electric field is measured. This drift time depends on the ion's size, shape, charge, and collision cross section (CCS) with gas molecules (Giles & Williams, 2018).

In IMS systems, ions typically pass through an ion gate and traverse a uniform electric field while undergoing continuous collisions with gas molecules. Small, compact ions travel more rapidly through the drift region, whereas ions with larger surface areas or extended conformations exhibit slower drift. This principle allows the differentiation of compounds that share the same mass-to-charge (m/z) ratio but exhibit distinct three-dimensional structures-such as structural isomers or proteins in different folding states (Kanu & Hill, 2008).

The combination of ion mobility with mass spectrometry (IMS-MS) plays a critical role in modern structural biology and analytical chemistry. In such hybrid systems, IMS provides conformational information, while MS supplies mass and compositional data. Consequently, both structural (conformational) and chemical

(molecular) characteristics of analytes can be obtained simultaneously within a single instrument (Gabelica & Marklund, 2018). IMS-MS is particularly valuable in the analysis of complex biological samples in fields such as proteomics, metabolomics, lipidomics, and drug interaction studies, where it provides high specificity in the separation of intricate mixtures (Hale et al., 2024).

Recent advances in high-resolution IMS technologies-such as Travelling Wave Ion Mobility Spectrometry (TWIMS) and Trapped Ion Mobility Spectrometry (TIMS)-have enabled the differentiation of conformational variants at the nanometer scale (Giles & Williams, 2018). In these systems, experimentally measured CCS values can be correlated with spatial molecular models, facilitating the integration of IMS data with structural bioinformatics tools (Kanu & Hill, 2008).

4. HYBRID NMR–MS ANALYTICAL APPROACHES

In modern analytical chemistry, the combined use of Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) is considered one of the most powerful and complementary strategies for molecular structure elucidation (Leitner et al., 2019). While NMR provides detailed information on bonding sequences, molecular conformation, and functional groups, MS offers highly sensitive data on molecular mass and elemental composition (Emwas, 2015). The integration of these two techniques significantly enhances the reliability of structural verification in natural product chemistry as well as in metabolomic and pharmaceutical analyses (Li et al., 2020).

In hybrid NMR–MS analytical workflows, MS analysis is typically conducted first. MS reveals the molecular formula, isotopic distribution, and fragmentation pattern of a compound, providing essential initial insights into its atomic composition and potential structural candidates (Gross, 2017). Subsequently, NMR spectroscopy is employed to deliver definitive structural information regarding atomic connectivity, conformation, and stereochemistry (Emwas, 2015). Together, the two datasets form a complementary whole: MS indicates “how heavy” the molecule is, while NMR reveals “how it is connected.”

The value of this hybrid approach is particularly evident in fields such as metabolomics and natural product chemistry. For example, in the identification of metabolites present in complex biological mixtures, mass data obtained from LC-MS analyses can be matched with NMR databases, enabling rapid identification of unknown compounds (Kuhlisch & Pohnert, 2015). Moreover, the combination of high-resolution MS (HRMS) with multidimensional NMR

(e.g., HSQC, HMBC) offers substantial advantages for distinguishing structural isomers (Li et al., 2020).

In recent years, integrated data-processing software and automated correlation algorithms have made it possible to evaluate NMR and MS datasets within the same analytical platform. This development reduces manual interpretation time while increasing the accuracy of structural assignments (Emwas, 2015). Additionally, open-access NMR-MS correlation databases-such as HMDB, GNPS, and NMRShiftDB-enable researchers to compare experimental spectra with reference datasets from the literature (Wishart, 2021).

4.1 Applications in Coordination Complexes

In the structural characterization of coordination complexes, the combined use of NMR and MS provides complementary information on the stoichiometry, binding sites, and electronic environment of the compound. For example, in Ni(II)-Schiff base complexes, mass spectrometry (MS) directly confirms the molecular mass and stoichiometric ratio of the complex, whereas two-dimensional NMR techniques such as HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) experiments elucidate the specific binding sites of the ligand to the metal ion in detail (Atkins & Jones, 2021).

In such systems, the analysis of $^{1}H–^{13}C$ and $^{1}H–^{15}N$ correlations is particularly useful for confirming coordination at imine nitrogen and phenolic oxygen sites (Leitner et al., 2019). Additionally, isotopic distribution patterns obtained from MS play a crucial role in distinguishing whether a complex adopts a monomeric or dimeric structure (Hale et al., 2024).

In NMR spectra, chemical shift variations and signal broadening induced by coordination reflect the influence of the metal center on local electron density. These effects are especially pronounced in complexes containing paramagnetic ions (e.g., Ni (II), Co (II)) (Gabelica & Marklund, 2018). Therefore, the joint application of NMR and MS offers comprehensive insight into both the bonding environment and the stability of coordination complexes.

4.2 Biomolecular Complexes

Understanding the structural and dynamic properties of biomolecular complexes lies at the core of modern biochemistry and structural biology research. In this context, native mass spectrometry (native MS) and nuclear magnetic resonance (NMR) spectroscopy represent two highly complementary

techniques for elucidating the natural conformations of biomolecules such as proteins, nucleic acids, and metal ions (Hale et al., 2024).

Native MS enables the analysis of molecules under conditions close to physiological environments. This approach allows the investigation of protein–protein, protein–ligand, and protein–metal complexes without disrupting their native structures (Robinson, 2020). Owing to the soft ionization nature of electrospray ionization (ESI), protein complexes are transferred into the gas phase while preserving their solution-phase conformations and binding characteristics. As a result, direct information can be obtained regarding complex stoichiometry, binding affinity, metal-ion coordination, and conformational stability (Leney & Heck, 2017). Native MS is highly effective for metalloproteins, providing quantitative insights into the number of bound metal ions and their relative affinities (Hale et al., 2024).

In contrast, NMR spectroscopy is unrivaled in its ability to probe the dynamic behaviour and conformational changes of proteins at atomic resolution. For small and medium-sized proteins, NMR offers amino acid–level dynamic information (Wüthrich, 2003). Multidimensional NMR techniques, such as ^{1}H–^{15}N HSQC and NOESY, play a crucial role in identifying protein backbone mobility, ligand-binding sites, and interaction interfaces (Kay, 2005). Thus, when the stoichiometric insights obtained from native MS are combined with structural NMR data, both the structural and thermodynamic aspects of biomolecular interactions can be elucidated in a more comprehensive manner.

Recent advances in hybrid NMR–MS methodologies have enabled multidimensional investigation of the dynamic behaviour of biomolecular complexes (Hale et al., 2024). In these hybrid systems, native MS defines the mass composition and binding stoichiometry of complexes, while NMR reveals atomic-level interaction details and dynamic variability. This integrated approach provides a powerful foundation for structural bioinformatics modelling, particularly in studies of metalloproteins, enzyme–substrate complexes, and multi-protein networks (Leney & Heck, 2017).

5. CONCLUSION

The fundamental physical principle of NMR spectroscopy is based on the interaction of nuclear magnetic moments with an external magnetic field, and the excitation of the resulting energy differences by radiofrequency (RF) radiation. Chemical shift, spin–spin coupling, and relaxation times provide detailed insight into the electronic and dynamic properties of molecules. Therefore, NMR is

regarded as one of the most reliable spectroscopic tools for molecular structure determination in both organic and inorganic chemistry (Hore, 2015).

When 1D and 2D NMR spectroscopies are employed together, both atomic connectivity patterns and spatial relationships can be elucidated in detail. In this way, multidimensional structural analysis surpassing the limitations of classical one-dimensional NMR is achieved (Claridge, 2016).

In the study of coordination complexes, multinuclear NMR spectroscopy offers a unique contribution to the identification of ligand–metal binding modes, coordination geometries, variations in electron distribution, and dynamic processes. This approach reveals not only structural formulas but also microscopic changes in the electronic environment that occur upon bond formation.

The MAS technique enhances the resolution and applicability of solid-state NMR spectroscopy, making it an indispensable tool for understanding both crystalline and amorphous structures. Today, new approaches such as dynamic nuclear polarization (DNP)-enhanced MAS and proton-detected ultrafast MAS have further expanded the use of solid-state NMR in both materials science and biomolecular systems (Ultrafast MAS, 2021).

The choice of ionization method varies depending on sample type, analyte characteristics, and the structural information sought. Ionization techniques such as ESI, MALDI, and APCI have been developed to meet different analytical needs in modern mass spectrometry and, when properly selected, provide high sensitivity and specificity (Ma et al., 2022).

MS/MS fragmentation analysis is one of the cornerstones of modern analytical chemistry in terms of molecular structure determination and compound verification. The integration of various ionization and dissociation techniques has made this method an indispensable tool in both biochemical and pharmaceutical research.

Ion mobility spectrometry (IMS) has introduced a new dimension to analytical chemistry by revealing not only the mass of ions but also their spatial structure in the gas phase. The integration of IMS with mass spectrometry represents a breakthrough particularly in the investigation of isomers, protein conformers, and complex biomolecular structures (Hale et al., 2024).

The hybrid NMR–MS approach enables multidimensional and highly reliable characterization of molecular structure by combining both mass-based and magnetic resonance–based data. This integration represents the future of

structural elucidation in modern chemistry, biotechnology, and pharmaceutical research (Leitner et al., 2019).

In coordination compounds such as Ni (II) Schiff base complexes, MS reveals the overall compositional integrity of the structure, while NMR elucidates the binding topology, creating a powerful synergy in structural analysis (Atkins & Jones, 2021).

Native MS and NMR spectroscopy are two complementary analytical techniques that elucidate the structural integrity and dynamic nature of biomolecular complexes. Together, these methods generate significant synergy in interdisciplinary research aimed at understanding living systems at the molecular level (Hale et al., 2024).

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CHAPTER 2

BIOCHEMICAL AND MOLECULAR BASIS OF MICRORNA BIOGENESIS: BIOMARKER POTENTIAL IN PROSTATE CANCER

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Formation and roles of miRNAs in Human Cell

MicroRNAs are small ribonucleic acid molecules of approximately twenty-two nucleotides that modulate the classical model of molecular information flow, from deoxyribonucleic acid to ribonucleic acid to protein, originally described in 1958. Extensive research has examined the mechanisms through which microRNAs influence gene regulation, as well as the existence of alternative microRNA forms that modify transcript levels and functions across diverse biological systems (Bartel, 2018). Variability in gene expression is partly driven by differences in microRNA activity, their target genes and interactomes, associated signalling pathways, and additional yet-unidentified regulatory elements. This variability contributes to cellular identity and underlies cell type-specific patterns of microRNA regulation (Garg & Sharp, 2016; Maji et al., 2025).

MicroRNAs generally bind to the three-prime untranslated regions of messenger ribonucleic acids, where they inhibit translation or promote messenger ribonucleic acid degradation, resulting in reduced expression of specific genes (Nooreldeen & Bach, 2021). Over recent decades, the microRNA field has expanded substantially, with more than seventeen thousand microRNAs identified across one hundred forty-two species, including more than nineteen hundred documented in humans (Kozomara & Griffiths-Jones, 2010). MicroRNA expression is highly tissue-dependent and often reflects the physiological condition of the cell (Lim et al., 2005). Certain microRNA expression signatures have been proposed to possess predictive or diagnostic value due to their disease specificity. More refined profiling studies have demonstrated that distinct microRNA patterns are characteristic of cancer types, offering insights into tumor initiation and differentiation (Taghvimi et al., 2022; Vosough et al., 2023).

MicroRNAs play essential roles in the regulation of developmental processes in eukaryotic organisms, and because they control cellular differentiation and proliferation, they have been explored as potential therapeutic tools in oncology (Szymanski et al., 2005). Dysregulation of microRNA expression, whether through mutations, epigenetic silencing, or altered regulation of their targets, has been associated with clinically significant conditions, including autoimmune disorders and myocardial infarction (Solati et al., 2023; Vafadar et al., 2019). Compared with traditional biomarkers and therapeutic agents, microRNAs often exhibit superior diagnostic precision, faster detectability, and strong potential for individualized treatment strategies. In acute myocardial infarction, for example, certain microRNAs have demonstrated higher diagnostic accuracy than established biomarkers such as cardiac troponins and reach peak concentrations

more rapidly, enabling earlier clinical intervention (Rouhi et al., 2025; Wang et al., 2021). Although the initial costs of developing microRNA-based diagnostic and therapeutic platforms remain higher due to the novelty of these technologies, broader adoption is expected to lower costs over time and may ultimately yield substantial reductions in long-term healthcare expenditures (Hanna et al., 2019).

Multiple analytical methods are available for detecting and quantifying microRNAs, each offering distinct advantages and limitations. Northern blotting remains the reference method but is laborious and relatively inefficient (Jet et al., 2021). Reverse transcription quantitative polymerase chain reaction provides high sensitivity and specificity but is susceptible to amplification bias. Microarray-based platforms enable large-scale expression profiling; however, sensitivity and specificity can be compromised by sequence similarities among microRNAs. Next-generation sequencing yields comprehensive microRNA datasets but requires extensive computational resources. Droplet digital polymerase chain reaction enables precise quantification of low-abundance microRNAs (Siddika & Heinemann, 2021). Despite these methodological advances, several barriers hinder clinical translation, including low endogenous microRNA levels, variability across sample types, specificity challenges, and the absence of standardized laboratory protocols. Overcoming these constraints is essential for improving the utility of microRNA-based diagnostics (Alizadeh et al., 2025; Le & Nguyen, 2023).

Formation process of miRNA

MicroRNAs interact intimately with members of the Argonaute protein family, forming the core of gene-silencing pathways in diverse organisms (Lee et al., 1993; Wightman et al., 1993). These small regulatory molecules perform essential functions across numerous biological processes in both plants and animals. By directing Argonaute proteins to complementary messenger ribonucleic acid targets, microRNAs initiate translational repression, promote messenger ribonucleic acid deadenylation, or trigger endonucleolytic cleavage (Gebert & MacRae, 2019; Iwakawa & Tomari, 2022). A single microRNA can influence the expression of hundreds of protein-coding genes, thereby support cellular stability and enable dynamic adaptation to environmental stimuli. Consequently, the fidelity of microRNA biogenesis and activity is critical for maintaining normal physiological states, and perturbations in these pathways are closely linked to pathological conditions (Friedman et al., 2009).

In the canonical pathway, microRNA production begins with the transcription of a primary microRNA transcript by ribonucleic acid polymerase II (Cai et al., 2004). This primary transcript undergoes sequential processing by two

ribonuclease type III enzymes, Drosha and Dicer, which ultimately generate a duplex of approximately twenty-two nucleotides (Han et al., 2004; Ketting et al., 2001; Knight & Bass, 2001; Lee et al., 2002). The duplex is subsequently transferred to an Argonaute protein, where one strand is selectively discarded, yielding the mature gene-silencing assembly known as the ribonucleic acid-induced silencing complex (Hammond et al., 2001; Mourelatos et al., 2002). Non-canonical pathways, in contrast, circumvent Drosha, Dicer, or both, and employ alternative processing routes to produce mature microRNAs. Variations or errors in the processing of primary or precursor microRNAs give rise to distinct mature isoforms collectively referred to as isomiRs (Cheloufi et al., 2010; Ha & Kim, 2014; Jee et al., 2018; Yoda et al., 2013).

The endogenous biogenesis machinery in mammals can also be harnessed for experimental or therapeutic purposes by introducing externally designed small interfering ribonucleic acids or their precursors, including short hairpin ribonucleic acids. Through these approaches, small interfering ribonucleic acids effectively function as engineered microRNAs that direct targeted gene suppression via ribonucleic acid interference (Liu et al., 2004). Certain Argonaute proteins possess ribonuclease H-like catalytic domains, enabling them to cleave messenger ribonucleic acid molecules with perfect complementarity to small interfering ribonucleic acids. These molecular interactions and mechanisms are summarized in Figure 1 (Kim et al., 2025).

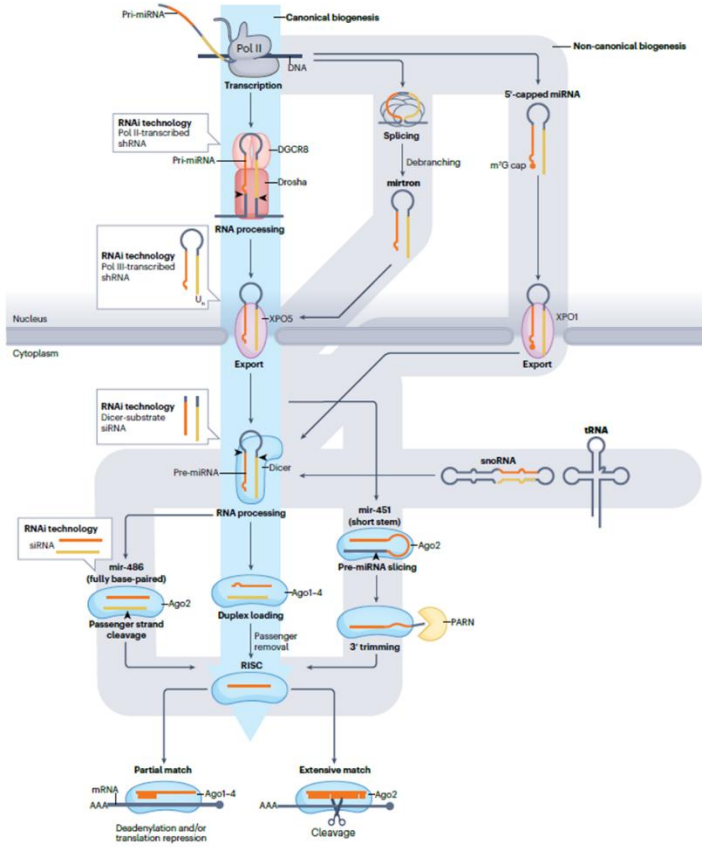

Figure 1. miRNA production process (Kim et al., 2025). Canonical microRNA (miRNA) biogenesis begins with RNA polymerase II (Pol II)–mediated transcription of primary miRNAs (pri-miRNAs), which are processed by the Microprocessor complex composed of Drosha and DiGeorge syndrome critical region 8 (DGCR8) to generate precursor miRNAs (pre-miRNAs). These are exported to the cytoplasm by exportin-5 (XPO5) and cleaved by the RNase III enzyme Dicer, yielding mature miRNA duplexes that are loaded onto Argonaute proteins (AGO1–4 in humans). One strand is retained to form the RNA-induced silencing complex (RISC). Several non-canonical pathways diverge from this route: mirtrons derive from debranched introns; some pre-miRNAs are generated as 5′-capped Pol II transcripts; selected small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs) bypass Drosha processing; pre-miR-451 is processed by Argonaute-2 (Ago2) and trimmed by the 3′–5′ exoribonuclease poly(A)-specific ribonuclease (PARN) instead of Dicer; and miR-486 requires Ago2-mediated cleavage for strand selection.The mature miRNA guides RISC to target messenger RNAs (mRNAs) primarily via the seed region (nucleotides 2–7), leading to translational repression and/or deadenylation, whereas extensive complementarity enables Ago2 (and in some contexts Ago3) to cleave the target mRNA between positions 10 and 11. Both canonical and non-canonical pathways can be exploited for RNA interference (RNAi) using Pol II- or Pol III-transcribed short hairpin RNAs (shRNAs) or chemically synthesized small interfering RNAs (siRNAs).

Over the last several decades, microRNAs have gained substantial attention as promising biomarker candidates in clinical research, complementing the established roles of proteins and messenger ribonucleic acids. These non-coding ribonucleic acids, typically twenty-one to twenty-four nucleotides in length, modulate gene expression at the post-transcriptional stage by binding to complementary regions within the three-prime untranslated regions of target messenger ribonucleic acids, thereby suppressing their translation. Beyond this regulatory activity, microRNAs participate in numerous fundamental biological processes, including cellular development, lineage differentiation, proliferation, cell-cycle control, apoptosis, and metabolic regulation (Bartel, 2004).

MicroRNA expression and function can be profoundly affected by chromosomal alterations, including structural deletions, amplifications, mutations, promoter methylation, and transcriptional regulatory mechanisms. Because a single microRNA can govern the expression of multiple messenger ribonucleic acids, disruptions in microRNA homeostasis may result in the misregulation of extensive gene networks, ultimately contributing to the development of a wide spectrum of human cancers (Calin & Croce, 2006; Kim & Kim, 2013). Indeed, accumulating evidence indicates that microRNA levels and expression signatures shift in accordance with tumour-related biological processes such as stage, size, invasiveness, and metastatic potential (Fendler et al., 2016).

MicroRNAs are detectable not only in tissue specimens but also in various body fluids, where they remain remarkably stable by virtue of their encapsulation in extracellular vesicles or association with ribonucleoprotein complexes (Boerrigter et al., 2020). This stability suggests that molecular alterations occurring within tumour tissues are often mirrored in circulating microRNAs, supporting their potential utility in liquid biopsy applications for early diagnosis, prognostic assessment, monitoring of minimal residual disease, and prediction of therapeutic outcomes.

The increasing availability of high-throughput platforms, including next-generation sequencing, ribonucleic acid sequencing, and microarray technologies, combined with extensive bioinformatic interrogation of large public datasets, has identified numerous microRNA candidates with potential biomarker value in andrological malignancies across diverse clinical contexts. Many of these microRNAs have subsequently undergone evaluation or validation through targeted, cost-efficient analytical methods such as real-time polymerase chain reaction or droplet digital polymerase chain reaction (Constâncio et al., 2023).

Prostate Cancer and MicroRNAs

Prostate cancer represents the second most frequently diagnosed malignancy in men, following skin cancer, and remains the fifth leading cause of cancer-related deaths worldwide (Daniyal et al., 2014). Although its precise molecular origins are not yet fully defined, prostate cancer is widely considered a multifactorial disease driven by genetic susceptibility, endocrine influences, and various environmental exposures (Attard et al., 2016). Androgenic hormones, particularly testosterone and its more active derivative dihydrotestosterone, exert essential regulatory functions on prostatic epithelial cells. Consequently, therapeutic strategies that inhibit androgen signalling, including androgen receptor antagonists or surgical/chemical castration, can induce apoptosis and regression of prostatic tissue (Hsing, Reichardt, & Stanczyk, 2002).

Most individuals with prostate cancer remain asymptomatic during the early stages. When symptoms do occur, they often resemble obstructive or irritative urinary complaints commonly attributed to benign prostatic hyperplasia, leading to frequent misdiagnosis among older men. Historically, early detection efforts relied on digital rectal examination, transrectal ultrasonography, and biochemical assessments (Mettlin et al., 1991). Today, prostate-specific antigen measurement is the predominant biomarker used to estimate prostate cancer risk and disease burden. Nevertheless, patients may develop clinically significant malignancy despite low prostate-specific antigen levels, while numerous benign conditions, including hyperplasia, inflammation, mechanical manipulation, and certain medications, can artificially elevate prostate-specific antigen concentrations and mimic malignancy during screening (Pron, 2015).

Current clinical decision-making incorporates nomograms that combine pathological and clinical factors such as the Gleason score, tumour stage, and prostate-specific antigen levels. However, these tools still struggle to non-invasively distinguish aggressive, metastatic-prone tumours requiring immediate intervention from indolent cancers that are suitable for active surveillance, or from benign conditions such as prostatitis or benign prostatic hyperplasia (Constâncio et al., 2023). Thus, there is an urgent need for reliable, non-invasive diagnostic markers capable of accurate disease stratification.

Several molecular assays have been developed to identify aggressive prostate cancer. Elevated circulating tumour DNA and increased numbers of circulating tumour cells correlate with poor survival outcomes, but technical limitations related to their reliable detection hinder routine clinical use, especially in localized disease (Danila et al., 2007; Cortese et al., 2012). Urinary protein biomarkers also show diagnostic promise, yet challenges associated with mass-

spectrometry assay standardization continue to limit clinical translation (Kim et al., 2016).

An alternative and increasingly explored avenue involves urinary microRNAs. These small regulatory ribonucleic acids contribute to prostate cancer initiation and progression, modulate therapeutic responsiveness, remain stable under extreme chemical conditions, and are readily measurable in urine (Korpela et al., 2015; Korzeniewski et al., 2015). Their characteristics and biological relevance position urinary microRNAs as attractive non-invasive biomarkers for diagnostic and prognostic applications. Nonetheless, progress in the field has been restricted by limited microRNA panels in earlier studies, small sample sizes, and a lack of systematic validation. Additionally, the extent of intra- and inter-individual microRNA variability is poorly defined due to the absence of repeated measurements. Because urinary microRNA levels correlate with those found in matched tumour tissue, they may serve as a surrogate liquid biopsy (Salido-Guadarrama et al., 2016; Fredsøe et al., 2018). Longitudinal analyses indicate that an individual's urinary microRNA signature remains stable over more than a year, suggesting that candidate biomarkers should be prioritized based on intra-individual temporal stability and tissue representativeness. Despite this stability, variability still exists among individuals and may reflect dietary differences, medication use, comorbidities, and additional epidemiological influences. Comparable intra-individual variability has been reported in placental messenger ribonucleic acid transcriptomes and in tissue-based messenger ribonucleic acid and methylation studies (Cowley et al., 2009; Turan et al., 2010).

