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A REVIEW OF ROLE OF X-RAY DIFFRACTION IN STRUCTURAL ELUCIDATION OF BIOLOGICAL MOLECULES

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ABSTRACT

The importance of structural elucidation via X-ray diffraction (XRD) in drug discovery cannot be overstated. It plays a pivotal role in advancing drug development and design by providing valuable insights into the three-dimensional structures of biological macromolecules, such as proteins and enzymes. This review will investigate the different XRD techniques employed in studying the structures of biological macromolecules and explore its role in drug discovery, including target identification, validation, rational drug design, and lead optimization. The review aims to systematically analyze the strengths and limitations of XRD in revealing the structural details of biological molecules. It will also highlight the contribution of XRD in understanding the structural and mechanism aspects of proteins and enzymes. In conclusion, it will identify the potential areas of growth and development in X-ray diffraction techniques for studying complex biological systems.

Keywords: X-ray diffraction (XRD), rational drug design, lead optimization, proteins, enzymes.

INTRODUCTION

X-ray diffraction (XRD) is a potent analytical technique pivotal for unveiling the atomic and molecular structure of crystalline materials. Rooted in the fundamental concept of wave-particle duality, XRD harnesses the power of X-rays, electromagnetic waves, to explore the hidden architectures within crystal lattices. It commences with the generation of a monochromatic X-ray beam, typically originating from an X-ray tube, which is then directed onto the crystalline sample under scrutiny. The sample may take the form of a single crystal or a powdered crystalline substance, meticulously prepared to be devoid of impurities and defects. When these X-rays collide with the electron clouds surrounding each atom within the crystal lattice, they scatter in diverse directions,

crafting a distinct diffraction pattern upon a detector screen. The recorded pattern, characterized by spots or peaks, holds critical angular and intensity data, forming the basis for subsequent analysis. By mathematically transforming the diffraction pattern, often via Fourier transformation, into an electron density map, precise details about atomic arrangements within the crystal emerge. This electron density map facilitates the determination of unit cell dimensions, symmetry, and the three-dimensional disposition of atoms. XRD finds wide-ranging utility across disciplines, spanning chemistry, materials science, biology, and geology, with profound applications in elucidating the structures of biological macromolecules, thereby advancing drug discovery and our comprehension of life's molecular foundations.

Historical evolution of XRD technology for studying biological molecules

The historical timeline of X-ray diffraction techniques in studying biological molecules reveals a remarkable journey of discovery **[1]** and innovation. Initiated by Wilhelm Conrad Roentgen's X-ray discovery in the early 20th century, Max von Laue's 1912 demonstration of X-ray diffraction by crystals **[2]** laid the groundwork for peering into their internal structures. In 1915, William Henry Bragg and William Lawrence Bragg formulated Bragg's law **[3]**, linking diffraction angles to crystal plane spacing. This ushered in the era of unraveling complex structures **[4]**, exemplified by their work on common minerals **[5]**. The 1930s saw proteins enter the scene, with James B. Sumner determining the urease enzyme's structure followed by Dorothy Crowfoot Hodgkin's breakthroughs in the 1930s and 1940s **[6]**.

The 1950s brought Rosalind Franklin's DNA X-ray images **[7]**, integral to the discovery of its double-helix structure. The 1960s witnessed automation and synchrotron radiation, enhancing crystallography's efficiency and data quality **[8, 9]**. By the 1980s, crystallography expanded to nucleic acids and carbohydrate **[10]**, while the Protein Data Bank (PDB) emerged **[11-13]**.

Late 20th-century advances yielded electron density maps **[14]**, culminating in Herbert Hauptman and Jerome Karle's Nobel Prize for their mathematical contributions **[15-17]**. The 21st century ushered dynamic insights, capturing transient interactions **[18, 19]** and ligand binding **[20-22]**, bolstered by X-ray free-electron lasers (XFELs) **[23-26]** for non-crystalline samples and rapid reactions. This journey integrates ingenuity, technology, and determination, revolutionizing our understanding of biology. From crystal structures to complex biomolecules, these developments have shaped fields like drug discovery and structural biology. Table 1 shows a brief list of research conducted on the role of XRD crystallography in structure elucidation of biological molecules.