The first comprehensive microRNA profiling in prostate cancer, published in 2007, examined cell lines, mouse xenografts, benign prostatic hyperplasia tissue, and human prostate cancer samples. This study identified fifty-one microRNAs with differential expression between benign and malignant states (Porkka et al., 2007). Furthermore, hierarchical clustering of tumour samples based on their microRNA signatures distinguished androgen-dependent from androgen-refractory cancers, emphasizing the biological and clinical value of microRNA profiling. These findings initiated the exploration of microRNAs as potential adjuncts or alternatives to serum prostate-specific antigen testing (Constâncio et al., 2023).

A 2018 meta-analysis by Song et al., which evaluated 104 studies across tissue, serum, and urine samples, identified ten upregulated and fourteen downregulated microRNAs with informative diagnostic and predictive capacity for differentiating prostate cancer from benign prostatic hyperplasia or healthy states (Song et al., 2018). Interestingly, microRNA expression patterns in blood differed markedly from those in tissue, and both diverged even further from

urinary microRNA profiles. These discrepancies reflect differences in microRNA release and transport mechanisms from tumour tissue to bodily fluids, reinforcing the necessity of validating tissue-derived biomarkers in liquid biopsies. Yet, the use of urine for prostate-specific microRNA analysis remains controversial due to potential contamination by exfoliated cells originating from the bladder or kidneys, which may dilute or obscure tumour-derived microRNA signals (Song et al., 2018; Constâncio et al., 2023).

Prostate cancer is characterized by extensive genomic heterogeneity and multifocality (Korpela et al., 2015), complicating the development of personalized biomarker tools. To address this, Jeon et al. examined the temporal stability of 673 urinary microRNAs in sequential samples from ten patients with localized disease. Their analysis revealed that each patient exhibited a distinct and highly stable urinary microRNA profile over time. They identified seven microRNAs, miR-3195, let-7b-5p, miR-144-3p, miR-451a, miR-148a-3p, miR-512-5p, and miR-431-5p, that were consistently stable within individuals. Incorporating these microRNAs into machine-learning models enabled the classification of high-risk patients with Gleason scores greater than seven in both the training cohort (n=99, area under the curve=0.72) and validation cohort (n=40, area under the curve=0.74) (Chen et al., 2008; Jeon et al., 2020). This work underscores the critical role of artificial intelligence in generating accurate and reliable biomarker strategies for prostate cancer (Constâncio et al., 2023).

New biomarkers in prostate cancer diagnosis: miRNAs

Multiple investigations have demonstrated that alterations in microRNA expression levels, identified through comprehensive microRNA expression profiling, are closely linked to the development and progression of prostate carcinoma. Consequently, there is a critical need to identify biomarkers with greater sensitivity than prostate-specific antigen to enhance clinical outcomes for individuals affected by prostate carcinoma. Increasing evidence indicates that aberrantly expressed microRNAs within tumor tissues, as well as circulating microRNAs detected in biological fluids such as plasma, serum, and urine, serve as valuable diagnostic, prognostic, and therapeutic biomarkers in prostate carcinoma (Goto et al., 2015).

miRNA-3195

In a Dutch cohort investigated by Rönnau and colleagues, the analysis included tissue samples from twenty-three individuals with benign prostatic hyperplasia, seventy-six cases of primary prostate carcinoma, and thirty-five specimens derived from castration-resistant prostate carcinoma. Within this

dataset, miRNA-3195 exhibited the most pronounced elevation among the examined groups, demonstrating a 5.6-fold increase in castration-resistant prostate carcinoma specimens with strong statistical support ($p < 0.0001$) (Rönnau et al., 2021). In contrast, a separate investigation reported reduced expression of miRNA-3195 in prostate carcinoma tissues from younger patients when compared with normal epithelial tissue, although the decrease did not reach statistical significance (Valera et al., 2020). Furthermore, a study conducted in a Turkish population revealed that miRNA-3195 expression was significantly higher in prostate carcinoma tissues relative to benign prostatic hyperplasia tissues, with statistical significance reported as $P = 0.0031$ and an odds ratio of 2.69 (Sancer et al., 2022). Conversely, a bioinformatic analysis utilizing microRNA expression data from prostate adenocarcinoma cases in The Cancer Genome Atlas demonstrated markedly diminished levels of miRNA-3195 (Yang et al., 2019).

Another investigation showed that melatonin treatment increased miRNA-3195 expression in hypoxic prostate cancer-3 cell lines. Importantly, forced overexpression of miRNA-3195 reduced messenger RNA abundance of angiogenesis-associated genes, including hypoxia-inducible factor 1-alpha, hypoxia-inducible factor 2-alpha, and vascular endothelial growth factor in hypoxic prostate cancer-3 cells (Sohn et al., 2015).

Collectively, one of these prostate cancer–related studies indicated that miRNA-3195 elevation was associated with a 5.6-fold increase in prostate cancer risk (Rönnau et al., 2021), whereas another demonstrated that miRNA-3195 suppressed key angiogenesis-related transcriptional targets in the presence of melatonin. These findings suggest that the functional role of miRNA-3195 may vary depending on the molecular environment in which it is expressed (Sohn et al., 2015).

let-7b-5p

The let-7 microRNA family participates in the regulation of developmental timing and cellular growth, modulates immune system activity, and helps shape inflammatory responses. Members of this family have been implicated in controlling mechanisms of immune evasion by influencing the expression of various immune-regulatory molecules, including major cytokines such as interleukin-6, interleukin-10, and tumour necrosis factor-alpha. Rong and colleagues reported that let-7b-5p may influence polarization toward the M2 macrophage phenotype through modulation of the suppressor of cytokine signalling 1 / signal transducer and activator of transcription signalling axis. They also demonstrated that inhibiting let-7b-5p, thereby reversing M2-type

differentiation, enhanced macrophage phagocytic capacity and ultimately suppressed the proliferation of prostate carcinoma cells (Rong et al., 2020).

In another investigation, Sun and collaborators showed that the circulating level of let-7b-5p in the serum of individuals with prostate carcinoma differed markedly from that of healthy participants. Their findings suggested that let-7b-5p may support earlier detection of prostate carcinoma and could help reduce the false-positive rate associated with prostate-specific antigen screening (Sun et al., 2024). The tumour-suppressive let-7 microRNA family targets several central regulators of oncogenesis, including the RAS and MYC genes. Moustafa and colleagues analysed microdissected indolent prostate carcinoma tissue (Gleason score 6) alongside matched normal prostate epithelium and identified let-7b-5p as one of the most strongly down-regulated microRNAs, with an approximate fourteen-fold reduction. In addition, let-7b-5p is predicted to regulate signalling pathways associated with RAS, cyclin D1, MDM4, MYB, BCL2-like-1, and nerve growth factor (Moustafa et al., 2017). Moreover, Li and coworkers demonstrated that the relative serum expression of let-7b-5p in one hundred twelve prostate carcinoma patients and healthy controls was significantly reduced ($P < 0.001$). Furthermore, a separate investigation conducted in colorectal carcinoma demonstrated that let-7b-5p suppressed cellular proliferation, migratory capacity, and invasive behaviour by directly targeting naked cuticle homolog 1. This molecule is recognized as a modulator of the Wnt/beta-catenin signalling pathway and has been reported to facilitate the proliferation of colorectal carcinoma cells (Dai et al., 2023).

miRNA-144-3p

MicroRNA-144-3p has been characterized as a tumour-suppressive regulator in multiple malignancies, including glioblastoma and hepatocellular carcinoma. Zheng and colleagues demonstrated that microRNA-144-3p expression was substantially reduced in prostate carcinoma tissues and corresponding cell lines when compared with matched adjacent non-malignant tissues and normal prostate epithelial cell lines. In addition, enforced expression of microRNA-144-3p in prostate cancer-3 and DU145 cell lines through transfection with microRNA-144-3p mimics resulted in a significant reduction in cellular proliferation, as assessed by methyl thiazolyl tetrazolium assays and colony formation experiments in vitro. Furthermore, microRNA-144-3p overexpression markedly inhibited tumor growth in vivo using a nude mouse xenograft model (Zheng et al., 2018).

In a separate investigation by You and Zhang (2018), the expression level of miR-144-3p was found to be markedly reduced in castration-resistant prostate

cancer (CRPC) tissues and corresponding cell lines when compared with androgen-dependent prostate cancer (ADPC) samples. Functional assays demonstrated that enforced expression of miR-144-3p in CRPC cells significantly restrained cell proliferation and colony-forming capacity, while simultaneously enhancing apoptotic activity. Mechanistically, miR-144-3p was shown to directly bind to centrosomal protein 55 (CEP55), resulting in the suppression of CEP55 expression. Consistently, silencing of CEP55 recapitulated the effects of miR-144-3p overexpression by inhibiting proliferation and inducing apoptosis in CRPC cells, suggesting that miR-144-3p regulates CRPC cell growth and survival through negative modulation of CEP55 (You and Zhang, 2018). Furthermore, dysregulation of miR-144-3p has also been reported in osteosarcoma tissues, where its ectopic expression markedly attenuated cellular migration and epithelial–mesenchymal transition (EMT). These findings imply that miR-144-3p may contribute to the regulation of bone metastatic processes in prostate cancer, and that therapeutic delivery of miR-144-3p mimics could represent a potential strategy for the treatment of prostate cancer bone metastasis (Cao et al., 2017; Luo et al., 2021). In another study, Tan et al. demonstrated that enhancer of zeste homolog 2 (EZH2), the catalytic core component of the polycomb repressive complex 2 (PRC2), mediates transcriptional repression through trimethylation of histone H3 at lysine 27 (H3K27). This EZH2-driven modification constitutes an independent epigenetic mechanism contributing to the silencing of tumor suppressor genes in cancer (Tan et al., 2014). Supporting this notion, Sun et al. reported that miR-144 overexpression significantly decreased EZH2 expression, reduced cell viability, and induced apoptosis in prostate cancer cells, whereas miR-144 suppression produced opposite effects. In addition, miR-144 was shown to inhibit the epithelial–mesenchymal transition in prostate cancer cells, further underscoring its tumor-suppressive role (Sun et al., 2021).

miR-451a

miR-451a is a relatively recently characterized microRNA that plays important roles in tumor development and progression in several malignancies, including breast, renal cell, and gastric cancers. Experimental studies have demonstrated that suppression of the proteasome subunit beta type 8 (PSMB8) markedly inhibits glioma growth both in vitro and in vivo through modulation of the ERK1/2 and PI3K/AKT signalling pathways. Elevated PSMB8 expression has also been shown to correlate with deeper tumor invasion, lymph node metastasis, and poorer survival outcomes. In prostate cancer (PCa), Liu et al. observed significantly reduced miR-451a levels accompanied by increased PSMB8 expression in PCa cell lines compared with the normal prostate epithelial

cell line RWPE-1. Functional analyses further indicated that enforced miR-451a expression suppresses PCa cell proliferation, colony formation, and invasion while inducing apoptosis. These findings suggest that miR-451a exerts tumor-suppressive effects in PCa at least partly through direct targeting of PSMB8 (Liu et al., 2020).

Consistent with these results, Fan and colleagues analyzed 78 prostate cancer tissues and matched adjacent normal tissues and reported decreased miR-451a expression in both tumor specimens and patient serum. Receiver operating characteristic (ROC) curve analysis revealed a strong diagnostic potential for miR-451a in PCa, with an area under the curve (AUC) of 0.921. Moreover, patients with low miR-451a expression exhibited significantly poorer five-year overall survival compared with those showing higher expression levels (Fan et al., 2021). Amplification and/or overexpression of the oncogene c-Myc is frequently observed across multiple cancer types, whereas miR-451a has been widely described as a tumor suppressor. Members of the heterochromatin protein 1 (HP1) family, including HP1α, HP1β, and HP1γ, are key regulators of higher-order chromatin structure and are involved in heterochromatin organization, DNA repair, transcriptional regulation, and telomere maintenance. Chang et al. demonstrated that in PCa cells, c-Myc induces upregulation of HP1γ, which in turn represses miR-451a expression, revealing a negative correlation between HP1γ and miR-451a levels. Since miR-451a can directly inhibit c-Myc, this regulatory axis contributes to PCa progression; however, miR-451a expression remains suppressed in PCa due to HP1γ-mediated transcriptional repression (Chang et al., 2018). In contrast, Li et al. (2021) reported that miR-451a expression was significantly increased in PCa patients and proposed its potential utility as a biomarker for distinguishing PCa patients from healthy individuals (Li et al., 2021). In a separate investigation, Li and colleagues reported a marked increase in urinary exosomal miR-451a levels in patients with prostate cancer. Furthermore, they proposed that a urinary exosomal miR-451a–based biomarker panel could discriminate prostate cancer from benign prostatic hyperplasia, demonstrating moderate diagnostic performance with an area under the ROC curve of 0.726 (Li et al., 2021). In a study conducted by Zabegina and colleagues, miR-451a isolated from cancer cell–derived small extracellular vesicles (SEVs) was proposed as a potential diagnostic biomarker for prostate cancer (Zabegina et al., 2021).

miR-148a-3p

miR-148a-3p exhibits heterogeneous expression patterns across different cancer types, showing increased levels in certain malignancies while being reduced in others. This miRNA is closely associated with cellular differentiation

and development and may contribute to tumorigenesis through modulation of relevant signalling pathways. Functionally, miR-148a-3p facilitates the differentiation of activated B lymphocytes into plasma cells and enhances plasma cell survival by suppressing the transcription factors BACH2 and MITF, as well as the pro-apoptotic molecules BIM and PTEN (Dybos et al., 2018). Elevated expression of miR-148a-3p has also been reported in other cancers, including glioma and osteosarcoma. In prostate cancer (PCa), Dybos et al. demonstrated increased serum levels of miR-148a-3p in affected patients. In contrast, Coman et al. observed a marked reduction of miR-148a-3p in plasma samples from PCa patients compared with those with benign prostatic hyperplasia (BPH), and ROC curve analysis indicated its potential value as a diagnostic biomarker for differentiating between these two conditions (Coman et al., 2024). Furthermore, miR-148a-5p has been characterized as a tumor-suppressive miRNA through its ability to target oncogenes or key components of tumor-promoting pathways (Li et al., 2016). Accordingly, its reduced expression in malignant tissues relative to normal counterparts highlights its promise as a diagnostic marker in cancer. In contrast, another investigation demonstrated that miR-148a-3p levels were elevated in both tissue and plasma samples from patients with prostate cancer (PCa) compared with healthy individuals (Paunescu et al., 2019). Consistently, increased miR-148a-3p expression has been detected in the serum and urine of PCa patients (Dybos et al., 2018; Stuopelyte et al., 2016), as well as in prostate tumor tissues relative to adjacent non-tumorous prostatic tissues (Szczyrba et al., 2010). However, lower miR-148a-3p expression has been reported in castration-resistant PCa (CRPC) cell lines, including PC3 and DU145, compared with therapy-responsive model cell lines (Fujita et al., 2010; Coman et al., 2024). In a similar manner, they found decreased serum miR-148a-3p levels in PCa patients when compared with benign prostatic hyperplasia (BPH) samples (Osiecki et al., 2025).

In a separate study, Li et al. (2023) showed that methyltransferase-like 3 (METTL3), an RNA methyltransferase involved in mRNA maturation, stability, and translational regulation via N6-methyladenosine (m6A) modification, enhanced miR-148a-3p expression by inducing m6A modification of pri-miR-148a-3p. Through targeting thioredoxin-interacting protein (TXNIP), disruption of METTL3 activity suppressed PCa cell proliferation, migration, and invasion, promoted apoptosis, and reduced tumor growth in nude mouse models. Collectively, these findings indicate that miR-148a-3p contributes to the establishment of a tumor-suppressive phenotype by limiting cell survival, including in PCa cells. Consequently, miR-148a-3p may function both as a dependable marker of tumor progression and as a potential biomarker for monitoring therapeutic response in PCa (Li et al., 2023).

Additionally, DNA methyltransferases (DNMTs) are responsible for catalyzing the transfer of methyl groups to genomic DNA (Lyko, 2018). Dysregulation of DNMT genes can promote malignant transformation, worsen clinical outcomes, and hinder effective treatment. Inhibition of DNMT activity has been shown to suppress tumor development by restoring the expression of tumor suppressor genes (Subramaniam et al., 2014). Phosphatase and tensin homolog (PTEN) is a well-established tumor suppressor that controls cell proliferation and survival through the phosphoinositide-3-kinase (PI3K) signaling pathway (Maehama et al., 2001). Loss of PTEN expression occurs in approximately 70% of PCa cases (Sun et al., 2019). Notably, Gürbüz et al. (2021) reported that miR-148a expression was associated with increased DNMT1 levels and concomitant suppression of PTEN in PCa patients compared with healthy controls.

miR-512-5p

miR-512-5p has been shown to promote apoptotic pathways in lung and gastric cancers and to directly target hTERT in head and neck squamous cell carcinoma. In basal-like breast cancer cells, miR-512 plays a critical role in regulating hTERT-mediated telomere integrity and resistance to apoptosis. Specifically, miR-512-5p facilitates the degradation of hTERT mRNA, leading to diminished telomerase activity. Clinical analyses have demonstrated that miR-512-5p expression is significantly reduced in breast cancer tissues. Notably, elevated expression levels of hTERT and genes targeted by miR-512-5p have been associated with unfavorable survival outcomes in patients with basal-type breast cancer (Buemi, 2017).

They identified a urinary miR-512-5p–based signature capable of distinguishing high-risk prostate cancer patients with considerable accuracy (AUC = 0.74, 95% CI: 0.55–0.92), and this signature remained stable over longitudinal assessments (Jeon et al., 2020). Furthermore, a study in a Turkish cohort reported decreased serum levels of miR-512-5p in individuals diagnosed with prostate cancer compared with healthy controls (Babazade, 2018).

In addition, the androgen receptor (AR) has been identified as a direct target of several androgen-responsive miRNAs, including miR-488*, miR-644, miR-149, and miR-512-5p. These andro-miRs interact with specific binding sites within the 3′ untranslated region of AR, resulting in reduced luciferase activity in target validation assays as well as suppression of endogenous AR expression. Consequently, post-transcriptional modulation of AR by andro-miRs highlights their potential utility as miRNA-based adjunct therapeutic strategies in the management of castration-resistant prostate cancer (CRPC) (Ebron et al., 2014).

miR-431-5p

Retrotransposon-like 1 (RTL1) is implicated in a wide range of essential biological functions. Loss of Rtl1 leads to multiple developmental abnormalities, including obesity, blepharophimosis, skeletal dysplasia, increased serum lipid metabolites, and placental hypoplasia, and is associated with a markedly elevated lethality rate. RTL1 plays a critical role in placental development (Fan et al., 2017). In melanoma cells, RTL1 overexpression has been shown to enhance cellular proliferation, facilitate progression through the G1 phase of the cell cycle, and upregulate genes involved in cell cycle regulation. Importantly, Rtl1 expression is modulated by an RNA interference mechanism mediated by RTL1 antisense (RTL1-as), which encodes at least seven microRNAs. The RTL1 gene and miR-431-5p are located at the same genomic locus on opposite DNA strands, indicating a close regulatory association between miR-431-5p and RTL1 (Yurikova et al., 2019).

Recent studies have increasingly linked RTL1 to the pathogenesis of various cancers. In lung cancer tissues, the promoter region of RTL1 exhibits significantly reduced methylation compared with normal lung tissues, accompanied by elevated expression of microRNAs located within the RTL1 exon. Moreover, high RTL1 expression has been observed in mouse liver tumors induced by the Sleeping Beauty (SB) transposon system. Notably, RTL1 was identified as the only gene consistently dysregulated in all SB-induced tumors containing Dlk1–Dio3 integrations, suggesting that aberrant RTL1 activation may serve as a driving event in hepatocarcinogenesis (Riordan et al., 2013). Additionally, a significant upregulation of RTL1 expression has been reported in head and neck squamous cell carcinoma (Hsu et al., 2016). Functional studies further demonstrated that silencing RTL1 in melanoma cells suppresses proliferation, induces G1-phase cell cycle arrest, and reduces the expression of key regulators such as E2F1, Cyclin D1, cyclin-dependent kinase 6 (CDK6), and c-MYC. These findings indicate that RTL1 promotes melanoma cell growth, at least in part, through modulation of the Wnt/β-catenin signaling pathway (Fan et al., 2017).

In a separate study, miR-431-5p was found to be significantly downregulated in prostate cancer tissues relative to tumor-adjacent histologically normal samples (Lu et al., 2021). Furthermore, DAB2 interacting protein (DAB2IP), a Ras GTPase-activating protein with tumor suppressor activity, was identified as a direct target of miR-431. Consistently, miR-431 has been shown to enhance metastatic potential in pancreatic neuroendocrine tumors by repressing DAB2IP expression (Zhang et al., 2020). In addition, Qu et al. reported that colorectal cancer patients exhibiting low miR-431-5p expression experienced significantly

reduced overall survival, highlighting its potential prognostic relevance (Qu et al., 2022).

Conclusion

Considering all this information, it is understood that miRNAs, after being synthesized, play a leading role in regulating the expression (down- or up-regulation) of many genes, thus being key players in the execution of various cellular processes. Recently, many studies have suggested that miRNAs could be biomarkers in various types of cancer. It is known that the stability of miRNAs in blood, serum, plasma, and urine is very low over time. However, recently it has been reported that the stability of miRNAs conserved in exosomes obtained from urine is maintained over time. It has been suggested that the miRNAs mentioned above remain stable in the urine of individuals over time, and therefore these miRNAs could be used as biomarkers in the diagnosis and prognosis of prostate cancer patients. Studies are ongoing, especially in finding differential diagnostic markers for the early diagnosis and treatment of diseases. It is known that the gray-zone values of prostate serum antigen (PSA), used in the diagnosis of prostate cancer, can cause misleading predictions in prostate cancer diagnosis. Therefore, for these miRNAs, which have attracted attention especially in recent years, to be used as biomarkers for diagnostic purposes, this information needs to be validated by studying them in large cohorts of prostate cancer patients and in different populations. With technological advancements, the easier detection and quantification of miRNAs indicate that they could be a promising biomarker in the diagnosis and treatment of many diseases.

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Chapter 3

DRINKING WATER SAFETY: SCIENTIFIC ASSESSMENT OF PHYSICAL, CHEMICAL AND MICROBIOLOGICAL PARAMETERS

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Water

Water is a fundamental compound that plays a central role in origin, evolution, and continuity of life. The emergence of life in aquatic environments and the extensive presence of water in cells, tissues, and whole organisms demonstrate its indispensable biological significance. Approximately 70% of the human body consists of water, although this proportion varies with age, being higher in children and lower in the elderly. Water is essential for maintaining circulatory, digestive, and excretory functions in both humans and animals (Çalık, Menteş, Dayıoğlu, & Karadağ, 2004).

To sustain vital physiological processes, an individual must consume an average of 1.5–2 liters of clean, safe water daily. This requirement forms the basis of the concept of hygienic water, which is necessary not only for drinking but also for cooking, food preparation, and household cleaning (Dayıoğlu, Özyurt, Bingöl, & Yıldız, 2004).

In residential areas, including provinces, districts, towns, and villages, spring water is typically collected in reservoirs and distributed to households through channels and pipelines. During this journey, water may become contaminated at various points: at the source, within storage structures, or throughout the distribution network. Additional contamination may arise from domestic sewage, industrial effluents, and agricultural runoff. The absence of adequate treatment systems or the discharge of untreated wastewater into rivers and lakes further exacerbates this issue (Erkman, 2011).

Water is an exceptional solvent, a property that offers both advantages and disadvantages for living systems. While essential minerals that the body requires from external sources dissolve readily in water, toxic and harmful elements such as cadmium (Cd), mercury (Hg), nickel (Ni), lead (Pb), and arsenic (As), are also water-soluble. Primary bioelements (C, H, O, N, S, P) form the molecular structures essential for life, whereas secondary essential elements (Na^+, K^+, Mg^{2+}, SO_4^{2-}, Cl^-, PO_4^{3-}), which maintain osmotic and electrical balance, also occur in water-soluble forms (Tofan, 2008).

Wastewater is known to contain high concentrations of pathogenic microorganisms that may infiltrate drinking water systems. Once introduced, these microorganisms can adhere to and proliferate on pipe surfaces and within storage tanks, posing significant public health risks (Edberg, 2000).

Water is a colorless, tasteless, and odorless liquid composed of two hydrogen atoms and one oxygen atom. It supports biological life across all scales, from microscopic organisms to complex multicellular systems. The water molecule has

a bent geometry, with an angle of 104.5° between the hydrogen atoms. Because oxygen lacks two electrons in its outermost orbital, it shares electrons with hydrogen atoms, completing a stable octet configuration. Water is considered a remarkable molecule due to its polarity, hydrogen-bonding capability, intermolecular cohesion, excellent solvent power, adhesive and cohesive forces, and its unique ability to freeze from the surface downward (Mutluay, 1996).

Water's high heat capacity, resulting from hydrogen bonding, allows it to absorb and release large amounts of heat with minimal temperature change. It also exhibits a high heat of vaporization compared with other hydrides formed by Group VIA elements. Its boiling and melting points are higher than expected based on molecular weight alone. Significant heat is required to disrupt hydrogen bonds in ice. Water reaches its maximum density at +4°C; above and below this temperature, its density decreases. It contracts as it cools to 4°C (density: 1.00000 g/cm³) and expands from 4°C to 0°C (density at 0°C: 0.99987 g/cm³) (Mutluay, 1996). This unique behavior prevents lakes and rivers from freezing solidly, enabling life to survive beneath the ice layer.

Water's solvency arises from its high dielectric constant, its polarity, and its ability to form hydrogen bonds. The dielectric constant reflects a solvent's ability to separate opposite electrical charges, allowing water to dissolve ionic and polar substances effectively (Mutluay, 1996).

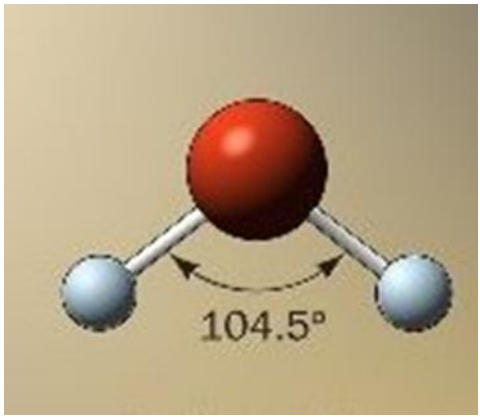

Figure 1. Water Molecule (Angle between H bonds)

Water Distribution in World and Turkiye

The distribution of water on Earth varies slightly among scientific assessments; however, the most widely accepted estimate indicates a total of approximately 1,386,000,000 km³ of water. Of this volume, 97.5% consists of saline water found in oceans and seas, while only 2.5% constitutes freshwater.

Furthermore, merely 0.4% of global freshwater is readily accessible and suitable for human use.

Oceans represent the largest source of evaporative freshwater input into the hydrological cycle. Water that evaporates from oceans enters the atmospheric system and precipitates across terrestrial surfaces, sustaining ecological and human life. However, the uneven global distribution of precipitation and water resources has resulted in severe disparities. Currently, an estimated 1.2 billion people lack access to safe drinking water, while 2.4 billion individuals live without adequate sanitation or clean water access. Global freshwater resources continue to diminish, primarily due to escalating population growth, which proportionally reduces per-capita water availability and accelerates resource depletion (Gemci, Akarsu, Zıba, & Dolaz Mustafa, 2016).

According to United Nations data published in November 2022, the world population is approaching 8 billion. This value represents an approximation, as population counts remain incomplete or imprecise in certain regions.

At the World Water Forum held in Brazil, critical issues were highlighted, including global water scarcity, sustainable water-use management, institutional governance, accountability mechanisms, financing of water infrastructures, and the need for environmentally and socially viable solutions (UNESCO–UN WATER, 2022).

Reports published by the United Nations emphasize that population density and freshwater availability are not proportionally distributed across global landmasses. Some regions enjoy abundant water resources, whereas others experience chronic water scarcity. Projections indicate that this disparity will intensify in the coming decades due to climate change, population increase, and environmental degradation (UN WATER, 2022). Numerical data is provided in Table 1.

Table 1. Continental Distribution of Population and Water Resources

Continents	Population (%)	Water Resources (%)
North America	8	15
South America	6	26
Europe	13	8
Africa	13	11
Asia	60	36
Australia and the Islands	1	5

Currently, the total annual renewable water potential of Turkey is estimated at 98 billion cubic meters, of which 95 billion cubic meters originate from internal surface waters, while 3 billion cubic meters flow from transboundary sources.

National water consumption is distributed among domestic, industrial, and agricultural sectors, with agriculture constituting the largest share of total usage. By the year 2025, per-capita water availability in Turkey is projected to decline to 1,237 m^3, placing the country in the category of water-stressed nations. Therefore, it is essential to implement protective measures before water scarcity becomes an unavoidable environmental and socioeconomic challenge (Anaç & Çeliker, 2004).

Over the past century, several factors have contributed to the deterioration of water quality in Turkey. These include global warming, unplanned industrial expansion, rapid and uncontrolled urbanization, destruction of natural ecosystems, and the contamination of drinking water resources from untreated or inadequately treated domestic and industrial wastewater. Significant deficiencies remain in the management of existing water treatment facilities, many of which either do not function effectively or lack the necessary regulatory oversight. Furthermore, agricultural and livestock activities exert substantial pressure on water resources, increasing pollutant loads on both surface and groundwater systems. For this reason, systematic monitoring of agricultural practices and proper regulation of wastewater discharge are essential for protecting water quality (Güloğlu, 2023).

Restoration and purification of a contaminated water source require substantial financial resources. From an economic standpoint, identifying pollution sources early and implementing preventive measures is significantly more cost-effective than remediation after degradation has occurred (Güloğlu, 2023).

Drinking and domestic-use water must possess specific characteristics to ensure safety, potability, and suitability for human consumption. According to established standards, drinking water is required to meet the following criteria (Şengül & Türkman, 1998):

- It should be **colorless, odorless, and palatable**.
- The **water temperature** should ideally be **below 15°C**.
- It must **not contain pathogenic microorganisms** capable of causing disease.
- It should **not exhibit corrosive properties**, as corrosion may damage distribution systems and release harmful metals.
- It must be **free from toxic substances**.

- Water sources should be **sufficient, accessible, and economically viable** for sustained use.

Beyond these general characteristics, the quality of drinking and household water is evaluated under **four main categories**:

1. **Physical properties**
2. **Chemical properties**
3. **Bacteriological (microbiological) properties**
4. **Radioactive (radiological) properties**

Each of these parameter groups has significant implications for human health, and their individual effects are examined in the following sections.

Physical Properties of Water

The physical properties of water refer to characteristics that can be perceived through the human senses. These include color, turbidity, odor, taste, and temperature, parameters that influence the sensory quality of drinking water and shape public perception regarding its safety. Any deviation in these properties alters the appearance and taste of water, often leading consumers to believe that the water is of poor quality.

Color in water typically results from dissolved organic substances or other contaminants introduced through natural or anthropogenic processes. Although color alone is not generally considered a direct health risk, it is regarded as an indicator of aesthetic deterioration and visual pollution (Gemci et al., 2016). Reddish or dark-brown coloration often signifies the presence of iron or manganese, whereas brown or yellow hues indicate organic matter. Greenish coloration usually suggests lime deposits or algal growth. Additionally, algal layers, clay particles, and industrial discharges can affect water coloration and contribute to pollution (Mutluay, 1996).

Turbidity is caused by suspended solids such as sand, clay, manganese, iron, and microorganisms. For drinking water, turbidity should ideally remain between 0 and 1 NTU to ensure hygienic safety (Mutluay, 1996). High turbidity may result from wastewater infiltration, sewage contamination, or other pollutants entering the water supply. Because turbidity may impair disinfection efficiency and indicate microbial risk, its presence is considered a health concern (Akar, 2000).

Odor in water is most detected by the human sense of smell. The presence of odor is frequently associated with contamination. Phenolic compounds, chlorine

derivatives, petroleum residues, and gases such as hydrogen sulphide (H_2S), carbon dioxide (CO_2), and methane (CH_4) can dissolve in water and produce unpleasant odors (Şengül & Türkman, 1998).

Taste abnormalities occur when undesirable or harmful substances enter the water supply. Heavy metal salts, humic acids, chlorides, petroleum derivatives, manganese, and iron can alter the natural taste of water (Mutluay, 1996). Any change in taste serves as an indicator of potential contamination.

Temperature provides insight into the physical and environmental conditions surrounding a water source. The ideal temperature for drinking water is approximately +14°C. Groundwater is expected to maintain stable temperatures; deviations may signal external contamination (Karpuzcu, 1988).

Conductivity is an important parameter used to assess the presence of dissolved ions. Elevated conductivity values typically reflect higher concentrations of minerals, salts, or pollutants, making conductivity a useful indicator for detecting contamination (Gemci et al., 2016).

Chemical Properties of Water

The chemical properties of water encompass a wide range of dissolved inorganic and organic substances, including heavy metals (e.g., Hg^{+}, Pb^{2+}, Cr^{3+}, Mn, Fe, Zn, Cu, Cd, Se), essential ions (e.g., Ca^{2+}, Mg^{2+}, Cl^{-}, SO_4^{2-}, PO_4^{3-}), nitrite–nitrate compounds, alkyl-benzene sulfonates, phenolic substances, and pH levels. These parameters collectively determine the chemical quality of drinking and domestic-use water (Gemci, Akarsu, Zıba, & Dolaz Mustafa, 2016). When present above threshold limits, many of these constituents, particularly heavy metals, pose serious health risks through chronic exposure.

The presence of **aluminium** in drinking water is often associated with acid rain, and its solubility is influenced by soil pH. Elevated aluminium concentrations may also result from corrosion of water distribution lines constructed with lead, iron, plastic, or cement-based materials (Snoeyink, Schock, & Sarin, 2003).

Mercury is among the most toxic heavy metals. Widely used in metal plating and the iron–steel industry, mercury can enter drinking water systems via industrial discharges. Upon ingestion, mercury accumulates primarily in the kidneys, causing severe toxicity. It may also contaminate aquatic organisms, thereby posing ecological and human health risks (Özbolat & Tüli, 2006).

Cadmium contamination generally arises from wastes associated with mining operations, battery production, paint manufacturing, vehicle emissions, plastic

production, and industrial plating processes (Samsunlu, 1999). Chronic cadmium exposure can impair renal function and bone metabolism.

Chromium, particularly hexavalent forms, is a recognized carcinogen when found in drinking water. Chromium contamination frequently originates from mining and industrial waste. Although trace levels in daily exposure may not cause acute toxicity, elevated concentrations pose significant health risks (Kara, Pırlak, & Özdilek, 2004).

Lead and its compounds have adversely affected human health for centuries. Lead enters the human body via inhalation, ingestion of contaminated food, and consumption of polluted water. Today, major sources include industrial activities and vehicle emissions. Lead released from petroleum-based fuels contaminates air, soil, and eventually water supplies. Its use in the paint and plastics industry further increases the likelihood of exposure (Öztürk, 2004).

Iron contamination in drinking water may result from corroded pipelines or the presence of iron minerals in the geological structure surrounding water sources. Industrial or domestic waste also contributes to elevated iron levels (Snoeyink et al., 2003).

Manganese enters water resources through the dissolution of soil minerals, particularly during rainfall, as well as through contamination from organic, industrial, and domestic waste. Elevated manganese levels impair water quality and may cause staining and adverse taste (The American Well Owner, 2002).

Nickel is widely used in electroplating, battery production, pigment manufacturing, coin making, magnet production, and medical prosthetics. Industrial waste discharge is the primary source of nickel contamination in drinking water (Çağlaırmak & Hepçimen, 2010).

The presence of **nitrate** in drinking water typically results from agricultural fertilizers, livestock waste, human sewage, and runoff following precipitation. Nitrite/nitrate compounds form through oxidation–reduction reactions mediated by bacteria. Pathogenic microorganisms can promote the conversion of nitrite to nitrate, accelerating nitrite reductase activity (Garay, 2008; Cemek, 2007). Elevated nitrate levels pose serious health risks, including methemoglobinemia.

Copper (Cu) contamination occurs due to corrosion of copper or brass pipes and industrial discharge. Copper is vital for hemoglobin synthesis and numerous enzymatic functions (Baysal, 1999). While low concentrations are harmless, levels above 1 mg/L impart a metallic taste and may cause toxicity. WHO

guidelines set allowable concentrations between 0.05 and 1.5 mg/L (WHO, 2008).

Zinc (Zn) is an essential trace element involved in enzyme function, protein synthesis, and immune system regulation. Daily adult requirements may reach 15 mg. WHO limits zinc in drinking water to a maximum of 3 mg/L, above which taste alterations occur (Belce, 2002; WHO, 2008). Industrial waste is a common source of contamination.

Nitrite and nitrate compounds may react with secondary amines to form nitrosamines, potent carcinogenic and teratogenic substances. These compounds also contribute to chemical hazards in processed foods where they inhibit *Clostridium botulinum* toxin formation (Özdestan & Nüren, 2010).

Calcium concentrations in water generally reflect the geological structure of the surrounding region. Gypsum, dolomite, and limestone formations contribute calcium to groundwater. Calcium in drinking water poses no known health risks (Güler & Çobanoğlu, 1997).

Magnesium, another contributor to water hardness, exists as dissolved salts. It plays essential physiological roles in bone, muscle, and nerve tissues and has no reported adverse effects at levels commonly found in drinking water (Güler & Çobanoğlu, 1997).

Phosphate (PO_4^{3-}) is essential for human metabolism and occurs naturally in spring waters. It may also enter water from fertilizers, industrial discharge, or geological dissolution. Elevated phosphate levels promote algal growth in reservoirs and pipelines, causing undesirable taste and odor (Güler & Çobanoğlu, 1997).

Sulfate (SO_4^{2-}) is a naturally occurring ion found in all drinking water sources. Industrial contamination can increase sulphate concentrations beyond natural levels. High sulphate levels are associated with toxicity, corrosion, unpleasant taste, and gastrointestinal disturbances. Concentrations exceeding 250 mg/L of magnesium sulphate or sodium sulphate may cause diarrhoea (Tofan, 2008).

Fluoride (F^-) has both beneficial and harmful effects depending on its concentration. Approximately 1 mg/L is recommended for dental health, as it reduces the risk of tooth decay. Excessive fluoride intake, however, may lead to fluorosis (Tofan, 2008).

Chloride (Cl^-) contamination originates from soil minerals or cleaning agents. Chloride introduced through natural soil usually does not pose a health risk; however, concentrations above 250 mg/L impart a salty taste to water.

Harmful chloride sources include sewage effluents, oil well drainage, refinery discharge, and road salt runoff (Samsunlu, 1999).

Microbiological Properties of Water

Microbiological contamination of water has been increasing substantially, particularly in agricultural regions, due to the influences of sewage discharge, human activities, and runoff generated by rainfall. Wastewater contains a wide range of pathogenic microorganisms, many of which pose significant public health risks when introduced into drinking water supplies (Alemdar, 2009). The primary sources of microbiological pollution in potable and domestic-use water are human and animal feces, which may harbour pathogenic bacteria, viruses, protozoa, and helminths (Alemdar, 2009; Erkman, 2011).

The intensity and type of precipitation strongly influence the transport of contaminants. Heavy rainfall can mobilize suspended solids and carry both anthropogenic and naturally occurring chemicals into surface waters or groundwater systems through runoff and infiltration (Depla, Jung, Baures, Clement, & Thomas, 2009; Pordue et al., 2005).

Short-term increases in pathogen concentrations significantly elevate the risk of infectious diseases and may lead to waterborne outbreaks such as diarrhoea, malaria, dysentery, and cholera (WHO, 2008). Thus, the **effective disinfection** of drinking water is essential for minimizing the presence of pathogenic microorganisms and ensuring the microbiological safety of water resources (Schoenen, 2002).

Because routine detection of all potential pathogens is challenging, time-consuming, and costly, **indicator organisms**, particularly **coliform bacteria such as *Escherichia coli* (E. coli)**, are widely used to assess water quality. The presence of coliforms provides a reliable indication of fecal contamination and reflects whether water is safe or unfit for consumption (Muslu, 1985).

According to national regulations and the World Health Organization (WHO) standards, drinking and domestic-use water should contain **0/100 CFU/mL total coliforms** under normal conditions (Ministry of Health, 2005; WHO, 2008). Detection of *E. coli* in water is considered definitive evidence of fecal contamination, as these organisms are naturally present in the intestines of humans and animals (Edberg, 2000).

Radioactive Properties of Water

Radioactive substances present in water pose significant health risks due to the ionizing radiation they emit. These materials may contaminate both drinking

water and domestic-use water through various environmental and anthropogenic pathways. The principal sources of radioactive contamination in water include:

1. Nuclear industrial facilities
2. Nuclear testing sites
3. Radioactive materials used in medical, research, or industrial applications
4. Uranium deposits and mining activities

Exposure to radioactive contaminants depends on concentration, duration of exposure, and the radionuclide involved. Radiation doses in water are quantified in **microcuries**, and regulatory standards indicate that the permissible level in drinking water should be **less than 10^{-9} microcurie/m^3** (Akar, 2000).

Contamination of water resources with radioactive elements can have severe consequences. Ionizing radiation damages biological tissues, increases the risk of cancer, and disrupts cellular processes. For this reason, the monitoring of radiological parameters in drinking water supplies is crucial, particularly in regions near industrial, military, or geological radiation sources.

To ensure the protection of public health, national and international regulatory agencies require routine testing of drinking water for radioactive isotopes such as radium, uranium, radon, and thorium. Consistent monitoring and proper management of radioactive waste are essential for preventing long-term radiological hazards in water resources.

Water Quality Parameters

The quality of drinking and domestic-use water is evaluated based on four principal categories of parameters: **physical, chemical, microbiological, and radioactive** characteristics. The European Union first established comprehensive water quality standards in 1998, followed by the World Health Organization (WHO), which published its own guideline values in 1999. In Turkey, national drinking water standards were formally defined through the "Regulation on Water Intended for Human Consumption," which was published in the Official Gazette in April 2005 and subsequently came into force (TBMM, 2005).

Current values and limit parameters from these regulatory and international guidelines are presented in **Table 2, Table 3, and Table 4**, respectively, based on updated datasets (Ethicwater, 2024).

Table 2. Drinking Water Chemical Quality Parameters

PARAMETER	TS 266	WHO STD.
Fluoride (mg/L)	1.5	1.5
Chloride (mg/L)	250	250
Nitrate (mg/L)	50	50
Sulfate	250	250
Phosphate	*	*
Bromate (uq /l)	10	25
Nitrite (mg/L)	50	50
Copper (mg/L)	2	--
Sodium (mg/ml)	200	200
Potassium (mg/L)	*	*
Calcium (mg/L)	*	*
Lead (mg/L)	0.01	0.05
Chromium (mg/L)	0.05	0.05
Manganese (mg/L)	0.05	0.5
Magnesium (mg/L)	*	*
Nickel (mg/ml)	20	20
Iron (mg/L)	0.2	--

Table 3. Drinking Water Physical Quality Parameters

PARAMETER	TS 266 APRIL 2015	WHO STD.
pH	6.5-9.5	6.5-8.5
EC (µS)	2500	2500

Table 4. Drinking Water Microbiological Quality Parameters

PARAMETER	TS 266 APRIL 2015	WHO STD.
Coliform (EMS/100ml)	0	0

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Chapter 4

NEXT-GENERATION CHEMICAL PLATFORMS: THE SCIENCE AND APPLICATIONS OF IONIC LIQUIDS

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Ionic Liquids

Ionic liquids are a new generation of ionic substances that have attracted significant attention in chemistry in recent years and are challenging the classical understanding of solvents. Their ability to exist in liquid form at room temperature, despite being composed entirely of ions, demonstrates their flexible and easily modifiable structure. The combination of an organic cation with other organic or inorganic anions allows their properties to be tailored to desired shapes; for this reason, ionic liquids are often described as "molecular LEGOs." Properties such as low volatility, remaining liquid over a wide temperature range, and high chemical resistance have made these solvents environmentally safer options (Wilkes, 2002).

The growing prominence of ionic liquids in the scientific community cannot be attributed solely to the introduction of a new class of solvents. Their inherently design-flexible structures have facilitated the development of task-specific ionic liquids (TSILs), engineered to perform a particular function. This capability has enabled the synthesis of ionic liquids that can accelerate targeted catalytic reactions, selectively coordinate metal ions, stabilize biomolecules, or enhance electrochemical performance. As a result, ionic liquids have evolved from simple solvent systems into active functional components within chemical processes. Rogers and Seddon (2003) notably emphasize that ionic liquids shift solvent chemistry “from a passive medium to an active engineering tool.”

Another important development in recent years is the rapidly expanding use of ionic liquids in biotechnology and biocatalysis. Their tunable hydrophobic and hydrophilic balance allows enzymes to maintain greater structural stability in microenvironments that resemble their native conditions. Consequently, many hydrolases, oxidoreductases, and isomerases have been reported to exhibit enhanced catalytic activity as well as increased thermal and chemical stability in appropriately selected ionic liquids. In particular, hydrophobic imidazolium-based ionic liquids have been shown to substantially improve reaction yields in enzyme immobilization applications, highlighting the emergence of ionic liquids as strategic media for bioengineering (Moniruzzaman & Goto, 2011).

Another prominent branch of ionic liquid research involves deep eutectic solvents (DESs). DESs are considered lower-cost, biocompatible, and environmentally sustainable alternatives to conventional ionic liquids. These systems, typically formed by combining hydrogen-bond acceptors such as choline chloride with hydrogen-bond donors including urea, glycerol, or organic acids, have attracted considerable interest due to their low toxicity and high chemical tunability. They are rapidly gaining momentum, particularly in biomass

conversion, natural product extraction, and green technology applications (Zhang et al., 2012). In this way, the field of ionic liquids has expanded into a broader domain of chemical design that extends beyond classical cation–anion solvent systems.

Collectively, these developments indicate that, while the interactions of ionic liquids with chemical and biological systems are not yet fully elucidated, these materials present substantial opportunities for both fundamental research and industrial applications. Across diverse fields, from advanced materials synthesis and pharmaceutical manufacturing to environmental remediation and enzyme engineering, ionic liquids are increasingly employed as highly flexible and tunable molecular media. In the subsequent section, the fundamental concepts, classification, physicochemical properties, and principal application areas of ionic liquids will be examined, with a specific focus on their roles in biotechnological processes.

Physico-chemical properties of ionic liquids

Ionic liquids, unlike conventional organic solvents, are tunable systems whose physical and chemical properties are directly dictated by the structures of their ion pairs. The sizes of the constituent cations and anions, their charge distributions, and the noncovalent interactions between them, such as hydrogen bonding, π–π interactions, and van der Waals forces, collectively determine the fundamental properties of these materials. As a result, parameters such as density, viscosity, polarity, conductivity, and thermal behavior can be modulated across a broad range simply by altering the ionic composition. This versatility has led to ionic liquids being described as “designable solvents” (Parvulescu & Hardacre, 2007).

One of the most striking characteristics of ionic liquids is their exceptionally low vapor pressure. The strong electrostatic forces between the constituent ions prevent these materials from readily transitioning into the gas phase. As a result, the environmental risks typically associated with volatile organic compounds are markedly reduced in ionic liquid systems. For instance, certain imidazolium-based ionic liquids exhibit vapor pressures as low as 10^{-9} atm at room temperature (Earle & Seddon, 2000). This property enables ionic liquids to remain stable even at elevated temperatures and to function as essentially loss-free solvents in processes such as distillation.

Viscosity is a critical physicochemical parameter that governs the behavior of ionic liquids in a wide range of applications. These materials are generally more viscous than conventional organic solvents because their ionic structures create

highly ordered networks of strong intermolecular interactions. Factors such as anion size, cation alkyl chain length, and water content have pronounced effects on viscosity. Large, charge-diffuse anions (such as $[NTf_2]^-$) tend to reduce viscosity, whereas small, high–charge-density anions (such as Cl^-) increase it (MacFarlane et al., 1999). Consequently, precise control of viscosity is essential, particularly in biocatalysis and electrochemical processes.

Polarity is another fundamental property arising from the complex structural organization of ionic liquids. These materials do not exhibit purely ionic behavior; dispersive and other noncovalent interactions also play significant roles. According to Reichardt's E_T(30) solvent polarity scale, certain imidazolium-based ionic liquids can display polarity levels comparable to those of methanol and ethanol. Moreover, hydrophobic microdomains may form within the same ionic liquid system (Reichardt, 2003). This dual character enables ionic liquids to dissolve both hydrophilic and hydrophobic compounds.

In terms of thermal stability, ionic liquids surpass many conventional organic solvents. Thermogravimetric analyses indicate that most ionic liquids begin to decompose only at temperatures exceeding 300 °C. However, their thermal robustness is highly dependent on the identity of the anion. For example, the PF_6^- anion undergoes rapid hydrolysis in the presence of moisture, whereas ionic liquids containing the $[NTf_2]^-$ anion exhibit exceptional resistance to both thermal degradation and hydrolytic processes (Ngo, LeCompte & McDermott, 2000). Consequently, careful anion selection is a crucial design consideration in chemical processes that require operation at elevated temperatures.