X-ray diffraction techniques employed in the study of structural biology

The investigation into various X-ray diffraction techniques utilized for studying the structures of biological macromolecules highlights the versatility and adaptability of this analytical approach.

Table 1: Summary of recent research articles on role of XRD in structural biology in chronological order

Year	Research Studies	References
2010	• Direct structure elucidation by powder X-ray diffraction of a metal–organic framework material prepared by solvent-free grinding. • High-resolution x-ray diffraction microscopy of specifically labeled yeast cells.	**[27, 28]**
2011	• Femtosecond X-ray protein nano crystallography • Mutual adaptation of a membrane protein and its lipid bilayer during conformational changes • Physiochemical characterization and release rate studies of solid dispersions of Ketoconazole with Pluronic F127 and PVP K-30 • Coformer Selection in Pharmaceutical Cocrystal Development: a Case Study of a Meloxicam Aspirin Cocrystal That Exhibits Enhanced Solubility and Pharmacokinetics • Structural Mechanism of the Pan-BCR-ABL Inhibitor Ponatinib (AP24534): Lessons for Overcoming Kinase Inhibitor Resistance	**[29-33]**
2012	• Characterization bone nanocomposites by X-ray Diffraction • Lanthanide Complexes of Macrocyclic Polyoxovanadates by VO4 Units: Synthesis, Characterization, and Structure Elucidation by X-ray Crystallography and EXAFS Spectroscopy • Structural elucidation of synthetic calcium silicates • The molecular architecture of the 26S proteasome holocomplex was determined by an integrative approach. • Development and characterization of a novel drug nanocarrier for oral delivery, based on self-assembled β-casein micelles. • Novel Furosemide Cocrystals and Selection of High Solubility Drug Forms • Structure Based Drug Design of Angiotensin-I Converting Enzyme Inhibitors	**[34-40]**
2013	• Demonstrated power of in situ XRD by elucidating the structure of Li4Ti5O12 upon addition of sodium • Crystal structure and functional mechanism of a human antimicrobial membrane channel • Antimicrobial Activity of CaO Nanoparticles • On the structural basis and design guidelines for type II topoisomerase-targeting anticancer drugs • A three-stage biophysical screening cascade for fragment-based drug discovery • The chemical and structural analysis of graphene oxide with different degrees of oxidation • Evaluation of Wound Healing Potential of β-Chitin Hydrogel/Nano Zinc Oxide Composite Bandage	**[41-47]**

2014	• Mechanism of action and epitopes of Clostridium difficile toxin B-neutralizing antibody bezlotoxumab revealed by X-ray crystallography. • Solidified Self-Nanoemulsifying Formulation for Oral Delivery of Combinatorial Therapeutic Regimen: Part I. Formulation Development, Statistical Optimization, and In Vitro Characterization • Coformer Selection in Pharmaceutical Cocrystal Development: a Case Study of a Meloxicam Aspirin Cocrystal That Exhibits Enhanced Solubility and Pharmacokinetics • Structural analysis of atovaquone-inhibited cytochrome bc1 complex reveals the molecular basis of antimalarial drug action. • Estimation of lattice strain in ZnO nanoparticles: X-ray peak profile analysis	**[32, 48-51]**
2015	• Simultaneous cryo X-ray ptychographic and fluorescence microscopy of green algae • Characterization of Ag@Fe3O4core-shell nanocomposites for biomedical application • Enhanced Oral Delivery of Curcumin from N-trimethyl Chitosan Surface-Modified Solid Lipid Nanoparticles: Pharmacokinetic and Brain Distribution Evaluations • Gold Nanoparticle Internal Structure and Symmetry Probed by Unified Small-Angle X-ray Scattering and X-ray Diffraction Coupled with Molecular Dynamics Analysis	**[52-55]**
2016	• Hydrogen atoms can be located accurately and precisely by x-ray crystallography. • Influence of crystallite size on the magnetic properties of Fe3O4 nanoparticles	**[56, 57]**
2017	• CuO-NiO Nano composites: Synthesis, Characterization, and Cytotoxicity evaluation • X-Ray Photoelectron Spectroscopic Characterization of Iron Oxide Nanoparticles	**[58, 59]**
2018	• The study verified that evaluation of iron oxides by using Cu and cobalt radiation for XRD analysis. • Unraveling the long-pursued Au144 structure by x-ray crystallography. • A Rare Lysozyme Crystal Form Solved Using Highly Redundant Multiple Electron Diffraction Datasets from Micron-Sized Crystals. • X-ray elemental mapping techniques for elucidating the ecophysiology of hyperaccumulator plants. • The CryoEM Method MicroED as a Powerful Tool for Small Molecule Structure Determination	**[60-64]**
2019	• X-ray Diffraction of Intact Murine Skeletal Muscle as a Tool for Studying the Structural Basis of Muscle Disease. • Importance of potassium ions for ribosome structure and function revealed by long-wavelength X-ray diffraction. • Phase Transformation Behavior and Stability of LiNiO2 Cathode Material for Li-Ion Batteries Obtained from In Situ Gas Analysis and Operando X-Ray Diffraction. • Inward- and outward-facing X-ray crystal structures of homodimeric P-glycoprotein CmABCB1 • Enantiomeric Amino Acid Schiff Base Copper (II) Complexes as a New Class of	**[65-70]**