Ionic conductivity is another key physicochemical property of ionic liquids. The flexible structural network formed by oppositely charged ions enables efficient ion mobility even at room temperature. Although their conductivities are generally lower than those of conventional electrolytes due to their relatively high viscosities, imidazolium–$[NTf_2]^-$ systems in particular exhibit conductivities in the range of 10^{-3}–10^{-2} S/cm and are widely employed in battery, capacitor, and sensor technologies (Sasaki et al., 2014). Moreover, the temperature-dependent flow behavior of ionic liquids deviates from the classical Arrhenius relationship and instead resembles a glass-transition-like relaxation mechanism.

Taken collectively, these properties yield a highly flexible, design-oriented chemical environment that has no true counterpart among conventional solvents. The capacity to independently tune key physicochemical parameters positions ionic liquids as a next-generation solvent platform for diverse fields, including chemistry, biotechnology, and materials science.

Environmental effects of ionic liquids

Ionic liquids have become a major research focus in chemistry, toxicology, and environmental sciences, paralleling the rapid expansion of their technological applications. Although they initially gained attention as "environmentally friendly solvents" because of their negligible volatility, subsequent studies have demonstrated that environmental safety cannot be assessed solely on the basis of vapor pressure. Actual environmental behavior depends on multiple factors, including biodegradability, toxicity toward living organisms, environmental persistence, and biochemical transformation pathways. Accordingly, current literature highlights that the environmental profiles of ionic liquids are highly heterogeneous and vary substantially with ionic structure (Pham et al., 2010).

The toxic effects of these substances on living systems are closely associated with the structures of their cation and anion components. Imidazolium-derived cations are known to interact with lipids in the cell membrane, thereby disrupting membrane permeability and adversely affecting energy metabolism. Furthermore, the elongation of alkyl chains increases lipophilicity, consequently elevating toxicity (Jastorff et al., 2003). Concerning the anion, it has been reported that large anions, particularly those containing fluoride, can decompose in aqueous environments to form toxic byproducts. Therefore, the behavior of ionic liquids in biological systems must be evaluated not only through fundamental physical parameters such as solubility but also by considering specific molecular interactions.

Studies on environmental persistence have shown that certain classes of ionic liquids exhibit considerable resistance to biodegradation. Those containing aromatic-ring-bearing cations are particularly recalcitrant to microbial degradation and can accumulate in sediments and biological tissues. Both experimental and modeling studies indicate that tetraalkylammonium- and imidazolium-based ionic liquids, in particular, possess significant bioaccumulation potential in aquatic organisms due to their high $\log K_o w$ values (Couling et al., 2006). These findings suggest that the environmental chemistry of ionic liquids presents a far more complex risk profile than that of volatile organic solvents.

In ecotoxicity studies, model organisms such as algae, the small crustacean *Daphnia magna*, and zebrafish are frequently employed. General findings indicate that while ionic liquids exert moderate toxic effects on aquatic organisms, the degree of toxicity varies significantly with their ionic structure. For instance, choline-based ionic liquids are notable for their lower toxicity profiles, whereas imidazolium-based species with long alkyl chains are reported

to exhibit significantly higher toxicity (Pretti et al., 2009). Consequently, design strategies aimed at enhancing the environmental compatibility of ionic liquids are gaining considerable importance.

Another notable trend in current research is the development of "green ionic liquids" characterized by low toxicity and enhanced biodegradability. These new-generation systems developed utilizing cations derived from choline, amino acids, and natural compounds, and anions based on organic acids, exhibit both low inherent toxicity and faster degradation rates in aquatic environments (Gomes et al., 2013). Thus, ionic liquid chemistry is evolving from a performance-focused field toward a new design approach that also prioritizes environmental sustainability.

Industrial uses of ionic liquids

The industrial applications of ionic liquids have expanded considerably in recent years, extending far beyond their initial use as solvents. Their readily tunable physicochemical properties, negligible volatility, high thermal stability, and broad solvation capabilities make them attractive for a wide variety of industrial operations. Ionic-liquid-based systems are now employed at pilot and semi-industrial scales across multiple sectors, ranging from petroleum refining and energy-storage technologies to polymer processing and pharmaceutical manufacturing. This high level of versatility has led to their characterization in the literature as “designable ideal solvents” (Santos et al., 2014).

Petroleum refining represents one of the areas where ionic liquids were adopted early on. Specifically, processes known as "ionic liquid alkylation units" offer a safer alternative to the long-established hydrofluoric acid and sulfuric acid-catalyzed methods. Technologies like ISOALKYL, developed by companies such as Chevron and UOP, achieve high efficiency in isooctane production by utilizing imidazolium or quaternary ammonium-based ionic liquids instead of volatile mineral acids (Rogers & Voth, 2006). These systems not only reduce equipment corrosion but also facilitate easier catalyst recovery.

Gas separation, particularly CO_2 capture, has gained significant importance due to the growing impact of energy and climate policies. Task-specific ionic liquids, such as amine-functionalized variants or those with amine-ether structures, offer higher CO_2 selectivity and lower regeneration energy compared to traditional monoethanolamine solvents. Consequently, they are now being evaluated in pilot-scale carbon capture studies at power plants and natural gas treatment facilities (Bates et al., 2002). Furthermore, their non-volatile nature

enhances operational safety and has a positive impact on overall process economics.

Metal separation and hydrometallurgy are additional fields in which ionic liquids provide substantial advantages. Phosphonium- and carboxylate-derived ionic liquids have demonstrated high selectivity in the recovery of nickel, cobalt, rare earth elements, and precious metals from ores and electronic waste streams (Papaiconomou et al., 2012). These systems can reduce the need for traditional organic ligands, simplify separation stages, and contribute to more sustainable and resource-efficient mining practices.

Polymer and materials science is another sector in which the use of ionic liquids is rapidly expanding. In the solubilization of natural polymers such as cellulose and chitin, ionic liquids provide an environmentally safer alternative to conventional high-risk solvents such as NMMO or DMAc/LiCl. They also offer a more controlled reaction microenvironment during polymerization processes, facilitating the production of polymers with narrower molecular-weight distributions (Vitz et al., 2009). Consequently, ionic liquids are being increasingly adopted in textile manufacturing and biopolymer production.

Energy storage technologies constitute one of the fastest-growing sectors for ionic liquids. Ionic liquid electrolytes used in lithium-ion and sodium-ion batteries are preferred due to their inherent stability at elevated temperatures and their non-flammability. Ionic liquids utilized in supercapacitors, furthermore, offer superior energy and power densities, owing to their wide electrochemical window (Liu et al., 2010). Consequently, the interest in ionic liquid-based electrolytes is rapidly increasing in the design of next-generation batteries.

In pharmaceutical and fine chemical production, ionic liquids are primarily employed as solvents to enhance solubility, regulate crystallinity, and improve catalytic processes. Biocompatible ionic liquids, particularly those derived from choline or amino acids, provide environmentally benign alternatives for pharmaceutical synthesis and drug delivery applications (Stoimenovski et al., 2012). These systems not only mitigate the environmental impact of chemical processes but also contribute to enhanced product quality.

In summary, these diverse applications underscore the fundamental rationale for the enduring adoption of ionic liquids in industrial settings. Their tunable chemical structures enable the design of task-specific solvents tailored to distinct processes. Looking forward, the broader industrial implementation of ionic liquids is anticipated to prioritize classes that are environmentally benign, exhibit low toxicity, and are readily recyclable.

Biochemical Uses of Ionic Liquids

Ionic liquids have constituted a unique research avenue in biochemistry over the last 15–20 years, thereby establishing a new discipline that combines enzyme chemistry and solvent engineering. The solubility, stability, and activity of enzymes, proteins, nucleic acids, and various biomolecules can vary significantly depending on the cation-anion composition of the ionic liquid utilized. Therefore, ionic liquids serve an important role as tunable reaction media and phase separation systems, replacing classical buffer-organic solvent mixtures. Key advantages, such as increased enzyme activity, enhanced stability, and improved substrate solubility, can be achieved within these environments (Moniruzzaman & Goto, 2010).

The most intensive research in this domain focuses on enzyme catalysis and biocatalytic applications. Numerous studies have demonstrated that enzymes, including lipases, esterases, oxidoreductases, and hydrolases, can exhibit enhanced activity while maintaining their operational and thermal stability in compatible ionic liquids. For enzymes immobilized in forms such as Cross-Linked Enzyme Aggregates (CLEAs), on solid supports, or within ionic liquid-based phases, these solvents provide a dual function: they create a stabilizing microenvironment for the enzyme while simultaneously acting as an efficient solvent for the substrate. This unique combination results in significant improvements in conversion rates, enantioselectivity, and reusability compared to conventional organic solvents (Clark et al., 2018).

From the perspective of proteins and nucleic acids, certain ionic liquids are utilized as additives for the stabilization of biological macromolecules. Cholinium-, imidazolium-, or amino acid-based ionic liquids can preserve the native structures of proteins, reduce their propensity for aggregation, and even aid in the refolding of some misfolded proteins. Similarly, the behavior of nucleic acids, such as DNA and RNA, within these environments opens new possibilities in applications including gene delivery, biosensor design, and nucleic acid storage (Reslan & Kayser, 2018; Veríssimo et al., 2022).

Ionic liquids also provide highly effective solvent systems for the extraction, purification, and analytical determination of biomolecules. Aqueous two-phase systems formed with phosphonium- or imidazolium-based ionic liquids enable the selective partitioning of proteins, peptides, amino acids, and natural products into a single phase. This approach offers higher separation efficiency and reduces solvent consumption compared with traditional polymer–salt systems (Louros et al., 2010; Ventura et al., 2017). Moreover, ionic-liquid-based preconcentration strategies provide substantial advantages for detecting low-abundance

biomarkers when coupled with mass spectrometry or chromatographic techniques (Clark et al., 2018).

In bioanalytical applications, ionic-liquid-supported biosensors and enzyme electrodes represent an important and rapidly growing research area. The high ionic conductivity and wide electrochemical window of these liquids provide both an efficient conductive medium and a soft, biocompatible matrix for enzyme immobilization on electrode surfaces. Ionic-liquid-gel-based biosensors incorporating enzymes such as glucose oxidase, horseradish peroxidase (HRP), and choline oxidase have been reported to exhibit enhanced storage stability and improved measurement reproducibility (Khorsandi et al., 2022).

In my doctoral research at the Institute of Science, Çukurova University, glucoamylase (GA) and glucose isomerase (GI) were co-immobilized as combined cross-linked enzyme aggregates (combi-CLEAs). The catalytic performance of these combi-CLEAs in the hydrolysis of starch and subsequent fructose production was evaluated in various imidazolium-based ionic liquid media, including [Bmim][Cl], [Bmim][PF_6], and [Bmim][OAc]. The highest enzymatic activity was observed specifically in [Bmim][Cl], demonstrating that certain ionic liquids can provide a multifunctional environment that enhances both operational stability and catalytic efficiency in complex biocatalytic cascades (Akkoç, 2016).

Sustainability of ionic liquids

While properties such as low vapor pressure, reusability, and tunable chemical structures may initially appear compatible with Green Chemistry principles, current literature reveals that many ionic liquids exhibit significant toxicity and poor biodegradability (de Jesus & Maciel, 2023). This indicates that assessing solvent sustainability based solely on process performance is insufficient; a comprehensive life cycle assessment (LCA), encompassing production, use, and disposal stages, is essential.

Life cycle assessment (LCA) is central to this debate. LCA studies, particularly those focused on ionic liquids for CO_2 capture, indicate that while these solvents enhance process efficiency, the energy and raw material inputs during their synthesis phase often contribute substantially to the overall environmental burden (Cuéllar-Franca et al., 2021; Zhang et al., 2012). Furthermore, methodological reviews highlight that LCA remains a developing methodology in this context, with challenges such as data gaps, scaling uncertainties, and scenario variability frequently complicating the assessments of ionic liquids (Maciel et al., 2019; Baaqel et al., 2023).

A substance's toxicological profile and environmental fate are critical components of its overall sustainability. Extensive research has established that many conventional ionic liquids, particularly those based on imidazolium and pyridinium cations, exhibit significant toxicity to aquatic organisms, persist in soil and sediments, and demonstrate poor biodegradability (Pham et al., 2010). More recent studies systematically compare the ecotoxicity, biodegradability, and potential environmental exposure of different cation-anion pairs, underscoring the necessity of integrating both computational structure-activity relationship models (QSAR, QSTR) and experimental data in risk assessments (Magina et al., 2021; Flieger et al., 2020). Consequently, the objective in sustainable ionic liquid design is increasingly shifting toward systems that fulfill the triad of low toxicity, rapid degradation, and controlled environmental release.

In this context, in recent years, research has focused on the development of biocompatible and more easily degradable ionic liquids. This class, called " Bio -ILs," is based on cation-anion pairs formed from choline, amino acids, organic acids, and other biobased compounds. Studies published between 2020 and 2022 indicate that such ionic liquids are increasingly preferred in catalysis, biomedical applications, and separation processes (Tzani et al., 2022). However, it is also emphasized that for these new compounds to be truly "green," their biodegradability must be verified through standardized testing; otherwise, solvents considered environmentally friendly may re-create similar problems (Zampeti et al., 2025).

Recent discourse has reinforced the perspective that ionic liquids must be evaluated alongside deep eutectic solvents (DES) and other green solvent alternatives. Evidence indicates that while certain DES exhibit advantages in terms of lower production costs and superior biodegradability, ionic liquids often remain superior in thermal stability and process performance (Atashnezhad et al., 2024). Consequently, modern solvent design should move beyond a reliance on a single solution and instead adopt a portfolio-based approach, strategically selecting solvents to achieve an optimal balance between functionality, environmental toxicity, and life cycle impact. This holistic perspective is fundamental to developing next-generation sustainable solvents that simultaneously mitigate environmental footprints and enhance process efficiency (Macário, 2020; de Jesus & Maciel Filho, 2022).

Conclusion

Ionic liquids represent versatile chemical platforms that are expanding the long-standing boundaries of solvent science, owing to their molecularly engineerable structures. The ability to combine cations and anions in diverse

configurations permits the precise tuning of their physical and chemical properties. This tunability facilitates the design of novel solutions across numerous fields, including catalysis, separation processes, biocatalysis, polymer technology, and electrochemistry. Particularly in biotechnology, their capacity to enhance enzyme stability, increase reaction yields, and facilitate the single-phase execution of complex biochemical processes underscores their strategic importance for future applications.

While properties such as non-volatility, reusability, and high thermal stability initially suggested that ionic liquids would be environmentally advantageous, subsequent studies on their toxicity and biodegradability have underscored the necessity for a more comprehensive assessment. Many conventional ionic liquids have been shown to pose significant environmental risks; in particular, those with poor biodegradability can lead to considerable ecotoxic effects. Consequently, the design of sustainable solvents demands a holistic approach that considers production cost, energy consumption, environmental fate, and toxicological profile. The recent development of novel ionic liquids derived from biologically sourced cations and anions, which offer reduced toxicity and enhanced biodegradability, represents a promising advancement in this field.

In conclusion, ionic liquids offer significant flexibility and considerable development potential across both fundamental research and industrial applications. The primary focus in the coming years will be on designing high-performance, economically viable, and safe ionic liquids with a minimized environmental footprint. Achieving this objective will critically depend on interdisciplinary collaboration, the application of advanced characterization techniques, and the integration of a full lifecycle assessment into the design process. The successful convergence of these elements will undoubtedly solidify the position of ionic liquids as indispensable materials from both a scientific and technological standpoint.

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Chapter 5

THE ROLE OF LACCASE IN INDUSRIAL PROCESSES

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Laccase is an oxidoreductase enzyme containing copper that can oxidise a wide variety of phenolic and aromatic compounds (Figure 1). Its reaction mechanism involves reducing molecular oxygen to water through a four-electron transfer. Consequently, laccase is regarded as a 'green catalyst' that facilitates environmentally clean oxidation processes. The enzyme plays a critical role in the breakdown of complex polymers such as lignin and is an important component of the carbon cycle in ecosystems (Chauhan & Gothwal, 2022).

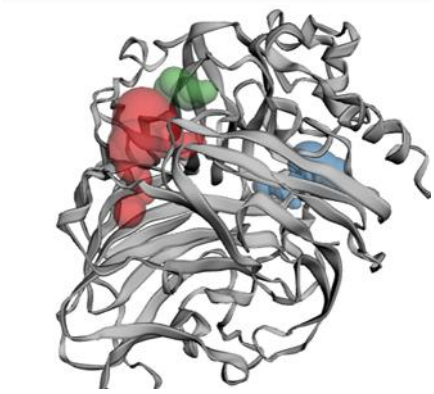

Figure 1. The image displays the active sites that contain four copper ions, which are essential for the laccase's catalytic activity (CASTp server DB ID:1V10)

Laccase copper centres:

The active site of laccase contains four copper ions and can be classified into three types:

T1 copper (type 1): This is the copper ion that accepts electrons from the substrate. It is usually located near the enzyme surface and is referred to as 'blue copper'. It binds directly to phenolic or aromatic compounds and carries out electron transfer.

T2 copper (type 2) and T3 copper (type 3):T2 and T3 together form a trinuclear cluster. This centre reduces molecular oxygen (O_2) to water (H_2O).It forms the second part of the electron transfer chain.

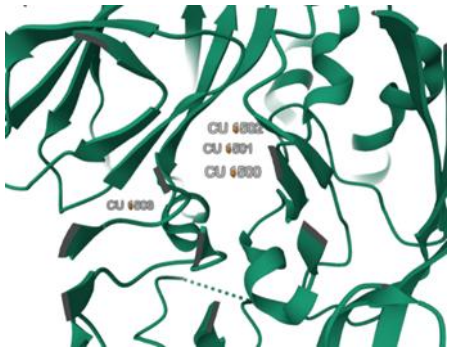

Figure 2. This is a representation of the copper metals in the laccase structure. (PDB ID: 1V10)

Electron Transfer Mechanism:

The substrate is oxidised by the T1 copper centre, with electrons being transferred from T1 to the T2/T3 centre. The T2/T3 copper cluster then reduces the molecular oxygen, converting it to water. During this cycle, the substrate is converted into either a phenoxyl radical or a quinone derivative.

Structural features of the active site

Substrate binding site: Hydrophobic and polar amino acid residues are found around the T1 copper atom (e.g. histidine, cysteine and aspartic acid). Oxygen binding region: The His and Asp residues are located near the T2/T3 triple copper cluster.

Important amino acids:

His: copper coordination and electron transfer

Cys provides the blue colour of T1 copper.

Asp and Glu stabilise electron transfer in the T2/T3 copper group.

Laccase's importance on an industrial scale is further enhanced by its high substrate diversity and low cofactor requirement. It is widely used in wastewater treatment due to its ability to oxidise recalcitrant pollutants. Laccase-based bioremediation strategies have been found to be highly effective in the biological removal of synthetic dyes, phenolic compounds, pesticide residues and pharmaceutical waste (Arregui et al., 2019; Shraddha et al., 2021).

Laccase is frequently used in biotechnological applications, particularly in fields such as bioenergy, biopolymer synthesis and biosensor design. It can enhance the efficiency of biofuel production by facilitating the pretreatment of

lignocellulosic biomass. Furthermore, laccase-mediated cross-linking reactions offer significant potential for developing biodegradable polymers and functional materials. The enzyme's high electron transfer capacity also enhances the performance of electrochemical biosensors (Mate & Alcalde, 2017; Upadhyay et al., 2023).

In the food, textile, and paper industries, laccase is primarily used for decolourisation, improving taste and aroma, bleaching, and stabilisation. It can improve the rheological properties of flour, reduce phenolic turbidity in fruit juices and perform environmentally friendly bleaching processes in textiles (Kudanga & Le Roes-Hill, 2021; Munk et al., 2020). This wide range of applications demonstrates that the enzyme is an important biocatalyst in environmental and industrial technologies.

In summary, laccase has a variety of applications.

1. Environmental and waste management:

- Treatment of paint and textile wastewater
- Removal of phenolic pollutants and pesticide residues
- Degradation of pharmaceutical waste
- Soil and water bioremediation

2. Biotechnology and industrial applications

- Pre-treatment for lignin modification and biofuel production
- Development of biopolymers and composite materials
- Design of biosensors and biofuel cells (electrochemical applications)

3. Food industry

- Fruit juice clarification
- Flavour and aroma improvement
- Improvement of flour rheological properties
- Reduction of undesirable phenolic-induced discolouration

4. Textile and paper industry

- Dye removal and environmentally friendly bleaching
- Bleaching of paper and cellulose

- Post-treatment colour stabilisation in textile products

5. Medical and Pharmaceutical Research

- Development of antimicrobial surfaces and coatings

- Biomaterial modification

- Conversions of phenolic drug derivatives (for research purposes)

6. Organic Synthetic Chemistry

- Oxidation of aromatic compounds

- Enzymatic cross-linking and polymerisation reactions

1. POLLUTANTS AND WASTEWATER

The industrialisation and urbanisation of societies has led to significant environmental problems caused by pollutants carrying 'micro-pollutants' and 'coloured/organic load' in surface water and wastewater systems. Phenolic compounds, pesticides, pharmaceuticals and paint residues originating from the paint and textile industries, agricultural activities and medical and pharmaceutical sources are commonly found in water systems. These pollutants are often difficult to break down biologically, can be ecotoxic and are not always adequately removed by conventional treatment systems (e.g. physical or chemical treatment methods may not always be sufficiently efficient). In this context, introducing biocatalysts such as laccase offers a clean, environmentally friendly and potentially highly efficient alternative (Chauhan & Gothwal, 2022; Peña-Gómez et al., 2022).

Treatment of dye and textile wastewater

Wastewater from the textile industry usually contains various stable, high-molecular-weight aromatic dyes. When discharged into water, these dyes cause colour and visible pollution, posing a risk of toxicity and harming ecological balance and living organisms. Laccase is recognised as an effective biocatalyst in the oxidative degradation of such complex aromatic dyes. Recent studies have shown that 'immobilised laccase' systems are highly efficient at reducing pollution, particularly from azo, reactive and acid dyes. For instance, the removal of up to 98% of Remazol Brilliant Blue R, an azo dye, has been achieved using laccase immobilised on nanocomposite carriers (Naghdi et al., 2023).

Furthermore, treating toxic dye solutions with laccase has been demonstrated to remove colour and reduce the ecotoxicity of decomposition products. This demonstrates that bioremediation is a meaningful strategy for improving both

appearance and water quality and ensuring the safety of ecosystems (Kübra & Yüksel, 2021).

Removal of phenolic contaminants and pesticide residues

Phenolic compounds and pesticide residues generated in industry, agriculture and domestic use can degrade water quality and have toxic effects on biota when they enter water systems. The laccase enzyme is ideal for bioremediating such pollutants as it can oxidise a wide range of phenolic and aromatic compounds. Numerous experimental studies have demonstrated the effectiveness of immobilised (carrier-bound) laccase systems in removing phenols, chlorophenols and aromatic pollutants from aqueous solutions (Arregui et al., 2019).

Additionally, recent studies on the removal of pesticides from aquatic environments are promising. For instance, Pizzul et al. (2022) reported that when 29 different pesticides (herbicides, insecticides and fungicides) were added individually or in mixtures to wastewater using laccase produced by Pleurotus dryinus in biosolids, nearly all high-priority pesticides and around 90% of others were removed.

These findings suggest that laccase could be a viable 'green bioremediation' tool for removing pesticide residues from water. However, careful evaluation of parameters such as the optimal pH level, the stability of the enzyme, the selection of the carrier and the toxicity of potential by-products is crucial in these systems (Zhou et al., 2023; Zhang et al., 2020).

Degradation of pharmaceutical waste and microcontaminants

Microcontaminants such as pharmaceuticals, antibiotics and hormones contaminating water sources pose a serious threat to the environment and human health today. Traditional wastewater treatment plants (WWTPs) are not always able to break down these components sufficiently. However, laccase enzymes derived from fungi or bacteria show promise in the oxidative conversion of pharmaceutical waste. Recent literature has demonstrated the effectiveness of laccase in breaking down various pharmaceuticals, including analgesics, antibiotics, and steroid hormones. Furthermore, it has been shown that laccase can provide total or partial mineralisation of certain components (Tran et al., 2023).

Additionally, unlike chemical oxidation processes, findings suggest that laccase-based biotransformations often produce lower-toxicity byproducts. For instance, oxidation products formed after chemical pretreatments may be

ecotoxic, but post-oxidation with laccase can effectively reduce this risk (Suda et al., 2023; Li et al., 2023).

The applicability of laccase in bioremediation: advantages and challenges

One of the most significant advantages of laccase is its ability to address a broad spectrum of substrates, supporting the 'single enzyme – multiple pollutants' approach. It can perform oxidative conversion on a variety of chemical groups, such as aromatic, phenolic and azo-based dyes, chlorophenols, pesticides and pharmaceuticals. Furthermore, the fact that the by-products released are water ('green catalyst') and that it produces a smaller environmental footprint than chemical processes is a significant advantage (Upadhyay et al., 2023; Peña-Gómez et al., 2022).

However, there are also some practical challenges: the stability, recovery and reusability of laccase in large-scale applications present significant obstacles. Consequently, recent studies have focused on 'immobilisation' — binding laccase to carriers such as nanocomposites, magnetic particles, or activated carbon — thereby enhancing enzyme stability, durability, and applicability to discharge waters (Naghdi et al., 2023; Arregui et al., 2019).

Additionally, a 'redox mediator' is sometimes necessary for oxidation processes involving laccase, which increases system complexity and requires careful mediator selection. The formation of by-products and their ecotoxicity are also important issues that need to be investigated (Peña-Gómez et al., 2022; Li et al., 2023).

Future perspectives and research recommendations

Studies and reviews published in recent years have demonstrated the high potential of laccase in areas such as wastewater treatment, pesticide removal and the degradation of pharmaceutical residues (Peña-Gómez et al., 2022; Tran et al., 2023). However, certain issues need to be addressed for this potential to be fully realised on an industrial scale:

Further optimisation of immobilised laccase systems is required, including improvements to properties such as carrier material, binding stability, number of reuses and pH/heat tolerance.

- Careful evaluation of mediator selection criteria, mediator waste and the toxicity of by-products in systems requiring redox mediators.

- Analysis of the persistence, ecotoxicity and biodegradability of post-treatment products in nature.

- Integration of laccase-based bioremediation processes into existing treatment facilities, including economic and operational feasibility analyses.

2. BİOTECHNOLOGY AND INDUSTRİAL APPLICATIONS

Lignin modification and pretreatment for biofuel production

Efficiently converting lignocellulosic biomass for biofuel production is complicated by the structural integrity and chemical stability of lignin. Laccase is an important oxidative enzyme which facilitates the modification of lignin and its partial depolymerisation by generating phenolic radicals. Recent studies have demonstrated that laccase increases the efficiency of enzymatic hydrolysis by loosening the structure of lignin, thereby facilitating the production of sugars suitable for biofuel production (Mate & Alcalde, 2021; Gupta et al., 2022). Laccase-based pretreatments are important for sustainable biofuel technologies as they require less energy and chemicals than chemical processes.

In this context, laccase–mediator systems (LMS) have proven effective, particularly in the modification of Kraft lignin and lignin fractions with a high aromatic content. The presence of mediators enables laccase to convert non-phenolic lignin components, thereby increasing biomass accessibility (Aracri & Vidal, 2021). These results suggest that laccase could become a key biocatalyst in the biofuel industry in the future.

Biopolymer and composite material development

The laccase has a wide range of applications in biopolymer synthesis thanks to its ability to catalyse the oxidative cross-linking of phenolic and aromatic monomers. Notably, laccase-mediated cross-linking of materials such as chitosan, cellulose, lignin and bio-based phenolic compounds offers significant advantages in developing environmentally friendly composite materials (Zerva et al., 2022). These biopolymers are being evaluated for use in biodegradable packaging, wound dressings, controlled release systems and low-toxicity structural composites.

Furthermore, it has been reported that the mechanical strength, thermal stability and water resistance of composites synthesised using laccase are enhanced. This is attributed to laccase's ability to form permanent covalent bonds within polymer matrices (Wang et al., 2023). Consequently, laccase has become a critical biocatalyst in developing environmentally friendly materials as an alternative to petroleum-derived plastics.

Biosensor and biofuel cell design (electrochemical applications)

Due to its four-electron redox mechanism that catalyses the reduction of oxygen in water, laccase is an ideal enzyme for electrochemical applications. This property enables its use as a cathode catalyst, particularly in oxygen reduction reaction (ORR)-based biofuel cells. Recent studies have demonstrated that immobilising laccase on nanomaterials such as carbon nanotubes, graphene oxide and metal-organic frameworks can provide high current density and stability (Ramírez-Núñez et al., 2023). Consequently, laccase-based biofuel cells are regarded as a cost-effective and biocompatible means of energy production.

In biosensor technologies, laccase is widely used for the electrochemical detection of phenolic pollutants, biomarker molecules, and pharmaceutical residues. Its broad substrate range, high redox activity and stability when immobilised enable sensitive and selective biosensor design (Gugliuzza et al., 2022). Consequently, laccase is at the forefront of emerging biosensor technologies for environmental monitoring, quality control, and clinical diagnostics.

3. FOOD INDUSTRY

Fruit juice clarification

The main components that cause turbidity in fruit juices are insoluble polysaccharides and phenolic compounds. The laccase enzyme in particular oxidises phenolic compounds, forming larger, precipitable polymeric structures that increase filtration efficiency and clarify the fruit juice. Recent studies have shown that using laccase alongside pectinase and other auxiliary enzymes can significantly improve the clarification of apple, grape and cranberry juices (Pan et al., 2021; Osma et al., 2020). Consequently, laccase is regarded as a key enzyme in 'cold process' clarification technologies, which do not require heat treatment and preserve the sensory properties of the product.

Flavour and aroma improvement

In the food industry, the quality of flavour and aroma is directly related to the oxidation of phenolic compounds. Laccase reduces bitterness and undesirable astringent flavours by enabling the controlled polymerisation of phenolic compounds. Studies have shown that using laccase increases aroma stability and slows down oxidative deterioration, particularly in products with a high phenolic content, such as wine, beer and coffee (Morales et al., 2022). Furthermore, laccase can extend product shelf life by providing oxidative protection to aroma components.

Improving the rheological properties of flour

Laccase oxidises ferulic acid and arabinoxylan-like phenolic compounds in wheat flour. This leads to the formation of cross-links that strengthen the dough structure. This reaction has a positive effect on the viscoelastic properties of the dough, increasing its gas retention capacity, strength, and volume (Reed et al., 2019). In modern baking applications, laccase is in demand as a natural alternative to chemical additives for improving dough. Indeed, in laccase-supported dough systems, the gluten matrix becomes more stable and the texture of the finished product is more consistent.

Reducing phenolic-ınduced undesirable browning

In the fruit and vegetable processing industry, browning caused by the oxidation of phenolic compounds negatively affects product quality and is a significant problem. Laccase can help to reduce these reactions by enabling the controlled polymerisation of phenolic compounds, thereby preserving product colour and extending shelf life (Kumar et al., 2023). Applications of laccase have been found to increase colour stability, particularly in products with a high phenolic content, such as apples, potatoes and avocados.

Industrial applicability and process integration

Using laccase in industrial food processes has several advantages, including low energy consumption, minimal chemical usage requirements and suitability for sustainable production practices. Furthermore, studies with immobilised laccase systems show that the enzyme can be used many times and reduces process costs (Fernandes et al., 2022). However, it is important to optimise limiting factors such as pH and temperature stability for the wider applicability of laccase-based technologies.

Future perspectives

In recent years, the development of new laccase formulations using nanotechnology, microencapsulation and biopolymer carriers has further increased their potential for use in the food industry. Given that laccase by-products are non-toxic and classified as natural ingredients in many products, enzyme-assisted processes are expected to become more widely adopted in the future (Zerva & Topakas, 2022). Laccase therefore holds an important place as a sustainable biocatalyst in processes such as clarification, colour control, aroma stabilisation, and texture improvement.

4. TEXTİLE AND PAPER INDUSTRY

Dye removal and environmentally friendly bleaching

Conventional treatment methods cannot adequately remove synthetic dyes used in the textile industry due to their high aromatic structure and chemical stability. Laccase is considered an environmentally friendly biocatalyst that provides colour removal by oxidising phenolic and aromatic dyes. In recent years, studies have focused on the degradation of azo, anthraquinone and reactive dyes with laccase (Chhabra et al., 2021). Due to its reduced use of chemical oxidants and lower energy requirements, laccase-assisted bleaching has gained an important place among sustainable textile treatment technologies.

Enzyme–mediator systems for paint degradation

The "laccase–mediator system" is frequently used to enable laccase to exhibit higher activity on certain synthetic paints. In this system, the mediator enhances the indirect oxidation power of laccase and enables the degradation of even non-phenolic dyes. Dye degradation with a mediator is particularly critical for the removal of high-molecular-weight reactive dyes (Bilal et al., 2022). This approach demonstrates that environmentally friendly bleaching processes can be a powerful alternative to chemical-based methods.

Bleaching of paper and cellulose

Chlorine-based chemicals are commonly used in the paper industry to remove lignin and bleach cellulose. However, these methods pose significant environmental risks. Laccase contributes to the bleaching of paper pulp by breaking down coloured chromophore groups through the oxidation of phenolic structures in lignin (Fillat et al., 2020). Laccase–mediator systems are less toxic and more environmentally compatible than chlorine-based chemicals, especially for bleaching kraft pulp.

Improving cellulose quality

It has been reported that laccase applications contribute to bleaching and improve the surface properties of cellulose fibres. Enzyme applications facilitate the breakdown of lignin residues, helping fibres to achieve a more homogeneous structure. This can increase paper strength, surface smoothness and print quality (Munk et al., 2021). Consequently, laccase is regarded as a component that enhances quality and is environmentally beneficial in paper production processes.

Post-processing colour stabilisation in textile products

One of the biggest problems after dyeing textiles is the subsequent decrease in colour stability and fading over time. Laccase can enhance colour stability by catalysing oxidative cross-linking reactions that strengthen the dye-fibre bond. Studies have shown that using laccase on textiles dyed with natural dyes significantly improves washing and light fastness values (Yang et al., 2022). This makes laccase an increasingly popular choice, particularly for eco-textile applications.

Industrial applicability and future perspectives

The most significant advantages of using laccase in the textile and paper industries are its low chemical consumption and energy requirements, and its compatibility with sustainable production models. Furthermore, immobilising laccase increases its stability and enables reuse (Fernandes et al., 2023). As nanomaterial-based immobilisation systems become more prevalent in the future, laccase is expected to find much broader industrial applications.

5. MEDİCAL AND PHARMACEUTİCAL RESEARCH

Antimicrobial surface and coating development

Laccase oxidises phenolic and aromatic compounds to form reactive radicals, which can inhibit biofilm formation and exhibit antimicrobial effects against microorganisms. In recent years, laccase-based antimicrobial coatings have been developed for use in medical and clinical environments. These coatings reduce the risk of infection by decreasing microbial colonisation, particularly on surgical instruments, implant surfaces and medical devices (Bilal et al., 2023). Laccase can also facilitate cross-linking reactions in biopolymer matrices to provide long-term antimicrobial activity.

Biomaterial modification

The use of laccase is increasing in order to enhance the mechanical, surface and biocompatibility properties of biomaterials. Laccase forms cross-links by oxidising polymers with phenolic end groups, thereby enhancing the durability and stability of biopolymers (Topakas & Zerva, 2022). For instance, modifying collagen, chitosan and cellulose-based biomaterials with laccase creates functional surfaces for tissue engineering and controlled release systems.

Conversion of phenolic drug derivatives

In pharmaceutical research, laccase is used as a model enzyme for the oxidative conversion of drug candidates with phenolic structures. This process

mimics the biotransformation of drug metabolites and enables the prediction of toxic by-products (Gupta et al., 2022). In particular, in vitro laccase reactions involving antioxidant, antibacterial and anti-inflammatory phenolic derivatives provide valuable insights into biotransformation pathways in drug development processes.

Antimicrobial and anti-biofilm applications

The ability of laccase-based coatings to inhibit biofilm formation is crucial for preventing hospital-acquired infections. Laccase attacks the cell wall of microorganisms by converting phenolic compounds into radicals, thereby preventing colonization (Fernandes et al., 2022). This approach provides an environmentally friendly, long-lasting antimicrobial solution that reduces the need for chemical antiseptics.

Stability and functionality of biomaterials

Laccase-mediated cross-linking enhances the mechanical strength and thermal stability of biomaterials, as well as improving surface hydrophobicity and biocompatibility. These properties are essential for tissue engineering, wound dressings and drug delivery systems (Zerva et al., 2022). Furthermore, the use of immobilised laccase systems for biomaterial modification can make the process more controlled and reusable.

Future perspectives and research recommendations

The potential of laccase in pharmaceutical and medical applications is growing, particularly with regard to antimicrobial coatings and biomaterial modification. In the future, its stability and efficacy can be improved using nanomaterial-based immobilisation techniques and mediator-supported systems (Mate & Alcalde, 2021). Additionally, research into the oxidative transformations of phenolic drug derivatives will yield valuable data for safety and efficacy evaluations in the development of pharmaceuticals.

6. ORGANİC SYNTHETİC CHEMİSTRY

Oxidation of aromatic compounds and the role of laccase

Laccase is a copper-containing oxidoreductase enzyme with the capacity to oxidise phenolic and aromatic compounds. This property provides a biocatalytic approach to the controlled oxidation of aromatic compounds in organic synthetic chemistry (Rodríguez Couto & Toca-Herrera, 2020). Oxidation with laccase requires less energy and has a smaller environmental impact than traditional chemical methods.

Substrate diversity and reactivity

Laccase is particularly effective in the oxidation of highly reactive compounds, such as polyphenols, aromatic amines and hydroxyphenols. Its broad substrate range enables its application to numerous aromatic derivatives in laboratory and industrial syntheses (Bilal et al., 2021). In this respect, laccase supports the 'one enzyme – many reactions' approach in synthetic chemistry.

Green chemistry approach

Using laccase is consistent with the principles of green chemistry in organic synthesis. Enzymatic oxidation allows reactions to occur without the need for toxic metal ions or potent chemical oxidants. This reduces the toxicity of by-products and minimises the environmental impact of processes (Bilal et al., 2021). Furthermore, laccase generally operates at room temperature and under neutral pH conditions, resulting in energy savings.

Enzymatic polymerisation mechanism

Laccase oxidises monomers with phenolic end groups to form radicals, which initiate cross-linking and polymerisation. This mechanism offers a biocatalytic advantage in the synthesis of biopolymers and synthetic polymers (Mate & Alcalde, 2021). The controlled formation of radicals enables the regulation of polymer structure and increases polymerisation efficiency.

Production of functional polymers and materials

Laccase-mediated polymerisation is used to produce functional polymers and composite materials from aromatic monomers. Properties such as electrical conductivity, mechanical strength and biocompatibility can be achieved by cross-linking polyphenolic monomers (Rodríguez-Couto, 2020). This highlights the importance of laccase in laboratory and industrial-scale applications.

Mediator use and reaction efficiency

The oxidation of certain aromatic compounds may be insufficient when using laccase alone. In these cases, laccase–mediator systems are employed. Mediators extend the enzyme's electron transfer capacity, enabling the oxidation of non-phenolic aromatic compounds (Bilal et al., 2022). This strategy increases the scope and efficiency of laccase in synthetic chemistry applications.

Industrial application potential

Laccase is considered a sustainable, low-cost biocatalyst for aromatic oxidation and polymerisation processes. It is particularly important as a green

alternative to synthetic processes in the chemical and materials industries (Mate & Alcalde, 2021). Intensive research is being conducted on immobilisation techniques and enzyme stabilisation for industrial-scale use.

Nanomaterial-based systems

Nanomaterial-based immobilisation systems enhance the stability and reusability of laccase. They enable more controlled polymerisation reactions and allow for highly efficient industrial applications (Rodríguez Couto & Toca-Herrera, 2020). Nanocomposite carriers can also minimise by-product formation by optimising the use of laccase mediators.

Research and development perspective

Laccase-based oxidation and polymerisation are becoming increasingly important in fundamental research and applied organic synthesis. New substrate designs, enzyme engineering and mediator combinations can achieve higher yields and more specific products (Bilal et al., 2022). This approach offers a critical biocatalytic solution, particularly in the production of sustainable materials and polymers.

Future perspectives

In future, the stability, catalytic activity and industrial applicability of laccase can be enhanced further through immobilisation techniques and protein engineering. Laccase-based organic synthesis provides a sustainable, energy-efficient and cost-effective platform for the oxidation and polymerisation of aromatic compounds (Mate & Alcalde, 2021; Rodriguez-Couto & Toca-Herrera, 2020). In this regard, laccase emerges as a pivotal biocatalyst in both academic research and green synthetic chemistry processes.

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Chapter 6

NANOBIOCATALYSTS ENHANCED WITH GREEN SYNTHESIS NANOPARTICLES: ENZYME STABILITY, REUSABILITY AND ENVIRONMENTAL APPLICATIONS

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1.Introduction

Initially considered the source of inventions essential to modern life, chemistry is now largely seen by many as the primary cause of environmental pollution threatening our planet. However, focusing solely on dangerous and harmful products should not overlook chemical outputs that simplify and enrich our lives, such as medical products, plastics, textiles, cosmetics, pesticides, liquid crystals, and artificial organs. Rapidly developing technology has brought with it several problems, including ozone depletion, flue gases, global warming, eutrophication, acidification, carcinogens threatening human health, ecotoxicity, depletion of fossil fuels, and excessive land and water use. While chemistry is the source of many problems facing humanity, such as heating, technology, energy, transportation, and lighting, the solutions are also hidden within the science of chemistry. However, when considered in terms of time, effort, and energy, eliminating the factors that create the problem is a far more effective method than solving the problem itself. The method that aims to eliminate the negative consequences caused by traditional chemical methods at their source is called green chemistry (Moir et al., 2025). Hence, nanobiotechnology, a discipline that combines functional properties of biological systems with nanoscale materials, comes into being. This domain explores interactions between biological macromolecules and nanostructures to fabricate newer biosystems that are more efficient, selective, and stable, according to Khafaga (2024). Integration of nanomaterials with biocatalytic molecules such as enzymes has led to various path-breaking innovations in basic sciences as well as industrial biotechnology.

Nanobiocatalysts are hybrid systems formed by immobilizing enzymes onto nanoparticles, resulting in enhanced catalytic performance. Enzyme immobilization enables the preservation of enzyme conformation, improves stability under operational conditions, allows repeated use, and increases reaction efficiency (Cavalcante et al., 2024).

Nanoparticles, owing to their high surface area-to-volume ratios, magnetic or optical properties, facile surface functionalization, and biocompatibility, have become superior support matrices for enzyme immobilization (Nadar & Rathod, 2018).

Conventional methods of synthesizing chemical nanoparticles can be very energy intensive and involve toxic chemicals. Therefore, scientists have increasingly turned to green methods of synthesizing nanoparticles. Green methods are environmentally benign and involve the use of plant extracts, algae, fungi, bacteria, and biologically active molecules in making nanoparticles with lower toxicity and higher biocompatibility (Iravani, 2011). Biological capping

ligands can easily attach to green nanoparticles without denaturing because these nanoparticles have a biological capping ligand on their surface.

In this case, nanobiocatalysts produced using green synthesis methods of nanoparticles have a variety of advantages, such as

- Improved enzyme stability,
- Maximized catalytic efficiency,
- Enhanced tolerance to temperature, pH, and organic solvents,
- Reusability over multiple reaction cycles,
- Substantial environmental benefits in wastewater treatment, bioremediation, and industrial processes.

In this chapter, a brief insight into the scientific principles of immobilization methods, working properties, and applications of nanobiocatalysts based on green synthesized nanoparticles will be presented.

2.Basic Principles Of Green Synthesis

Green synthesis is a strategy that focuses on minimizing environment-associated side effects in chemical synthesis methods, using less poisonous material, and developing products from environmentally sustainable materials. The green synthesis strategy is based on the12 principles of green chemistry established by Warner and Anastas in 1998. Some of these principles include waste prevention, using renewable resources, using non-toxic solvents, and improving energy efficiency (Anastas & Warner, 1998).

Green synthesis protocols contrast with conventional chemical protocols in terms of using biochemical reducing agents such as plant extracts, enzymes, and bacteria, and natural stabilizing agents (Osman et al., 2024). Such protocols make it safe in terms of environments and improve biocompatibility when synthesizing these metal nanoparticles (Khafaga, 2023).

Plant extracts possess adequate components such as polyphenols, flavonoids, alkaloids, and terpenoids to stabilize these metal nanoparticles and reduce metal ions (Fotiadou et al., 2021). Hence, green synthesis assists in encouraging the usage of natural reducing agents and eco-friendly solvents in biotech applications. Green synthesis reactions are based on reactions using room temperature or low temperatures to conserve energy. Energy consumption will thus be reduced, and greenhouse gas emissions will decline. Recently, studies have indicated TiO_2, ZnO, and Fe_3O_4 nanoparticles, which were synthesized using green chemistry, have immense potential in environmental achievements, especially in wastewater treatment and biosensors production (Valls-Chivas et al., 2023; Cavalcante et al., 2024; Osman et al., 2024 & Fahim et al., 2024).

Generally, using green synthesis methods, systems can be produced with capabilities for energy sustainability, biodegradability, and reduction in ecotoxicity. Hence, green synthesis methods represent a basis for production technology in terms of both environment and biotechnology. A graphical illustration of these 12 principles is presented below (Khafega, 2024).

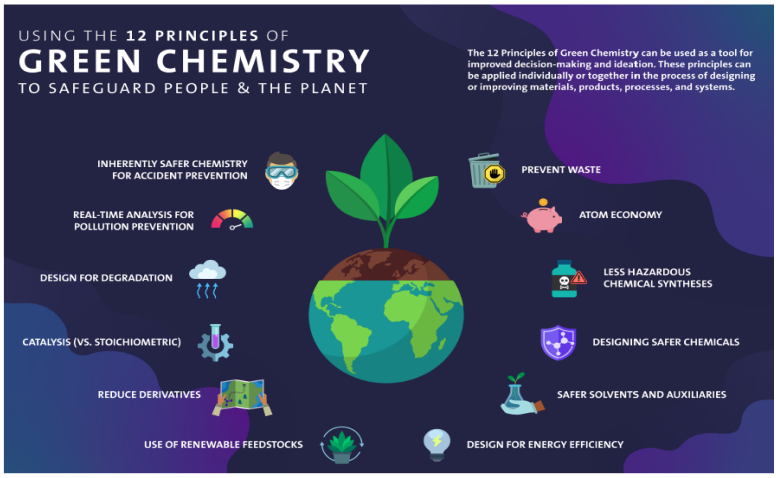

Figure 1. The 12 Principles of Green Chemistry (Anastas & Warner, 1998).

3.Methods for Nanoparticle Production via Green Synthesis

A wide variety of physical and chemical processes have been developed for the synthesis of nanoparticles (NPs), but these methods are expensive and require the use of toxic chemicals as reducing agents. Therefore, integrating them into green chemistry nanotechnologies is of paramount importance, especially when used in medical applications such as NP imaging, tissue repair, drug delivery, disinfection, and metal detection. For NP synthesis, plant extracts are easier to use than plant tissue. The biosynthesis of metal NPs consists of three main stages. First, reducing agents are selected; second, a solvent is selected; and third, stabilizing reagents are selected. Plant extracts generally stabilize and reduce the synthesis. Therefore, it is clear that plant extracts play a role in NP formation during synthesis. Flavonoids, coumarins, xanthones, anthraquinones, terpenoids and various other antioxidant compounds (reducing agents) can be found in many plant species. The processes required for the synthesis of NPs using plant extracts are easier and cheaper than methods based on microbial processes or the use of the whole plant (Armendariz et al., 2004; Beattie & Haverkamp, 2011; Iravani, 2011; Dhillon et al., 2012). Therefore, the synthesis of NPs using plant extracts has become more popular recently (Bar et al., 2009).

Green synthesis approaches represent an interdisciplinary field that combines biological, physical, and chemical methods used in the production of

environmentally friendly nanoparticles. These approaches involve the reduction of metal ions and the stabilization of nanoparticles using renewable biological materials (Osman et al., 2024). While traditional chemical syntheses utilize toxic reducing agents (e.g., $NaBH_4$ or hydrazine), green synthesis replaces these reagents with plant extracts, microorganisms, polysaccharides, and enzymes (Khafaga, 2023).

3.1. Synthesis via Plants (Phytochemicals)

As an environmentally friendly alternative to chemical and physical pre-treatment methods, it has the ability to synthesize NPs with different compositions using plants. Time-consuming and challenging processes such as cell culture preparation and microbial cell growth are more complex than plant extract. The method of preparing the plant extract can vary depending on the type and objectives of the study, and also greatly influences the nanoparticle morphology. However, the various compositions of plant extracts do not clearly determine the reason for the reduction of metal ions. Further research is needed to solve this problem. The benefits of plant-mediated biosynthesis are as follows (Iravani, 2011; Shankar et al., 2004).

Advantages include ease of use, safe operation, cost-effectiveness, simple one-step process, inclusion of various metabolites, elimination of detailed cell culture maintenance, rapid synthesis, environmental friendliness, obtaining more stable nanoparticles, better control over the size and shape of nanoparticles, and suitability for large-scale synthesis (Iravani, 2011; Shankar et al., 2004). For example, Fe_3O_4-ZnO nanoparticles synthesized using Psidium guajava leaf extract exhibit high surface area and biocompatibility for enzyme immobilization (Fahim et al., 2024).

In this method, particle morphology and size are directly affected by parameters such as pH, temperature, and extract concentration (Valls-Chivas et al., 2023).