	RNA-Targeted Metallo-Intercalators: Single X-ray Crystal Structural Details, Comparative in Vitro DNA/RNA Binding Profile, Cleavage, and Cytotoxicity Comparison of Antimicrobial, Antioxidant and Anticancer Activities of ZnO Nanoparticles Prepared by Lemon Juice and Citric Acid Fueled Solution Combustion Synthesis.	
2020	• The study involved synthesis of nano hydroxyapatite from natural courses and the synthesized compound was characterized by comparing XRD patterns for crystal size calculation. • Structural Elucidation of the Mechanism of Molecular Recognition in Chiral Crystalline Sponges • Structural elucidation of microcrystalline MOFs from powder X-ray diffraction. • Crystal structure of SARS-CoV-2 nucleocapsid protein RNA binding domain reveals potential unique drug targeting sites. • Eco-friendly synthesis of antibacterial zinc nanoparticles using Sesamum indicum L. extract.	**[71-75]**
2021	• Effect of lemon juice on microstructure, phase changes, and magnetic performance of CoFe2O4 nanoparticles and their use on release of anti-cancer drugs • X-ray linear dichroic ptychography	**[76, 77]**
2022	• Evaluated Crystal structure of two recent phenoxy benzaldehyde 4-(4-bromophenoxy) benzaldehyde and 4-(4-nitrophenoxy) benzaldehyde derivatives using XRPD. • In vitro anticancer activity and comparative green synthesis of ZnO/Ag nanoparticles by moringa oleifera, Mentha piperita, and citrus lemon • Some metal chelates with Schiff base ligand: synthesis, structure elucidation, thermal behavior, XRD evaluation, antioxidant activity, enzyme inhibition, and molecular docking studies	**[78-80]**

Here, we probe into several prominent techniques, each equipped with unique strengths and applications.

Single Crystal X-ray Diffraction (SXRD)

[81] stands as a cornerstone in the realm of structural biology. By analyzing well-ordered single crystals, SXRD provides atomic-level insights into complex biomolecules. For instance, the elucidation of the ribosome's structure **[82, 83]**, essential for protein synthesis, was achieved through SXRD. However, the challenges of obtaining suitable crystals and mitigating radiation damage persist as areas of refinement **[84]**.

X-ray Powder Diffraction (XRPD)

[85] offers a versatile approach, particularly beneficial when dealing with samples lacking well-ordered crystalline structures **[86**]. XRPD is pivotal in phase identification, exemplified by its use in identifying different crystal polymorphs **[87]** in drug formulations **[88]**. While its resolution might be lower compared to SXRD, its ability to handle complex mixtures and amorphous materials is undeniable **[89]**.

Small Angle X-ray Scattering (SAXS)

[90] ventures into the realm of solution-based studies, uncovering macromolecular

shapes and dimensions **[91**]. In the examination of protein conformational changes, SAXS played a pivotal role in revealing the dynamic structural transitions of proteins like calmodulin **[92]**. Despite the challenges of accurately determining particle shapes from scattering data, SAXS remains a powerful tool for understanding solution structures.

Fiber Diffraction

[93] offers insights into linear structures like collagen and DNA fibers. An exemplary application lies in the elucidation of the DNA double helix structure by Rosalind Franklin through fiber diffraction **[7]**. By exploiting the oriented nature of fibers, this technique uncovers periodic arrangements along the fiber axis.

Free Electron Laser (FEL)-based Serial Crystallography

[94] marks an advancement enabled by X-ray free-electron lasers. It shattered the limitations imposed by radiation damage on delicate crystals **[95]** For instance, researchers used serial crystallography to unveil the structure of G protein-coupled receptors **[96, 97]**, integral to cellular signaling, by studying microcrystals.

Time-Resolved X-ray Crystallography

[98] opens a dynamic window into structural changes over time. By initiating reactions within crystals and collecting diffraction data at different time points, researchers have captured fleeting moments. Notably, time-resolved crystallography illuminated the light-driven proton pump bacteriorhodopsin's intricate mechanism of action **[99]**.

In selecting a technique, researchers have navigated a nuanced landscape, considering factors such as sample characteristics, research goals, and available equipment. These varied X-ray diffraction methods exemplify the multidimensional nature of structural elucidation, enabling us to decipher the intricate blueprints underlying biological function.

Importance of structural elucidation via XRD in drug discovery

Among the myriad techniques available for elucidating macromolecular structures, X-ray crystallography stands out, especially in the context of drug discovery. This preference is grounded in its unique capacity to yield structures with remarkably precise atomic resolution. Moreover, X-ray crystallography excels at unraveling the architecture of macromolecules that encompass heteromeric complexes, such as the ribosome. It offers intricate insights into binding mechanisms, shedding light on the interplay of small molecules with target molecules, thus fostering a deeper comprehension of molecular-level mechanisms. In this vein, X-ray crystallography remains the quintessential means of attaining essential biological structural data through diverse experimental protocols, essential for pioneering new drug discoveries. The synergy of X-ray crystallography with automated methodologies, like high-throughput screening, assumes a pivotal role in initial

crystallization screening and hits optimization, an indispensable practice in myriad biological laboratories and research endeavors **[100, 101]**. Table 2 describes the comparison of XRD with NMR Spectroscopy and Electron Microscopy.

A notable research paper underscores X-ray diffraction's efficacy in the exploration of bone nanostructure. The non-destructive nature of X-ray diffraction has proven invaluable in uncovering the structural intricacies of mineral crystals within bone.

Table 2: Comparison of XRD with NMR Spectroscopy and Electron Microscopy

Parameters	X-ray Diffraction	NMR Spectroscopy	Electron Microscopy (EM)
Resolution and Size of Molecules	XRD provides high-resolution structural information for large molecules, such as proteins and nucleic acids. It excels at determining the arrangement of atoms within the macromolecule and revealing the overall three-dimensional structure.	NMR is better suited for smaller molecules and provides information about molecular dynamics and interactions in solution. It's effective for studying protein structures up to a certain size and can provide insights into dynamic processes.	While EM can capture images of large macromolecular complexes, it might not achieve atomic-level resolution for individual atoms within a molecule.
Sample Preparation	Crystallization of the biological molecule is a prerequisite for XRD analysis. This can be challenging and might not be feasible for all molecules.	NMR works well with molecules in solution, eliminating the need for crystallization. This is particularly advantageous for large and complex structures that are difficult to crystallize.	EM requires the molecule to be visualized in its native state, often in a thin layer, without the need for crystallization. It's particularly useful for studying large complexes.
Structural Information	Provides information on the electron density distribution within the molecule, offering details about atomic positions and bond lengths.	Offers insights into the spatial arrangement of atoms and the flexibility of molecules in solution. Can provide information on distances between atoms.	Provides images that reveal the overall shape and arrangement of macromolecular complexes but might lack the atomic-level detail of XRD.
Accuracy and Precision	Offers high accuracy in determining atomic positions and bond lengths, making it a gold	While accurate, NMR results can be influenced by factors like molecular dynamics and interactions	Provides accurate information about the overall shape and arrangement of complexes

	standard for precise structural information.	in solution.	but might have limitations in resolving atomic details.
Complementary Information	Provides static, averaged structures and is particularly effective for well-ordered crystalline samples.	Offers insights into dynamic processes, protein folding, and interactions in solution.	Provides information about the shape and organization of large complexes, often in their native environment.