3.2. Synthesis via Microorganisms

Microorganisms, including algae, bacteria, fungi, viruses, yeasts, and protozoa, play very important roles in the ecosystem. They act as decomposers in the recycling of nutrients. Microorganisms have the potential to synthesize nanoparticles intracellularly or extracellularly under ambient conditions without toxic chemicals and strict conditions. The properties of NPs synthesized in this way are quite similar to those of chemically synthesized NPs (Bäuerlein, 2003).

Bacteria have been the subject of much research due to their relatively simple modification for biological NP synthesis among other natural resources. Many

bacteria can produce nanoparticles with various morphologies from 0.1 nm to 1000 nm at room temperature and under mild culture conditions (Thakkar et al., 2010; Bai & Zhang, 2009).

Biosorption and biological reduction aided by proteins inside or outside bacterial cells are two examples of two known mechanisms. Particle size and morphology depend on various chemical and physical parameters such as the pH of the solution and incubation time, but the microbial biosynthesis of NPs is crucial. Bacteria have developed numerous detoxification methods to survive in hazardous metallic environments. Oxidation or reduction, complexation, precipitation and more are carried out by these mechanisms (Das & Marsili, 2011).

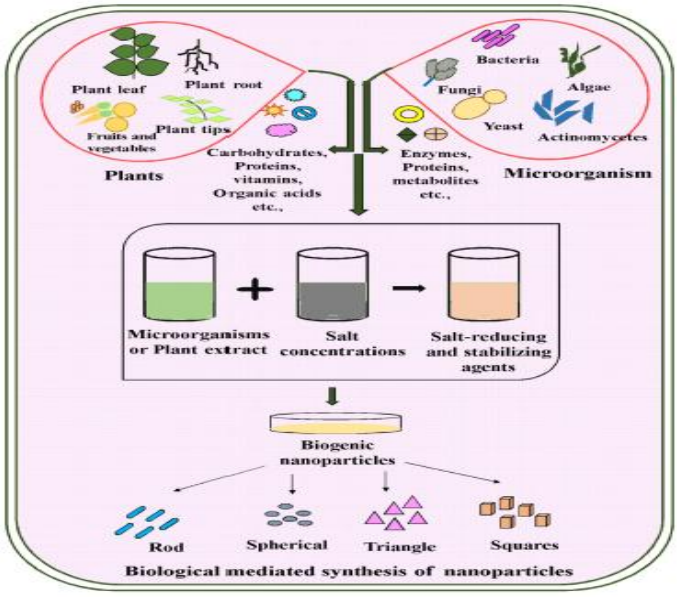

Figure 2. Synthesis of nanoparticles from biological materials (Karunakaran et al., 2023).

Membrane ion flow, mineral detoxification, and resistance to the most toxic heavy metals are based on the microbial capacity to produce NPs (Lengke et al., 2006).

Microbial synthesis enables the production of nanoparticles with high specificity, which prevents the emergence of harmful byproducts. For example, silver nanoparticles (AgNPs) synthesized using the fungus Fusarium oxysporum have yielded successful results in antimicrobial and catalytic applications (Osman et al., 2024).

3.3.Synthesis of Enzymatic and Biopolymer-Based Nanoparticles

The formation of nanoparticle is controlled by specific enzymes that catalyze the reduction of metal ions (Cavalcante et al., 2024). This technique is remarkable since it synthesizes high-purity and homogeneous nanoparticles. Moreover, the application of natural polymers, starch, cellulose, chitin, and chitosan as a reducing agent or coating agent makes it feasible to synthesize stable, nontoxic, and biodegradable nanoparticles (Fotiadou et al., 2021).

3.4. DNA and Protein-Based Templating Method

In this method, DNA, peptide, or protein scaffolds regulate the synthesis of NPs (Valls-Chivas et al., 2023). The synthesis of different kinds of materials termed "nanobiocatalysts" can be achieved by using enzyme synthesis and biocatalysis methods (Khafaga, 2024).

Nanostructures produced via this technique can be applied in Biosensors, Drug Delivery, and Environmental Detoxification because of their high specificity. Green synthesis methods not only counteract damage to the environment but can further aid in developing efficient and reusable nanostructures in biotech applications (Osman et al., 2024; Fahim et al., 2024).

4. Types and Surface Properties of Nanoparticles

Nanoparticles (NPs) exhibit size in the range of 1-100 nm, having different physicochemical properties due to a high surface-to-volume ratio, hence becoming highly valuable in applications such as biotechnology, catalysis, sensor technologies, and environmental ones. Generally, nanoparticles are classified according to the materials used in their synthesis and their size. Carbon-based NPs, organic NPs, and inorganic NPs are divided into three categories based on the materials used in their synthesis (Pal et al., 2011; Dhaka et al., 2023; Osman et al., 2024).

Some examples of carbon-based nanoparticles are fullers, carbon nanotubes, carbon nanofibers, graphene, and carbon black groups. Fullers can be conductive, semiconducting, and superconducting. Graphene is strong in terms of light absorption and electrical conductivity. Carbon nanotubes consist of elastic materials with high thermal and electrical conductivity. Carbon nanofibers have high electrical and thermal conductivity. Carbon black groups are materials resistant to ultraviolet radiation due to their high electrical conductivity (Çalişkan, 2020; Zahoor et al., 2021).

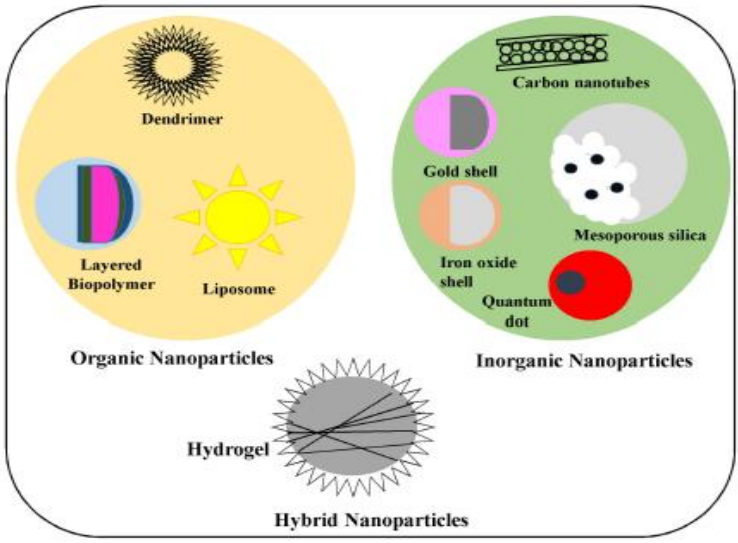

Figure 3. Schematic representation of different types of nanoparticles (Karunakaran et al., 2023).

Organic nanoparticles consist of molecules such as micelles, liposomes, and ferritin. These biodegradable nanoparticles have drug delivery capacity, are resistant to electromagnetic radiation, and do not have toxic properties (Yavuz & Yılmaz, 2021; Bulut & Ezgi, 2012; Wang et al., 2017). Non-carbon-based nanoparticles are inorganic nanoparticles. There are two categories between metal-based nanoparticles and metal oxide-based nanoparticles (Çalişkan, 2020).

Metal oxide nanoparticles can be used in imaging and pharmaceutical production. When synthesized with oxygen, metal oxide NPs such as aluminum oxide (Al_2O_3), silicon oxide (SiO_2), cerium oxide (CeO_2), zinc oxide (ZnO), iron oxide (Fe_2O_3), and titanium oxide (TiO_2) have higher reactivity and improved properties. Zinc oxide and titanium oxide are the most commonly used. These substances are used in many cosmetic products both to preserve the whiteness of foods and to protect the skin from sunlight (Osman et al., 2024; Yavuz & Yılmaz, 2021; Dhaka et al., 2023).

Metallic nanoparticles (MNPs) consist of metal particles of nanometer size. Almost any metal ion can be used in the fabrication of nanoparticles. Silver (Ag), copper (Cu), zinc (Zn), platinum (Pt), iron (Fe), aluminum (Al), lead (Pb), gold (Au), cobalt (Co) and cadmium (Cd) are commonly used metals in NP synthesis. These metals can be used to produce nanoparticles with various properties (Khafaga, 2023; Çalişkan, 2020).

Metal nanoparticles have a wide array of applications. Due to a high correlation existing among metallic nanomaterials and their physical, chemical,

and optical properties, these nanomaterials are used in different fields such as technology based on nanomaterials, medicine, drug, and chemistry delivery systems (Cavalcante et al., 2024; Lee & Jun, 2019).

Metal nanoparticles can easily be circulated in the body for a prolonged period because of their small size and surface modifications, and can successfully be used in cancer research targeting tumor cells. Targeted drug delivery systems often use metallic nanoparticles, especially gold and silver (Marangoz & Yavuz, 2020). Surface properties of nanoparticles are equally important in achieving stability and interaction with biological systems. Plant polyphenols, carboxylic, and hydroxyl functions are present on the surface of green-made nanoparticles. The presence of these functions enables binding with enzymes and protects them from oxidation degradation (Khafaga, 2023). Amino, carboxylic, thiol, or silane functions can be added to improve nanoparticle functionality. As a result, stability is attained in the nanoparticles, which can interact with target biological molecules (Cavalcante et al., 2024; Fotiadou et al., 2021).

Surface characteristics of NPs, especially the degree of hydrophobicity, charge distribution, roughness, and surface energy, can influence the immobilized enzyme activity and stability (Khafaga, 2024).

5.Enzyme Immobilization and Advantages

Biocatalysts, or enzymes, are substances that speed up the rate of chemical reactions in living things and can also offer 100% efficiency with no by-product formation in ideal conditions. Under the proper conditions, enzymes can be helpful in many aspects (Nelson & Cox, 2005).

Immobilization of enzymes in biotechnology refers to the practice of physically or chemically binding enzymes from solution onto a solid support material, which might help to enhance reusability, stability, and catalytic efficiency of the enzyme biocatalysts. This technique is imperative in various industrial biocatalysis applications related to economic and environmental sustainability (Telefoncu, 1997).

Numerous methods have emerged for immobilizing enzymes in recent years; these methods are more efficient and have a broad array of applications in interdisciplinary fields. Immobilized enzymes can also be used in a variety of application domains such as the food industry, metabolism of medication, production of bioethanol and biodiesel, production of antibiotics, production of biosensors, and bioremediation. Immobilized enzymes are widely used because they are affordable and eco-friendly (Zucca & Sanjust, 2014; Sirisha et al. 2016).

Another crucial step in enzyme immobilization is the choice of method. Of the various physical and chemical methods available, covalent bonding is the most preferred. Reasons for choosing covalent bonding include its ability to increase enzyme activity, the strength of the bonds, and the ease of enzyme-substrate interaction. Furthermore, cost, reliability, and the activity and stability of the enzyme are considered when selecting the immobilization method (Carlsson, Axén, & Unge, 1975).

5.1.Enzyme Immobilization Methods

Enzyme immobilization techniques, despite different classification methods, are generally divided into two categories:

5.1.1.Chemical Method

One of the chemical methods that can be used for enzyme immobilization is covalent bonding, which is a traditional method in immobilization (Nisha, Arun Karthick, & Gobi, 2012). Enzyme immobilization occurs by covalently binding to a support containing functional groups such as amino, hydroxyl or carboxyl (Alagöz, 2007). It generally occurs in aqueous environments and is difficult to achieve under moderate conditions (room temperature, neutral pH). Enzymes and support interact strongly, which can slow down enzyme activity (Kocatürk, 2008). The method has both advantages and disadvantages. Advantages of the immobilization method include strong bonds, very little enzyme leakage, and enzyme stability.

Covalent Bonding: The covalent bonding of the enzyme to the carrier occurs via the functional groups carried by the amino acids in the enzyme chain (Zaborsky, 1973). Enzyme immobilization by covalent bonding is one of the most widely used methods in enzyme immobilization because, due to the nature of the covalent bond, the bond between the enzyme molecule and the support is stable and therefore the enzyme can’t be easily separated from the support surface.

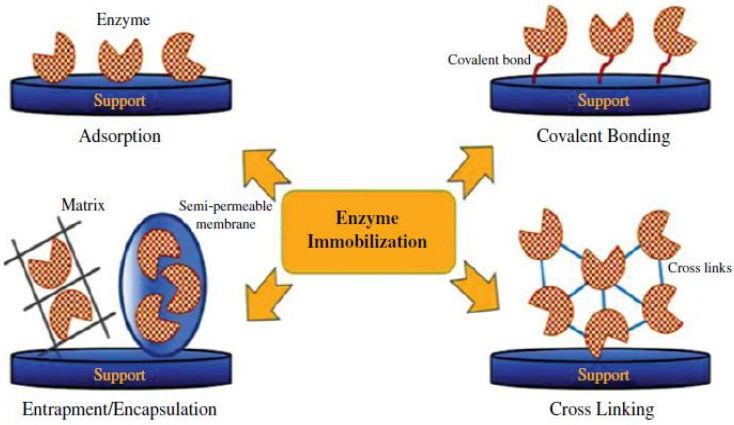

Figure 4. Enzyme immobilization methods (Nguyen & Kim, 2017).

Cross-linking: Small molecules with two or multiple functional groups form cross-links between enzyme molecules (Chibata, 1978). The degree of cross-linking and immobilization is highly dependent on the protein and reagent concentration, pH, and the enzyme to be immobilized. The most commonly used cross-linking reagents are glutaraldehyde, chloroformate and carbonyldiimidazole, heterocyclic halides, bioxirans, and epichlorohydrins. Glutaraldehyde is generally used as a cross-linking reagent because it is inexpensive and readily available commercially (Sheldon, 2007; Alptekin, 2009).

Ionic Bonding: This method is based on the principle of ionic binding of the enzyme to water-insoluble carriers that have the ability to exchange ions (Wang, 1993).

Enzyme immobilization by ionic binding is a simple method. However, it is difficult to find the conditions under which the enzyme binds strongly to the carrier and is highly active simultaneously (Bahulekar, Ayyangar, & Ponrathnam, 1991). Since ionic binding occurs under very mild conditions, It does not induce any alterations in the structure or active site of the enzyme. However, since the bond between the enzyme and the carrier is not as strong as a covalent bond, the enzyme may separate from the carrier (Telefoncu, 1997).

5.1.2.Physical Method

Adsorption: This is the oldest and simplest method used in enzyme immobilization. It is based on mixing a surfactant, water-insoluble adsorbent (such as activated carbon, porous glass, ash, silica gel, $CaCO_3$, starch, gluten) with the enzyme solution and washing away the excess enzyme (Nelson and Griffen, 1916; Klibanov, 1983).

Enzyme immobilization by adsorption is performed under mild conditions, eliminating the need for carrier activation, offering economic advantages, and generally preserving a large portion of enzyme activity after immobilization. Although the method appears simple, determining the optimization conditions is challenging. If the non-covalent interaction between the carrier and the enzyme is not sufficiently large, the enzyme can pass into the medium through desorption, thus causing product contamination (Telefoncu, 1997).

Immobilization by entrapment. This method involves holding the enzyme within a cage or polymer to prevent its entrainment. The difference from covalent bonding and cross-linking is that the enzyme is free in the solution. Enzyme leaching is minimized by entrapment, the stability of the enzyme is increased, but the binding of the substrate to the active site of the enzyme is limited. A suitable microenvironment for the enzyme is provided by the use of a capsule-forming material and appropriate pH and polarity (Spahn & Minteer, 2008).

The advantages of this method include its simplicity and efficiency, the absence of strong bonds between the enzyme and the polymer, and very little enzyme loss. Its disadvantages include leakage into the environment surrounding the enzyme, poor penetration into the surface gel matrix, and resistance to substance transfer to surfaces and species (Bayındırlı, 1995).

a) Microencapsulation: This method is based on the principle of encapsulating enzyme molecules within a semi-permeable membrane. The size of the microcapsules varies between 1 and 100 μm. The pore diameters of the semi-permeable membrane should be large enough to allow substrate molecules to enter the capsule and product molecules to exit (Chang, 1976).

b) Cage-type immobilization method: This method is based on the principle of retaining the enzyme in cages formed as a result of cross-linking, provided that the enzyme is also present in the environment where polymerization and cross-linking occur (Cremonesi & Angiuro, 1983).

This method has both advantages and disadvantages. The advantages of the method are that it is effortless to apply, it is a true physical method, and it requires a minimal amount of enzyme. Since neutral and water-insoluble carriers are used during the immobilization process, charged carriers are not required.

6.Environmental and Industrial Applications

Technologies for immobilizing enzymes and nanostructures produced via green synthesis have a huge potential for industrial efficiency and sustainability. These help enhance eco-friendly processes by decreasing the consumption of dangerous chemicals (Iravani, 2011; Ahmed et al., 2016). Furthermore, they

provide both economic and operational advantages by increasing catalytic efficiency and stability in sectors such as food, energy, biotechnology, and pharmaceuticals (Abuzeid, Julien, Zhu, & Hashem, 2023).

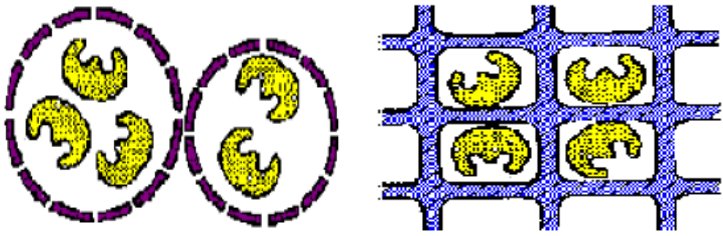

Figure 5. Microencapsulation and Cage-type immobilization method (Chang, 1976).

6.1.Environmental Applications

Application of green synthesized nanoparticles has immense potential in wastewater treatment and reduction in environmental pollution. More specifically, silver (Ag), iron oxide (Fe_3O_4), and titanium dioxide (TiO_2) nanoparticles are capable of degrading organics through photocatalysis (Sharma et al., 2019). Such nanoparticles can decompose pesticides, dyes, and phenolic compounds through the production of reactive oxygen species when exposed to light. In biotechnology applications for environmental conservation, the immobilization of enzymes plays an integral role in the formation of biocatalytic cleaning agents. Biodegradation of pharmaceutical residues, dyes, and phenolic compounds in wastewater can be done through the immobilization of enzymes like laccase or peroxidase enzymes (Zdarta et al., 2019). These systems enable longer-term catalytic performance by stabilizing enzymes and reducing the volume of generated waste. Nanobiocatalysts decrease energy consumption and give lower carbon footprints (Singh et al., 2020).

Therefore, immobilization technology and green synthesis are basic components in so-called “green chemistry” and “clean production” strategies.

6.2.Industrial Applications

On an industrial scale, green synthesis nanoparticles and immobilized enzyme systems have a wide range of applications. Today, green synthesis nanoparticles are widely used in biomedical fields and drug delivery, as catalysts, in agriculture, as antioxidants, as anticancer agents, as antibacterial agents and as antibiofilm agents, as well as in biofuel production, the food industry, pharmaceutical and biomedical applications, and the chemical industry (Castaneda et al., 2007; Chaloupka et al., 2010; Ganeshkumar et al., 2013; Prow et al., 2011; Maity et al., 2012; Nair et al., 2010; Niraimathi et al., 2013; Bhat et al., 2013; Inbakandan et al., 2013; Fahim et al., 2024).

Conclusions

The main challenges encountered in the widespread application of nanobiocatalysts developed through green synthesis approaches are standardization of synthesis processes, industrial-scale producibility, and insufficient determination of long-term ecotoxicological effects. In green synthesis methods carried out using plant extracts, microorganisms, or biopolymers, biological variability stands out as a significant factor limiting consistency between products.

In the future, metal-organic lattice structures, covalent-organic lattice structures, and hierarchical nanoflower morphologies have the potential to increase enzyme immobilization efficiency due to their high surface areas and structures rich in functional groups (Cavalcante et al., 2024). In particular, metal-organic lattice structures allow for the long-term maintenance of catalytic activity by preserving the conformational stability of enzymes (Nadar & Rathod, 2018).

However, a holistic assessment of the biodegradability, recoverability, and life cycle analysis of nanobiocatalysts to be used in environmental applications is necessary. Future research focusing on the design of non-toxic and economically sustainable nanocarrier systems compatible with green synthesis principles will be decisive in determining the effectiveness of nanobiocatalysts in industrial and environmental applications (Khafaga et al., 2024).

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Chapter 7

NATURE'S CHEMISTRY LABORATORY: MYSTERIOUS ORGANIC COMPOUNDS

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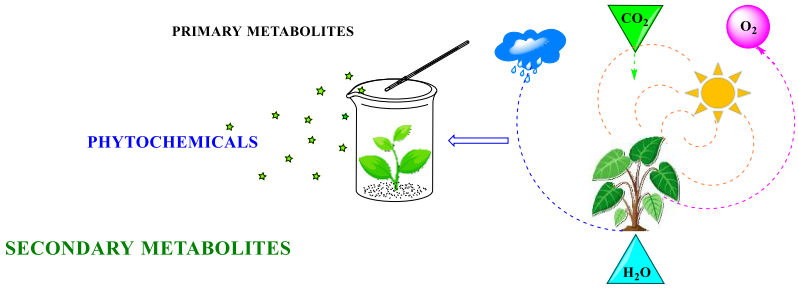


Plants, which play a crucial role in maintaining ecological balance and are among the most generous organisms in nature, sustain their growth, development, reproduction, and vital functions through metabolites produced as a result of biosynthetic processes. These natural compounds, known as plant chemicals, are converted through metabolic pathways into macronutrients such as nucleic acids, enzymes, carbohydrates, proteins, vitamins, and lipids, which are defined as primary metabolites (PMs) (Saha et al., 2024; Paudel et al., 2023). Secondary metabolites (SMs), which are not directly involved in the vital functions of plants, are biosynthetically derived from primary metabolites (PMs) and are characterized by their roles as micronutrients and by their contributions to taste, aroma, pigmentation, adaptation, and defense mechanisms against biotic and abiotic stresses.

Phytochemicals (PCs), produced through biochemical processes occurring in various plant parts such as fruits, leaves, seeds, roots, flowers, bark, and stems, comprise a wide array of compounds with substantial structural and functional diversity (Takemoto & Arita, 2009; Acikgoz & Yildiz, 2017). The use of plants, although one of the traditional methods of ancient civilisations for survival, such as nutrition, hunting and treatment, the isolation of morphine, the first natural biochemical, from opium poppy and the subsequent discovery of new PCs with the same chemical structure form the basis of important scientific studies. Grains, fruits, vegetables, nuts, spices, and green leafy plants consumed in the daily diet contain different types and amounts of SM (Elhussein, 2018).

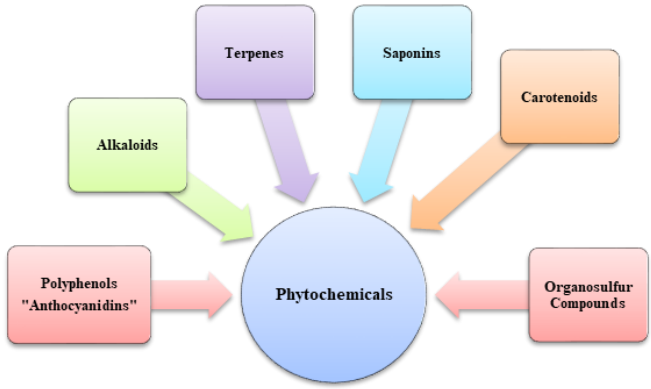


Figure 1. Classification of phytochemicals**.**

These bioactive chemical compounds, which can be used as functional food ingredients and nutritional supplements, have attracted attention in recent years, particularly in application areas such as medicine, pharmacy, cosmetics and agriculture.

The structural frameworks and diversity that confer medicinal and aromatic properties on PC require the classification of these compounds. Generally, PC are studied in six groups: Polyphenols, Alkaloids, Terpenes, Saponins, Carotenoids, and Organosulphur Compounds (Ibrahim et al., 2021).

1. Polyphenols

Polyphenols (PPs) are high-molecular-weight aromatic compounds characterized by a phenolic nucleus bearing one hydroxyl (-OH) group attached to an aromatic benzene ring or multiple substituted hydroxyl groups. Phenolic compounds, commonly found in cereals, fruits, and vegetables, represent the most abundant and widespread class of SMs. Variations in the concentration of PCs are influenced by factors such as soil composition, oxygen availability, climate conditions, and environmental factors where the plant is cultivated. Depending on their functional groups and the number of carbon atoms, PPs can be further classified into phenolic acids, stilbenes, lignans, coumarins, curcuminoids, anthocyanins, tannins, and flavonoids (Behl et al., 2021).