This technique's discernment of mineral crystal existence and organization has enabled a profound understanding of bone's structural composition. Moreover, X-ray powder diffraction (XRPD) emerges as a routine yet indispensable tool for the qualitative assessment of diverse mineral constituents. In another study, X-ray diffraction elucidated the mechanism behind stabilizing the amorphous form of tenapanor. Employing X-ray diffraction techniques-single crystal X-ray diffraction (SXRD) for the anhydrous crystalline form and XRPD for the 2HCl salt form-researchers probed the crystal structures of these systems. Tenapanor holds promise in treating irritable bowel syndrome linked to constipation. Recent advancements also showcased X-ray diffraction's role in revealing the crystal structure of the nucleocapsid protein RNA N-terminal binding domain of SARS-CoV-2, further affirming its indispensable contribution **[34, 74, 89, 102**].

In the contemporary realm of nanotechnology, XRD assumes a pivotal role in nanoparticle characterization. Researchers have harnessed X-ray powder diffraction (XRPD) to assess nanoparticle size and crystalline structure, a fitting choice given that nanoparticles are typically analyzed in powder form. This resonates particularly as nanotechnology gains prominence across diverse realms of nanoscience, including novel drug development, embodying an innovative global pursuit **[103]**.

Studying crystal structures of unknown molecules **[78]** is crucial for drug development and for various analytical processes which are mostly carried out using X-ray diffraction (XRD) techniques. Recent advancements in the drug design field have shown structural elucidation of biological macromolecules that are necessary for designing new drugs and improving the current drug molecules. With the fast increase in the spread of diseases, new medications are needed worldwide pepsin and conventional methods for drug development had played an important role in drug development they are time-consuming and costly as compared to the newly initiated advanced methods for drug development, these methods utilize rational drug designing methods that involve the

identification of various target macromolecules (proteins, nucleic acids, DNA, RNA) these molecules have been studied and their structures elucidated using X-ray crystallography and NMR spectroscopy; since then X-ray crystallography has shown many advancements with the time; the first molecule crystal was identified via XRD was pepsin **[104]** and the first protein whose structure was determined is myoglobin **[105]** precise role of XRD in drug design **[108-115]**. and followed by many other proteins including the enzyme lysozyme **[106]**. In 2012, Kobilka and Lefkowitz won the prize for studying G protein–coupled receptors (GPCRs), which are the most common targets for many drug molecules. These inventions gave much importance to XRD for its role in the structural elucidation of various unknown drug molecules and target macromolecules **[107].** Table 3 shows the

Table 3: Significance of XRD in Drug Design & Discovery.

Target Identification and Validation	• XRD enables researchers to determine the precise atomic arrangement of a target biomolecule, thereby identifying potential drug targets and validating their relevance in disease pathways.
Rational Drug Design	• Knowledge of the three-dimensional structure of a target allows for rational drug design. By understanding how a drug candidate interacts with the target, scientists can optimize its structure to enhance efficacy and specificity while minimizing off-target effects.
Lead Optimization	• XRD helps in lead optimization by providing structural information on drug-target complexes. This information is crucial for fine-tuning lead compounds to improve binding affinity and selectivity.
Understanding Binding Mechanisms	• XRD reveals the exact binding interactions between drug molecules and their target, shedding light on key molecular interactions critical for drug efficacy.
Prediction of Drug-Target Interactions	• XRD data can be used to predict potential drug-target interactions, guiding the screening of compound libraries and facilitating the discovery of new drug candidates.
Structure-Based Virtual Screening	• X-ray structures of drug targets can be used in structure-based virtual screening, allowing researchers to virtually dock small molecules and predict their binding affinity to the target.
Resolving Drug Resistance	• In cases of drug resistance, XRD can be employed to analyze the structural changes in the target biomolecule, enabling the development of more potent and resistant-resistant drugs.
Fragment-Based Drug Design	• XRD is instrumental in fragment-based drug design, where small molecular fragments are docked onto the target, and their interactions are used as building blocks to design more potent drug-like molecules.
Validation of Drug Binding Sites	• XRD helps validate the binding sites of drug molecules on the target, ensuring the accuracy of drug design efforts.
Crystallization of Drug-Target Complexes	• XRD guides the crystallization of drug-target complexes, facilitating their detailed structural analysis and providing a deeper understanding of the drug's mode of action.

Strengths and limitations of XRD in structural biology

A systematic analysis of the strengths and limitations of X-ray diffraction in unraveling the structural intricacies of biological molecules underscores both its significance and areas for improvement.

X-ray diffraction yields atomic-level resolution, enabling the determination of precise three-dimensional structures. This capability has been crucial in deciphering intricate biomolecular architectures like the ribosome and viral capsids. X-ray diffraction extends beyond proteins, offering insights into nucleic acids, carbohydrates, and small molecules. This versatility allows a comprehensive exploration of biological systems. It provides static snapshots that form the foundation for understanding dynamic processes. These structures serve as essential templates for computational simulations and mechanistic studies. X-ray diffraction elucidates the molecular interactions governing ligand binding, informing drug discovery and design efforts. Notably, it contributed to understanding the binding of antiretroviral drugs to HIV protease. The experimental verification of hypotheses derived from computational modeling is a forte of X-ray diffraction. This iterative process refines models and enhances their accuracy.

One of the most substantial limitations is the necessity for well-ordered crystals. Crystallization can be challenging for large or complex molecules, potentially hampering structural determination. High-energy X-rays can induce radiation damage, altering the sample's structure during data collection. This becomes particularly pertinent for sensitive samples and time-resolved experiments. X-ray diffraction provides static images, leaving dynamic processes and conformational changes unexplored. It necessitates complementary techniques like NMR spectroscopy for a complete picture. For non-crystalline or flexible samples, X-ray diffraction encounters limitations. Innovations like XFELs offer solutions, yet challenges persist in adapting this technology widely. Interpreting X-ray diffraction data demands sophisticated computational analysis and model refinement. This complexity can hinder the accessibility of the technique to non-experts. Particularly for metalloproteins and redox-active centers, X-rays can perturb the sample's native state, potentially leading to altered structures.

X-ray diffraction stands as an indispensable pillar in structural biology, providing unparalleled insights into biological macromolecules. Recognizing its strengths, such as high resolution and wide applicability, allows researchers to leverage its power effectively. Simultaneously, acknowledging its limitations, from crystallization challenges to the static nature of data, spurs the quest for innovation and the integration of complementary techniques.

CONCLUSION AND FUTURE DIMENSIONS

In conclusion, the future dimensions of the X-ray diffraction (XRD) technique in the structural elucidation of biological molecules hold great promise and potential for advancements across various fields. XRD has consistently proven its significance in drug discovery by providing detailed insights into the three-dimensional structures of biological macromolecules, enabling rational drug design, lead optimization, and target validation. As technology evolves, XRD is poised to continue its pivotal role.

Advancements in XRD instrumentation and data analysis techniques are expected to enhance its accuracy and efficiency. High-throughput screening approaches, coupled with XRD, are likely to expedite the initial crystallization screening process, enabling faster identification of potential drug candidates. Moreover, the integration of XRD with other complementary techniques, such as cryo-electron microscopy and nuclear magnetic resonance spectroscopy, will enable a comprehensive understanding of biomolecular structures and dynamics.

The exploration of dynamic processes through time resolved XRD and serial crystallography will unveil transient molecular interactions and mechanisms, offering a more complete view of biological processes. Additionally, the development of synchrotron facilities and X-ray free-electron lasers (XFELs) will enable researchers to study challenging samples, including large complexes and non-crystalline structures, further expanding the scope of XRD applications.

Furthermore, XRD's potential extends into the realm of nanotechnology, where it plays a crucial role in characterizing nanoparticles for novel drug delivery systems and therapeutic applications. As the field of nanomedicine continues to evolve, XRD will remain a cornerstone in understanding the structures of nanomaterials and their interactions with biological systems.

In sum, the future of X-ray diffraction techniques in the structural elucidation of biological molecules holds exciting possibilities. With continued technological advancements, interdisciplinary collaborations, and innovative methodologies, XRD is poised to revolutionize our understanding of complex biological systems and drive breakthroughs in drug discovery, materials science, and nanotechnology.

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