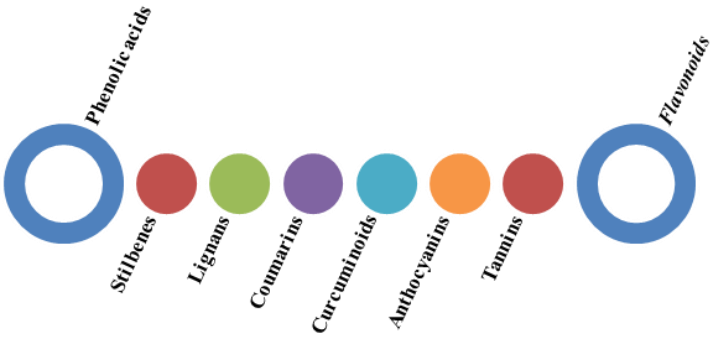
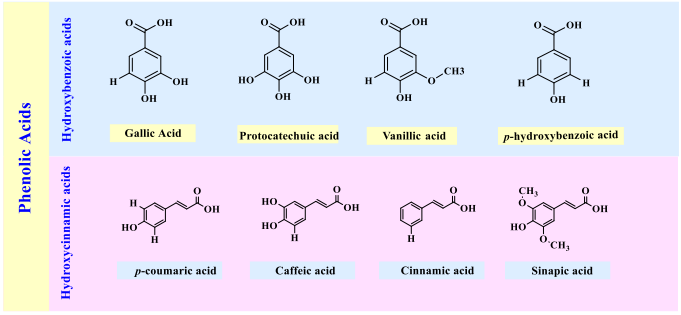


Figure 2. Classification of *Polyphenols.*

1.1. Phenolic Acids

Phenolic acids are derivatives of the phenol aromatic ring with a para-positioned carboxylic acid group. They are subdivided into different subgroups, including lignans, stilbenes, coumarins, curcuminoids, and quinones, with those bearing benzoic and cinnamic acid functional groups being the most prominent. Hydroxybenzoic acids and hydroxycinnamic acids are diversified through the substituents present on their structural backbone. Among the most well-known hydroxybenzoic acids are gallic acid, protocatechuic acid, vanillic acid, salicylic acid, and salicin (Kisiriko et al., 2021).

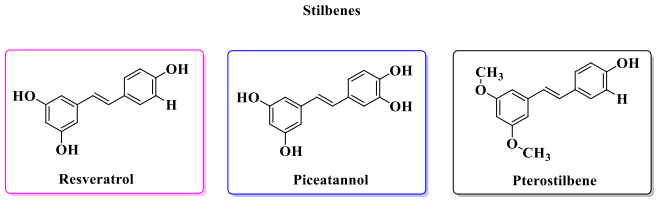


Some hydroxybenzoic acids can be obtained through the hydrolysis of tannins. Hydroxycinnamic acids, which are predominantly found in the bark and seeds of plants, include derivatives such as p-coumaric acid, caffeic acid, rosmarinic acid, ferulic acid, chlorogenic acid, caftaric acid, and sinapic acid. Notably, caffeic acid and ferulic acid are heat-sensitive compounds (King & Young, 1999).

1.2. Stilbenes

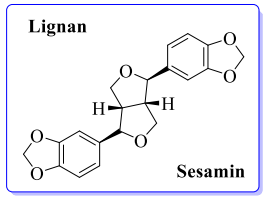
Stilbenes are PPs characterized by a 14-carbon 1,2-diphenylethylene backbone, in which two benzene rings are attached to an ethylene group.

Resveratrol, known as 3,4′,5-trihydroxystilbene, is a hydroxylated stilbene that exists in two diastereomeric forms, cis-(Z) and trans-(E) (Sepehri et al., 2024).



1.3. Lignans

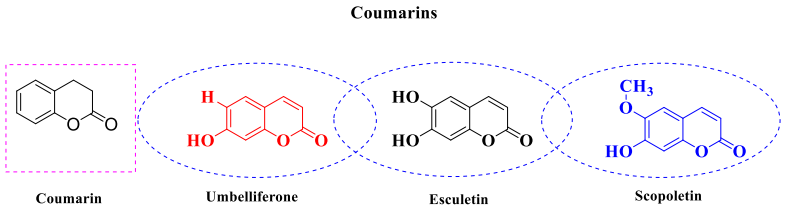
Lignans are molecules formed in the phenylpropanoid biosynthetic pathway through the linkage of two phenylpropane units via their β and β′ carbons, connected by ether, carbon, or lactone bonds. The presence of oxygen atoms, aromatic or aliphatic side chains, glycosidic or free forms, and the existence of the 8,8′-phenylpropanoid linkage in their structural backbone determine the abundance and diversity of lignans in natüre (Hassanein et al., 2024).



1.4. Coumarins

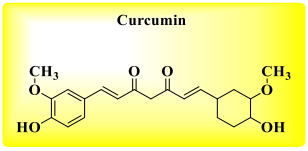
Coumarins are polar compounds in which a benzene ring is fused with a pyrone ring—a six-membered unsaturated ring containing an oxygen atom and a ketone group—commonly referred to as “2H-1-benzopyran-2-one” (Elhussein, 2018).

Modification of the simple coumarin molecule with various functional groups results in complex coumarins, which can exist in either free or glycosidic forms (Stringlis et al., 2019).



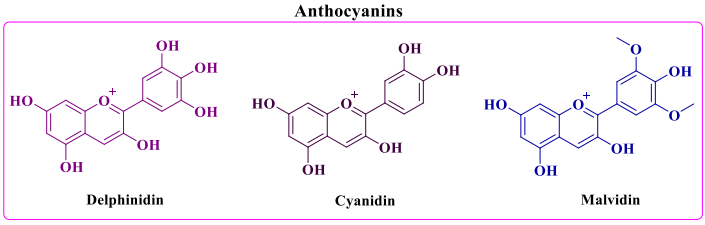
1.5. Curcuminoids

Curcumin is a compound characterized by a structural framework formed through the dimerization of 2-methoxyphenol rings via a keto–enol tautomeric unsaturated chain. Curcumin derivatives are produced by the removal of methoxy groups from the molecule (Kisiriko et al., 2021).



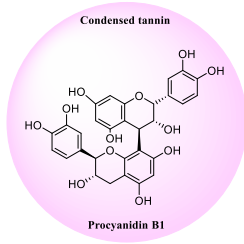
1.6. Anthocyanins

Anthocyanins are striking and visually appealing pigments responsible for the dark purple, deep blue, and various shades of red in plants. Commonly known anthocyanins include malvidin, peonidin, pelargonidin, and delphinidin. Notably, the cyanidin pigment is present at high concentrations in the seeds and skins of certain fruits (Behl et al., 2021).



1.7. Tannins

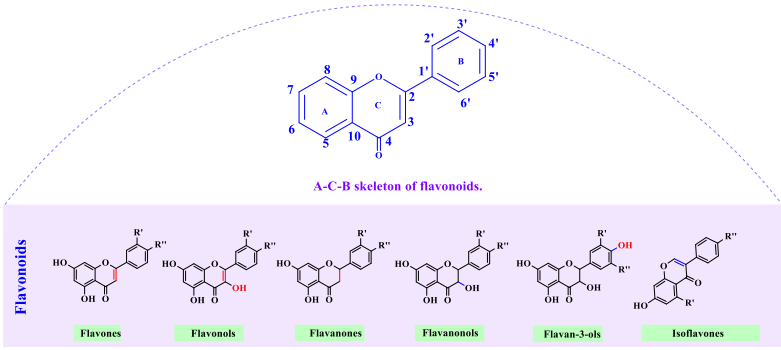
Tannins are protective macromolecules synthesized primarily in the external parts of plants in response to environmental stress and biological pathogens. Due to their hydrophobic and hydrophilic functional groups and their ability to form oligomers and polymers, tannins can readily bind to proteins at multiple sites. The astringent sensation experienced in the mouth from consuming certain plants and unripe fruits in the daily diet results from the formation of complex tannin compounds, in which SM units bind to salivary proteins (Behl et al., 2021; Agidew, 2022).



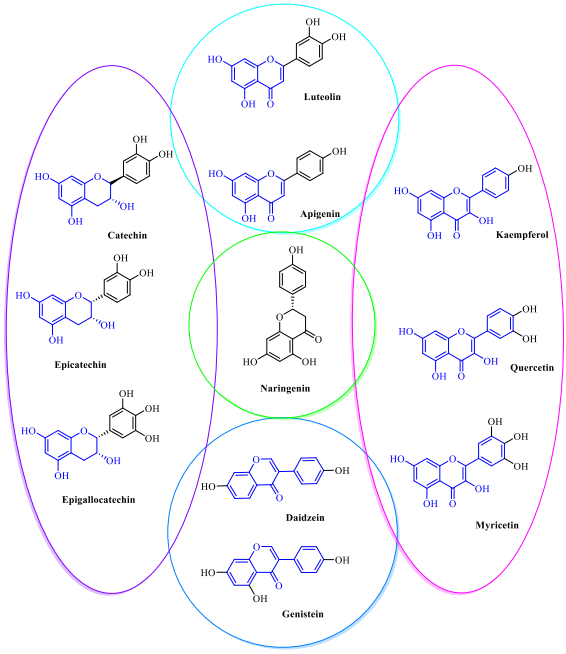
Based on their molecular structures, tannins are classified into two main types: hydrolyzable tannins and condensed tannins. Hydrolyzable tannins are primarily formed through esterification reactions of gallic acid or ellagic acid units. Condensed tannins, also known as proanthocyanidins, are polymerization products resulting from the linkage of flavonoid monomers, such as catechin and gallocatechin, in varying chain lengths (Behl et al., 2021; Agidew, 2022).

1.8. Flavonoids

Flavonoids, the most extensive and widespread class of PPs, possess a core structure containing γ-pyrone and benzoyl moieties that are often hydroxylated (Pawase et al., 2024). Due to their low molecular weight and hydrophilic nature, flavonoids can exist in glycosidic forms and are generally divided into two groups: colored molecules (anthocyanins) and colorless molecules (anthoxanthins) (Kalkan, 2007; Petric et al., 2015).

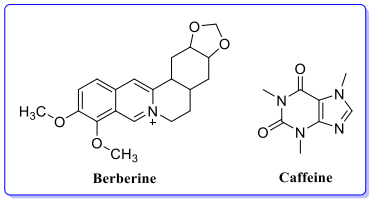


Various anthoxanthin molecules present in the daily diet, such as flavones (luteolin, apigenin), flavanols (catechin, epicatechin, epigallocatechin), flavonols (kaempferol, quercetin, myricetin), isoflavones (genistein, daidzein), and flavanones (naringenin, hesperidin), constitute the fundamental components of PC products. Structural analyses of PPs, particularly flavonoids, indicate that their diversity is determined by the positions of hydroxyl (-OH) groups on the aromatic rings, the presence of isomeric forms, and the existence of double bonds (Paudel et al., 2023; Behl et al., 2021; Pawase et al., 2024; Hui et al., 2013; Kaushik et al., 2021; Karabulut & Yemis, 2019).



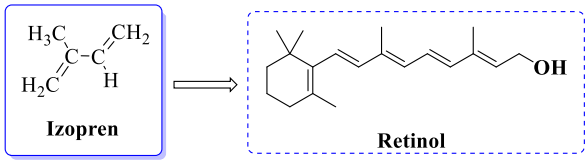
2. Alkaloids

Heterocyclic compounds containing one or more nitrogen atoms in the ring, including molecules with imidazole, quinoline, indole, pyrrolidine, pyridine, and piperidine scaffolds, represent a broad class of SMs (Paudel et al., 2023).



These PCs are characterized by low molecular weight, high stability, colorless crystalline structures at room temperature, and non-volatility. They are classified according to the amino acids used during their biosynthesis (Paudel et al., 2023; Behl et al., 2021; Agidew, 2022).

3. Terpenoids



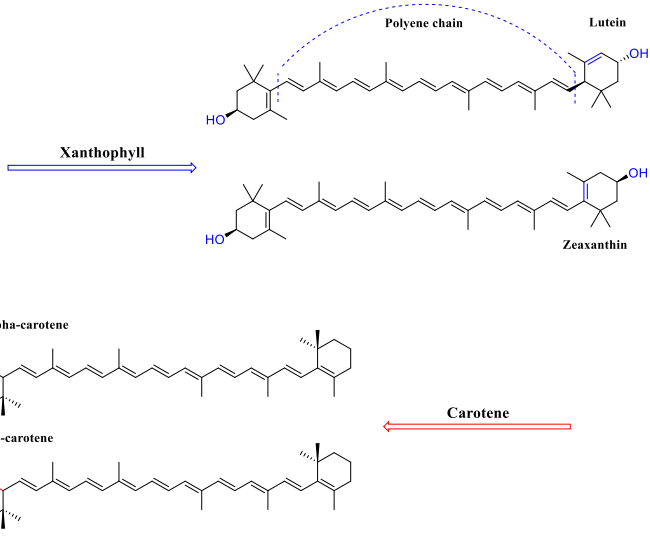
Isoprenoids are PC composed of isoprene units, each with the structural designation "2-methylbuta-1,3-diene." Variations in the length of the hydrocarbon chain and the number of isoprene units provide the basis for the classification of isoprenoids (Paudel et al., 2023; Elhussein, 2018; Behl et al., 2021; Agidew, 2022).

4. Saponins

Saponins are complex SMs composed of glycone and aglycone units. The aglycone portion, referred to as genin or sapogenin, does not contain sugar molecules within its hydrocarbon chain, whereas the glycone unit possesses a linear or branched structural backbone in which sugar moieties are linked via acetal bonds to hydroxyl groups. Triterpenoids, the most common members of saponins, are glycosides formed from three monoterpene units, each consisting of six isoprene units, with oxygen atoms linked through ether and ester bonds (Elhussein, 2018; El-Aziz et al., 2019).

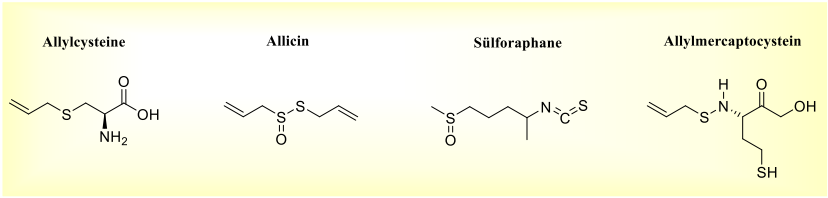
5. Carotenoids

Carotenoids are tetra-terpenoids composed of eight isoprene units with conjugated C=C bonds, making them the second most abundant pigments in plants after chlorophyll.



Based on their chemical structure, polarity, and pigmentation properties, carotenoids are divided into two main groups. The attachment of oxygen-containing functional groups, such as hydroxyl (-OH), carbonyl (C=O), carboxyl (COOH), or acetate (CH_3COO^-), to the head-to-tail linear polyene backbone produces polar, yellow-colored xanthophylls. In contrast, oxygen-free unsaturated hydrocarbon molecules form orange to deep yellow pigments known as carotenes (Pawase et al., 2024).

6. Organosülfür Compounds



Organosulfur compounds are aliphatic molecules containing one or more sulfur atoms within a linear carbon backbone and are derived from cysteine. Stable, odorless, and water-soluble organosulfur compounds, such as S-allyl mercaptocysteine and S-allyl cysteine, are biosynthetically converted into lipophilic, volatile, pungent, and highly reactive diallyl sulfides, including allicin (Fatima et al., 2021).

Conclusion

Plants, which have existed since the dawn of human history, are remarkable organisms that facilitate life due to their therapeutic properties and nutritional value. In addition to essential biological functions such as survival, growth and development, reproduction, and attraction, plants have evolved defense mechanisms that involve diverse biosynthetic processes. PCs, naturally synthesized in vegetables, fruits, and cereals, are considered alternative sources to synthetic organic compounds. The presence of various bioactive functional groups in their chemical structures necessitates their intake through the daily diet. Moreover, due to their therapeutic effects including anti-allergic, anti-thrombotic, anti-aging, antidiabetic, antioxidant, anti-neurodegenerative, anticarcinogenic, anti-inflammatory, and antiviral activities both the extraction of plant-derived compounds and the study of their synthetic derivatives have become prominent areas of research.

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Chapter 8

SCHIFF BASES: STRUCTURE, PROPERTIES AND APPLICATIONS

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INTRODUCTION

Schiff bases were first synthesized in 1864 by the German chemist Hugo Schiff through a condensation reaction between a primary amine and an aldehyde, and this class of compounds was subsequently named after its discoverer (Schiff, 1869). Structurally, Schiff bases are the nitrogen analogues of aldehydes and ketones, in which the oxygen atom of the carbonyl group is replaced by an azomethine (–C=N–) linkage. Owing to this characteristic structural feature, they are also commonly referred to as imines or azomethines (da Silva et al., 2010). Numerous studies have demonstrated that the presence of the azomethine moiety is closely associated with biological activity, as this functional group plays a critical role in a wide variety of natural, semi-synthetic, and synthetic compounds (Al-Sultani, 2015).

The chemical behavior of Schiff bases is largely governed by the lone electron pair on the nitrogen atom, which imparts basic character to these molecules and enables them to function as strong ligands. Schiff bases containing donor atoms such as nitrogen, oxygen, and sulfur exhibit a pronounced tendency to chelate metal ions, often forming stable four-, five-, or six-membered chelate rings (Cozzi, 2004). These metal complexes are of particular importance in coordination chemistry, where they contribute to the understanding of catalytic mechanisms and facilitate the development of novel metal-based functional materials. Moreover, due to their structural stability and intense coloration, Schiff base metal complexes have found widespread applications in the paint, pigment, and textile industries (Scovill et al., 1984). In addition to their industrial relevance, Schiff bases and their metal complexes display a broad range of biological activities, including antimicrobial, antifungal, anticancer, antiviral, antioxidant, and antitubercular effects. As a result, they are regarded as important molecular frameworks in drug design and biomedical research (Chen et al., 2003; Kumar, 2016; Roberts, 2017; Abu-Dief et al., 2019). Several studies have further shown that coordination with metal ions such as copper, iron, and cobalt can significantly enhance antimicrobial and antitumor activity, highlighting the role of metal chelation in improving biological efficacy (Chohan et al., 2002). Furthermore, the ability of certain Schiff bases to exhibit liquid-crystalline behavior, along with high thermal stability and electrical conductivity, has increased their significance in electronics, polymer science, and materials engineering (Cîrcu et al., 2012).

Today, Schiff bases continue to attract considerable attention in both academic research and industrial applications due to their simple and cost-effective synthesis, extensive structural tunability through functional group modification,

and high selectivity toward metal ions. Consequently, this versatile class of compounds is utilized across a wide range of fields, including pharmaceutical development, liquid crystal technology, dye chemistry, catalyst design, water purification systems, and bioinorganic modeling (Alfonso-Herrera et al., 2022; Shakir et al., 2009).

The History of the Schiff Bases

Schiff bases constitute an important group of organic compounds characterized by the presence of an azomethine (–C=N–) linkage. This class of compounds derives its name from Hugo Schiff, the chemist who first synthesized and systematically described them. Hugo (Ugo) Joseph Schiff, born on 26 April 1834 in Frankfurt am Main, Germany, was a distinguished organic chemist who received his education at the University of Göttingen under the supervision of Friedrich Wöhler. In 1863, Schiff relocated to Italy, where he continued his academic career and served as a faculty member in Florence for many years (Tidwell, 2008; Qin et al., 2013). The origin of Schiff base chemistry can be traced to 1864, when Schiff investigated the reactions between aromatic aldehydes and primary amines. During these studies, he reported that the condensation of these reactants led to the formation of a previously unknown class of organic compounds. These products were distinguished by the replacement of the carbonyl group with an azomethine bond, and Schiff initially referred to them as "organic bases." His early investigations laid the groundwork for understanding both the structural features and reaction patterns of Schiff bases, thereby introducing this compound class into the scientific literature (Fabbrizzi, 2020; Sangle, 2023).

In the years following Schiff's original publication, interest in Schiff bases expanded rapidly. Although early research focused primarily on their synthesis and role as reaction products, subsequent studies revealed their broader utility across multiple disciplines, including organic synthesis, coordination chemistry, and analytical chemistry. A major milestone occurred in the 1930s, when Schiff bases were recognized as effective ligands capable of forming stable metal complexes, a discovery that substantially extended their chemical and practical applications (Ambike, 2007). Beyond the discovery of Schiff bases, Hugo Schiff made several influential contributions to organic and analytical chemistry. He developed analytical methodologies related to amino acid determination and introduced well-known techniques such as the "Schiff test," which remains relevant in chemical analysis. The continued use of the term "Schiff bases" to describe compounds containing a carbon–nitrogen double bond reflects the lasting impact of Schiff's work and highlights the enduring significance of his scientific legacy (Qin et al., 2006; Qin et al., 2013).

Definition of Schiff Bases and Their Structural and General Properties

Schiff bases, also referred to as azomethines or imines in the chemical literature, are characterized by the presence of a carbon–nitrogen double bond (–C=N–) within their molecular structure. This class of compounds was first described in 1864 by the German chemist Hugo Schiff, after whom they are named (Schiff, 1869). Typically, Schiff bases are formed through the condensation of an aldehyde or ketone with a primary amine, accompanied by the elimination of water, making them nitrogen analogues of carbonyl compounds. The azomethine linkage generated in this reaction constitutes the key structural feature that largely dictates both the chemical reactivity and physical properties of these molecules (da Silva et al., 2010). Their general structural formula is represented as 'R–CH=N–R′', where the substituents 'R' and 'R′' can be either aliphatic or aromatic. Schiff bases derived from aromatic aldehydes usually exhibit enhanced stability due to resonance stabilization. The sp^2-hybridized carbon and nitrogen atoms in the azomethine bond confer planarity to the molecule, a feature that also facilitates the formation of metal chelates (Cozzi, 2004). The nonbonding electron pair on the azomethine nitrogen renders Schiff bases effective Lewis bases, enabling them to coordinate readily to metal centers and function as monodentate, bidentate, or polydentate ligands (Alfonso-Herrera et al., 2022). Their chemical behavior is predominantly governed by the electronic properties of the azomethine group, with the nitrogen atom acting as a high electron-density donor capable of forming coordination bonds with metals. Consequently, Schiff bases exhibit significant chelation tendencies, and the resulting chelate rings are commonly four-, five-, or six-membered, with five-membered rings typically providing the greatest stability (Moses et al., 1987; Xavier & Srividhya, 2014). Additionally, the azomethine bond can undergo both protonation and nucleophilic attack, rendering Schiff bases useful intermediates in a wide range of organic synthesis processes (da Silva et al., 2010).

The stability of these compounds is strongly influenced by the electronic and steric characteristics of their substituents. Schiff bases bearing aromatic groups are generally more resistant to hydrolysis, whereas aliphatic derivatives tend to be less stable. This increased stability arises from the delocalization of π-electrons in aromatic rings, which reinforces the double bond character of the azomethine linkage (Jia & Li, 2015; Karaca, 2016; Cordes & Jencks, 1962). Electron-withdrawing substituents tend to decrease azomethine stability, while electron-donating groups enhance it, leading to a wide variation in properties depending on substituent type and position. Functional groups other than the nitrogen atom may also participate in metal binding; for instance, Schiff bases derived from o-

hydroxybenzaldehyde typically act as O,N-bidentate ligands forming stable chelates (Cozzi, 2004).

These ligands are particularly significant in coordination chemistry due to their capacity to form complexes with transition metals. Polydentate Schiff bases incorporating N, O, and S donor atoms are additionally employed in biological contexts to model processes such as metal ion transport, storage, or inhibition (Vernekar & Sawant, 2023; Arun et al., 2024). Another notable feature of Schiff bases is the diversity of their electronic and optical properties. The electron density associated with the azomethine group influences UV–visible absorption, often resulting in colored compounds, which makes these derivatives valuable as pigments or intermediates in dye synthesis (Sıdır et al., 2015; Halmi & Tay, 2022). Furthermore, the permanent dipole moment of the azomethine bond can promote the alignment of molecules in mesophases, thereby enabling certain Schiff base derivatives to exhibit liquid crystalline behavior (Ahmed et al., 2020).

Synthesis and Reaction Properties of Schiff Bases

Schiff bases are organic compounds formed by the condensation of carbonyl compounds (aldehydes or ketones) with primary amines, containing a carbon-nitrogen double bond (-C=N-) in their structure. These compounds are important both for their chemical reactivity and their potential as ligands in coordination chemistry, due to their azomethine or imine group content (Al zoubi et al., 2018; Raczuk et al., 2022).

Synthesis Mechanism

The synthesis of Schiff bases is generally carried out via a condensation reaction. The reaction involves the following steps:

Interaction of the carbonyl group with an amine: An aldehyde or ketone reacts with a primary amine to form a carbinoamine (aminol), which is a temporary umbrella structure.

Water elimination: The carbinoamine loses water from the environment and converts to a Schiff base containing a C=N double bond.

Equilibrium and catalysis: This reaction can often be accelerated in acidic or basic environments; mildly acidic environments (e.g., a few drops of acetic acid) are generally preferred.

$$R_1CHO + R_2NH_2 \rightarrow R_1CH{=}NR_2 + H_2O$$

Here, R_1 represents the alkyl/aryl group of the aldehyde or ketone; R_2 represents the amino group(Cordes, & Jencks, 1962; Qin et al., 2013; Rezvan, et al., 2024).

Synthesis Methods

Several different methods are used in the synthesis of Schiff bases:

***1. Classic Condensation Method*:** An aldehyde or ketone is mixed with an amine, and the reaction is carried out, usually with gentle heating of the medium. Water formation occurs slowly as the reaction progresses, and the Schiff base precipitates**.** (Da Silva et al., 2011).

***2. Microwave-Assisted Synthesis*:** In modern laboratories, Schiff bases can be synthesized more quickly and with higher yield using microwave energy. This method stands out as an environmentally friendly and low-energy consumption alternative. (Manjare et al., 2022; Al-Hiyari et al., 2021).

***3. Solid-Phase Synthesis (Solidsupported*):** The reaction is carried out on silica gel or other solid supports, minimizing side reactions in the solution. This method is particularly suitable for obtaining high-purity products. (Sarkar, et al., 2025;).

Schiff bases are compounds formed as a result of the condensation (imination) reaction between aldehydes or ketones and primary amines, containing the characteristic azomethine (–C=N–) group. This reaction was first described by Hugo Schiff in 1864 and is now considered one of the fundamental reactions in both organic synthesis and coordination chemistry (Schiff, 1869). The synthesis mechanism proceeds in several sequential steps: the carbonyl carbon of the carbonyl compound undergoes a nucleophilic attack by the nitrogen of the primary amine; then the resulting carbinolamine intermediate loses water through dehydration and converts to the stable imine form. (Fabbrizzi, 2020; Raczuk, 2022). This process can be accelerated under acid or base catalysis; however, very strong acids can cause protonation of the amine, thereby inhibiting the reaction. For this reason, mildly acidic or neutral conditions are generally preferred. The yield of Schiff base syntheses is highly sensitive to the structure of the carbonyl compound and amine involved in the reaction. Aromatic aldehydes are more prone to imine formation due to resonance stabilization and yield more stable products, while ketones exhibit lower reactivity and the Schiff bases obtained from them are often less stable (Qin, et al., 2013). Aliphatic Schiff bases are more prone to hydrolysis and are less resistant to water compared to aromatic derivatives. For this reason, most literature reports Schiff base syntheses are typically carried out with benzaldehyde or its derivatives (da Silva et al., 2011).

Carbonyl compounds containing o-hydroxy or o-amino substituents stabilize Schiff base formation via intramolecular hydrogen bonding, yielding high yields in the synthesis of such structures. Salicylaldehyde derivatives, in particular, are

widely used in the synthesis of O,N-bidentate Schiff bases, which are highly suitable for forming metal complexes (Cozzi, 2004). Such structures are prone to both proton transfer reactions and keto–enol tautomerism; this is an important reaction feature that directly affects metal coordination behavior. The reaction properties of Schiff bases largely stem from the electronic structure of the azomethine group. The unshared electron pair on the nitrogen atom makes Schiff bases strong Lewis bases and provides a high coordination tendency towards metal ions. Therefore, Schiff bases readily form chelate complexes with various metal ions. During coordination, the azomethine bond can often be partially weakened or polarized by electron transfer to the metal center (Knittl, 2018). Schiff bazlarının reaksiyon davranışları, nükleofiller ve elektrofilik türlerle verdikleri reaksiyonlar açısından da çeşitlidir. Azometin bağı, hem protonlanmaya hem de indirgenmeye karşı duyarlıdır. Örneğin, Schiff bazları sodyum borohidrit gibi indirgen maddelerle reaksiyona girerek karşılık gelen aminlere dönüştürülebilir. Bu özellik özellikle organik sentezde koruyucu grup stratejilerinde ve çok adımlı sentezlerde önemlidir (da Silva et al., 2011). Additionally, although azomethine bonds exhibit weaker electrophilic character compared to carbonyl compounds, they can be attacked by certain nucleophiles, thereby enabling the formation of new C–C or C–X bonds. Another important reaction feature of Schiff bases is their tendency to undergo tautomeric transformations, depending on their structure. The tautomeric conversion commonly observed in O-hydroxy Schiff bases, hydroxymethyl–azomethine (OH–C=N–) ↔ oxime–enamine, affects the behavior of the compound in both acidic and basic environments and is particularly decisive in the formation process of metal complexes (Cozzi, 2004). Such tautomeric systems can also alter the optical and electronic properties of Schiff bases; therefore, they are significant in materials science. The formation of Schiff bases is a reversible reaction, and the presence of water reduces the stability of the product. Therefore, removing water from the reaction medium is a fundamental technique that increases yield in Schiff base syntheses. Methods such as azeotropic distillation using a Dean–Stark apparatus, the use of molecular sieves, or conducting the reaction in an inert atmosphere are commonly employed. (Godwin et al., 2024; In modern synthesis approaches, microwave-assisted synthesis and solvent-free methods offer significant advantages in the rapid and highly efficient preparation of Schiff bases (da Silva et al., 2010).

Electronic and Spectroscopic Properties of Schiff Bases

The electronic and spectroscopic characteristics of Schiff bases are strongly influenced by the electronic configuration of the azomethine (–C=N–) moiety, the nature and position of substituents, and the extent of molecular conjugation.

The partial double-bond character of the C=N linkage plays a central role in governing electronic transitions and the photophysical properties of these compounds. Furthermore, the inherent polarity of the azomethine bond renders Schiff bases electronically versatile, allowing them to function as both electron donors and acceptors in various chemical and photophysical processes. (da Silva et al., 2010).

Electronic Features

Schiff bases possess extensive electronic delocalisation, as they are typically synthesised from aromatic aldehyde or amine derivatives containing conjugated π-systems. The partial electrophilic character of the azomethine carbon and the nitrogen's unshared electron pair enable the molecule to participate in both nucleophilic and electrophilic reactions. This electronic structure also leads to tautomerism in Schiff bases. O-hydroxy Schiff bases, in particular, are prone to imine–enamine or keto–enol tautomerism, which significantly affects both the acid–base behaviour and electronic transitions of the compound. The electron density of the azomethine group can change during coordination with metal ions. The formation of metal complexes causes characteristic shifts in the UV–Vis spectrum by reducing the electron density of the C=N bond. Metal-nitrogen coordination generally leads to shifts in the wavelengths of π→π* and n→π* transitions; this is considered an electronic indicator of the high chelating ability of Schiff bases towards metal ions (Cozzi, 2004; Godwin et al., 2024; Sunju et al., 2022).

UV–Vis Spectroscopy

The UV–Vis spectra of Schiff bases typically contain two fundamental electronic transitions:

π→π* transitions: These originate from the conjugated system formed by the aromatic ring and the azomethine group and are often observed as strong absorption bands in the UV region.

n→π* transitions: These originate from the unshared electron pair of the azomethine nitrogen and, due to their lower energy, can cause absorption peaks closer to the visible region.

The energies of these transitions vary depending on the electron-withdrawing or electron-donating character of the substituents. Electron-donating groups (–OH, –OCH_3) increase conjugation, shifting π→π* transitions to lower energies, while electron-withdrawing groups (–NO_2, –CN) can affect the energy levels of the system in the opposite direction. When metal complexes form, new d–d transitions and ligand–metal charge transfer (LMCT) bands emerge due to

ligand–metal interactions. Consequently, Schiff base complexes often become characteristic coloured compounds, a property that enables their use in dye technology. (da Silva et al., 2010; Turan et al., 2025; Godwin et al., 2024)

Infrared Spectroscopy

One of the most distinctive spectroscopic features of Schiff bases is the C=N stretching vibration band observed in their IR spectra. It is typically seen as a strong band in the range of:

1640–1690 cm^{-1}.

This band is fundamental evidence of the formation of an azomethine bond. In the case of metal coordination, the C=N band typically shifts downwards by 10–30 cm^{-1}; this indicates that the azomethine nitrogen has formed a bond with the metal. Furthermore, in o-hydroxy Schiff bases, the shift or disappearance of the O–H stretching band is an important indicator that coordination with the metal has occurred via oxygen (da Silva et al., 2010; Cozzi, 2004).

Nuclear Magnetic Resonance Spectroscopy

^{1}H-NMR and ^{13}C-NMR are important tools for the structural confirmation of Schiff bases. In ^{1}H-NMR, the azomethine proton typically appears as a singlet in the range of:

8.0–9.0 ppm. This chemical shift arises from the deshielding effect of the proton attached to the azomethine carbon.

In ^{13}C-NMR, the C=N carbon typically gives a characteristic signal in the range of:

160–170 ppm. In O-hydroxy Schiff bases, broad bands may be observed if intramolecular hydrogen bonds exist between the OH proton and the azomethine proton. The formation of metal complexes causes shifts and intensity changes in the NMR signals (Berber, 2020; Perona et al., 2006).

Fluorescence and Photophysical Properties

Some Schiff bases exhibit strong fluorescent properties. The degree of conjugation in the aromatic system, the intramolecular proton transfer (ESIPT) mechanism, and tautomeric structures directly influence photophysical behaviour. Characteristic emission bands may appear in Schiff bases containing a hydroxyl group as a result of enol–keto phototautomerism. Therefore, Schiff bases have potential for use in optoelectronic applications, sensors, and luminescent materials. (Zhao et al., 2022).

The stability of the Schiff Bases

The stability of Schiff bases is governed by the interplay of several factors, including molecular structure, electronic characteristics, environmental conditions, and coordination with metal ions. The defining azomethine (C=N) linkage, which constitutes the core functional group of Schiff bases, exhibits lower polarity than carbonyl groups and is particularly sensitive to external conditions, especially the presence of water. Consequently, the stability of Schiff bases must be evaluated from both thermodynamic and kinetic perspectives (da Silva et al., 2011). One of the most critical processes influencing Schiff base stability is hydrolysis. Aliphatic Schiff bases are especially prone to hydrolytic degradation under acidic or basic conditions, readily reverting to their corresponding carbonyl compounds and amines due to insufficient conjugative stabilisation of the C=N bond. In contrast, aromatic Schiff bases demonstrate greater resistance to hydrolysis, as resonance delocalisation within the aromatic framework provides enhanced stabilisation of the imine functionality (Cordes & Jencks, 1962; Janz & Farrens, 2004). Because hydrolysis proceeds through re-formation of the carbinolamine intermediate followed by cleavage of the C–N bond in the presence of water, the removal of water generated during synthesis significantly improves both yield and long-term stability (da Silva et al., 2011). Substituent electronic effects also play a decisive role in determining the stability of Schiff bases. Electron-donating substituents such as –OH and –OCH_3 increase electron density around the imine linkage, thereby reinforcing its double-bond character and enhancing molecular stability. Conversely, electron-withdrawing groups including –NO_2, –Cl, and –CN increase the electrophilicity of the azomethine carbon, which may facilitate nucleophilic attack by water and accelerate hydrolytic processes. Additionally, extended π-conjugation within aromatic systems contributes significantly to imine stabilisation through electron delocalisation (Jhon, 2025; Bouznif et al., 2025).

Intramolecular hydrogen bonding represents another important stabilising factor. Schiff bases bearing ortho-hydroxy substituents, particularly salicylaldehyde-derived compounds, often exhibit strong O–H···N hydrogen bonds that promote cyclic stabilisation and enhance resistance to hydrolysis. These systems frequently undergo keto–enol tautomerism, which is typically stabilised in the enol form; the position of this equilibrium is strongly influenced by solvent polarity and temperature (Cozzi, 2004; Chatziefthimiou et al., 2006; Nazır et al., 2000). Complexation with metal ions further increases the stability of Schiff bases compared to their free ligand forms. Coordination to a metal centre alters the electron distribution within the azomethine bond, thereby reducing its susceptibility to chemical degradation. In particular, N,O-bidentate Schiff bases form five-membered chelate rings with metal ions, which exhibit high thermodynamic stability as a consequence of the chelate effect (Haddad,

2016). This enhanced stability significantly broadens the applicability of Schiff base complexes in catalysis, biological systems, and materials science.

Finally, Schiff bases especially those with aromatic or polymeric architectures often display high thermal stability. Polymeric Schiff base materials, characterised by extended conjugation and high cross-linking density, exhibit remarkable resistance to elevated temperatures. These properties render them particularly suitable for advanced applications such as liquid crystal technologies and high-performance functional materials (Nafee et al., 2019; Zhong et al., 2022).

Reactivity and Chemical Behaviour of Schiff Bases

Schiff bases exhibit pronounced chemical reactivity primarily due to the presence of the azomethine (–C=N–) functional group within their molecular framework. This group is generated through the condensation of a carbonyl compound with a primary amine and possesses an electronic structure that facilitates participation in both nucleophilic and electrophilic reactions. As a result of this dual reactivity, Schiff bases play a significant role in organic synthesis, coordination chemistry, and a wide range of biological applications (Mushtaq et al., 2024; Başak & Ersanlı, 2024; da Silva et al., 2010).

Acid–Base Behaviour and Lewis Basicity

The azomethine nitrogen atom exhibits basic character owing to the presence of a nonbonding electron pair. This feature enables Schiff bases to act as Lewis bases and readily coordinate with metal ions. During complex formation, the nitrogen atom donates its lone pair to vacant d-orbitals of metal centres, leading to the formation of thermodynamically stable coordination complexes. However, under strongly acidic conditions, protonation of the azomethine nitrogen reduces electron density around the C=N bond, thereby increasing susceptibility to hydrolytic cleavage (Cozzi, 2004).

Hydrolysis and Stability

Schiff bases are prone to hydrolysis, particularly in aqueous or acidic environments. Hydrolytic cleavage results in the regeneration of the parent aldehyde or ketone and the corresponding amine. Schiff bases derived from aromatic aldehydes generally display enhanced resistance to hydrolysis compared to their aliphatic counterparts, owing to resonance stabilisation of the imine bond. In addition, substituent effects play a crucial role in governing hydrolytic stability, as steric hindrance and electronic factors can significantly influence reaction rates (Cordes & Jencks, 1962; Gupta et al., 2025).

Nucleophilic and Electrophilic Reactions

The polarity and electron distribution of the azomethine linkage enable Schiff bases to participate in a variety of nucleophilic and electrophilic transformations. These include reactions commonly employed in organic synthesis, such as aldol-type condensations, Michael additions, and Diels–Alder cycloadditions. Moreover, the C=N double bond often serves as a reactive intermediate in subsequent reduction or oxidation processes, further broadening the synthetic utility of Schiff bases (da Silva et al., 2011; Cozzi, 2004).

Reduction and Oxidation Behaviour

Schiff bases are readily reduced using appropriate reducing agents. Treatment with sodium borohydride ($NaBH_4$) or lithium aluminium hydride ($LiAlH_4$) typically converts the imine functionality into primary or secondary amine derivatives. Such reduction processes are widely employed in pharmaceutical research and organic synthesis for the preparation of key intermediates. In addition, certain Schiff bases function as intermediates in oxidative pathways leading to structurally complex molecules (Sreenivasulu et al., 2004; Al Zoubi & Ko, 2016).

Coordination Chemistry and Chelate Formation

One of the most distinctive chemical properties of Schiff bases is their strong affinity for coordination with transition metal ions. Through the azomethine nitrogen and, in many cases, additional donor atoms Schiff bases form stable four-, five-, or six-membered chelate rings. These metal complexes are of considerable interest due to their catalytic efficiency, biological relevance, and industrial applicability (Abdallah et al., 2010).

Thermodynamic and Kinetic Properties

The reactivity of Schiff bases is closely related to thermodynamic and kinetic factors. Schiff bases containing aromatic or electron-withdrawing groups exhibit higher stability, while aliphatic or sterically hindered derivatives react more rapidly. Hydrolysis, reduction, and metal chelation reactions exhibit different rates and efficiencies depending on these factors (Da Silva et al., 2010; Cozzi, 2004).

The Biological Activities and Medical Applications of Schiff Bases

Schiff bases exhibit various biological activities due to the azomethine (-C=N) group present in their structure. This functional group contains a nitrogen atom that can easily donate an electron pair, enabling it to form complexes with metal ions and thus facilitate important interactions in biological systems.

Consequently, Schiff bases have a wide range of potential applications as antimicrobial, antifungal, antitumour, antioxidant, and antiviral agents (Ceyhan, 2011; Kumar, 2016).

Antimicrobial and Antibacterial Effects: Schiff bases and metal complexes exhibit activity against Gram-positive and Gram-negative bacteria. Complexes formed with Cu(II), Co(II) and Fe(III) in particular display higher antibacterial activity than the free ligand form. This activity stems from the inhibition of microbial metabolism by metal ions interacting with cell proteins and enzymes via ligands (Chohan et al., 2002; Adam, 2018).

Antifungal Effects: Schiff bases are also effective against fungal pathogens, exhibiting antifungal activity through mechanisms that disrupt cell wall synthesis and interfere with metabolic processes. Due to these properties, they can be used as fungicidal agents in agriculture and medicine (Ashraf, 2011).

Anticancer and Antitumour Activities: Schiff bases, and particularly transition metal complexes, exhibit cytotoxic effects on cancer cells. These complexes inhibit cell proliferation by interacting with DNA and proteins. It has been reported in the literature that Cu(II) and Fe(III) Schiff base complexes inhibit growth in tumour cells and, in some cases, reduce tumour development (Abd El-Halim, et al., 2018).

Antioxidant Effects: Certain derivatives of Schiff bases possess the capacity to neutralise free radicals. Due to these properties, they are considered potential agents in preventing cellular damage caused by oxidative stress (Ceyhan, 2011; Kumar, 2016).

Medical Applications: Schiff bases are used in classical drug development processes as enzyme inhibitors and biomolecular targeting agents via metal complexes. Gd(III)-based Schiff complexes also have important applications in diagnostic medicine, such as MRI contrast agents (Sankar, & Sharmila, 2023). Other medical applications include anti-tuberculosis agents and antiviral compounds (Redshaw, 2017; Zhang, 2019).

The biological activity of Schiff bases varies depending on the position of the azomethine group in their structure, the electronic and steric effects of the substituents, the complexation state, and the type of metal ion. Therefore, the synthesis and characterisation of different Schiff base derivatives is critical for enhancing their efficacy in biological applications (Chinnasamy, et al., 2025).

Industrial and Technological Applications of Schiff Bases

Schiff bases are organic compounds containing the azomethine (–C=N–) functional group, formed as a result of the condensation reaction between

carbonyl compounds and primary amines. Their ease of synthesis, structural diversity, and high coordination ability have made them an important molecular class not only in academic research but also in industrial and technological applications. Schiff bases and their metal complexes have a wide range of applications, from dyes and pigments to catalysts, and from electronic materials to sensors (Chaturvedi, et al., 2025). One of the most prominent areas of industrial applications is catalysis. Schiff base ligands form stable complexes with transition metals through coordination, and these complexes are used as effective catalysts in both homogeneous and heterogeneous catalytic reactions. In particular, their high thermal and moisture stability provides the potential to catalyse organic reactions with high yield and selectivity. This enables the use of Schiff base complexes in industrially critical processes such as organic synthesis, polymerisation, and oxidation reactions (Grupta, et al., 2009; Juyal, et al., 2023). Another important area of application is the paint and pigment industry. Schiff bases, and particularly transition metal complexes, are used in the dyeing of textiles, leather and other materials due to their strong and durable colour properties. Schiff base metal complexes containing azo groups are particularly suitable for producing long-lasting and lightfast pigments. These complexes can be used effectively not only in textile dyeing but also in inks, plastic surfaces and coating materials (Leonard, et al., 2024). Another industrial application of Schiff bases is in polymer and plastic additives. Polymers prepared from Schiff base monomers exhibit superior properties such as thermal stability and mechanical strength. These properties are particularly important in the development of high-performance materials and liquid crystal polymers (Fei, et al., 2023). Schiff bases are used as polymer stabilisers to slow down oxidative degradation and extend product life. In electronic and optoelectronic applications, Schiff bases are playing an increasingly important role. Schiff base ligands and their metals are used in advanced technology devices such as OLEDs (organic light-emitting diodes), photonic materials, sensors and solar cells. Schiff base metal complexes can exhibit photoluminescence and light emission properties due to the π-conjugation in their molecular structures and their interactions with metals, thereby playing an effective role in functions such as colour adjustment and efficiency enhancement in electronic devices (Kagatikar, & Sunil, 2021).

Additionally, Schiff bases have important applications in analytical chemistry and sensor technologies. In situations such as the selective detection of metal ions, monitoring pH changes, or analysing environmental pollutants, the optical or electroanalytical signals that change with the reactions of the azomethine functional group can be evaluated as sensors (Chaturvedi, et al., 2025).

The Use of Schiff Bases in Biochemistry

Schiff bases play an important role in biochemical systems and healthcare due to the azomethine (-C=N-) group present in their structure. This functional group acts both as a ligand and as an active component in biological activities due to the nitrogen atom possessing an electron pair. These properties make Schiff bases particularly valuable in antimicrobial, anticancer, antioxidant, and enzyme inhibitor applications (Curini, 2002; Chohan et al., 2002)

Antimicrobial and Antiviral Activity

Schiff bases demonstrate efficacy against bacterial and fungal infections. The electron density and structural conjugation of the nitrogen atom interact with proteins in the cell membrane, thereby inhibiting bacterial metabolism. Studies have observed that complexes formed by transition metals such as Cu(II), Co(II) and Ni(II) with Schiff bases exhibit high activity against Gram-positive and Gram-negative bacteria (Chohan et al., 2002; Kumar, 2016). Additionally, some Schiff bases have antiviral properties that inhibit viral protein function, thereby preventing viral replication (Redshaw, 2017).

Anticancer and Tumour Activity

Schiff bases and their metal complexes have the ability to inhibit the growth of cancer cells. Studies have shown that Cu(II) and Fe(III) complexes trigger apoptosis in tumour cells and reduce cellular proliferation. This effect is generally attributed to the inhibition of intracellular enzymes and interaction with DNA by complexes formed by metal ions of the azomethine group (Adam, 2018).

Antioxidant Properties

Schiff bases are used as antioxidants due to their ability to neutralise free radicals. The electron transfer capacity of the azomethine group protects biomolecules against oxidative stress and reduces cellular damage. This property highlights Schiff bases as potential therapeutic agents in cardiovascular and neurodegenerative diseases (Ceyhan, 2011).

Enzyme Inhibitors and Bio-inorganic Applications

Schiff bases and metal complexes can be used as enzyme inhibitors in biochemical systems. For example, copper complexes exhibit a similar structure to natural copper-containing enzymes (haemocyanin, tyrosinase) and can mimic enzymatic reactions. This property is of great importance in pharmacological applications, particularly in the modulation of enzymes that require metal ions (Sankar, & Sharmila, 2009)

Drug Delivery and Pharmaceutical Applications

The stable structure and biocompatibility of Schiff bases make them suitable for use as drug delivery systems. Metal complexes can enable the controlled release of drugs and enhance their pharmacological efficacy. Furthermore, some Schiff bases can easily diffuse through biological membranes to reach target cells (Roberts, 2017; Zhang, 2019).

CONCLUSION

Schiff bases represent a versatile class of organic compounds first identified in the mid-19th century and are defined by the presence of the azomethine (–C=N–) functional group formed through the condensation of carbonyl compounds and primary amines. Their structural flexibility and ease of synthesis make them valuable platforms for a wide range of chemical investigations. Due to the electronic characteristics of the azomethine linkage, Schiff bases exhibit distinct behaviour in UV–Vis, IR, and NMR spectroscopy, allowing detailed structural characterization that underpins studies of their electronic and photophysical properties. Spectroscopic analyses often reveal $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions in the UV–Vis spectra, characteristic IR C=N stretching bands, and diagnostic ^{1}H and ^{13}C NMR resonances, all of which contribute to understanding their reactivity and stability profiles. Schiff bases are generally less stable in the presence of water, undergoing hydrolysis, but stability is enhanced by conjugation and specific substituent effects. Chemically, they display broad reactivity, participating in nucleophilic and electrophilic transformations, as well as reduction and oxidation processes, and readily form chelate complexes with transition metals via Lewis base coordination, which can be described in terms of both kinetic and thermodynamic stability. The biological and medical relevance of Schiff bases and their metal complexes is well documented. These compounds have demonstrated antimicrobial, antibacterial, antifungal, antioxidant, and anticancer activities in numerous studies, with metal coordination often enhancing biological effects compared to the free ligands due to improved interaction with cellular targets and increased lipophilicity (Chinnasamy, et al., 2025). In analytical chemistry and sensor technology, the optical and electroanalytical responses arising from azomethine involvement in binding events allow Schiff bases to act as effective sensors for metal ions, pH changes, and environmental pollutants (Rao, et al., 2025). Additionally, many Schiff base derivatives exhibit potent binding to biological macromolecules, with applications ranging from enzyme inhibition to anticancer mechanisms mediated through DNA interactions (Chinnasamy, et al., 2025). Overall, the diverse synthesis methods, rich spectral features, and multifaceted reactivity of Schiff bases, coupled with their broad biological, analytical, and technological

applications, underscore their importance across disciplines. Continued research into structure–activity relationships, coordination chemistry, and polymeric and optoelectronic derivatives promises further advances in medicine, materials science, and environmental technology (Kumar, et al., 2023).

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