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(Review Article)

Bioanalysis of antisense oligonucleotides: Techniques, challenges, regulatory considerations, and future perspectives

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Abstract

Antisense oligonucleotides (ASOs) are a powerful class of therapeutics designed to target RNA sequences and regulate gene expression, offering promising treatment avenues for various genetic disorders, neurodegenerative diseases, cancers, and viral infections. The bioanalysis of ASOs presents unique challenges due to their distinct chemical and biological characteristics, requiring specialized analytical methods and regulatory oversight. This review explores the latest methodologies in ASO bioanalysis, assesses the current regulatory environment, and considers future directions, particularly in the context of critical and emerging technologies (CETs) relevant to national security strategies. ASOs, classified under biotechnology, play a crucial role in gene expression modulation and protein regulation. By integrating insights from CETs, this review emphasizes the strategic significance of ASO bioanalysis in driving advancements in medical science and contributing to national security.

Keywords: Antisense oligonucleotides; Bioanalysis; Analytical techniques; Chromatography; Mass spectrometry; Hybridization assays; Regulatory requirements

1. Introduction

Antisense oligonucleotides (ASOs) are short, synthetic strands of nucleic acids designed to bind complementary mRNA sequences, thereby modulating gene expression through mechanisms such as mRNA degradation, splicing alteration, or translation inhibition [1, 2]. By targeting RNA, ASOs tend to correct or inhibit harmful protein production, as highlighted by recent research on their therapeutic potential. ASOs are being explored for treating various genetic disorders, including those where traditional therapies are ineffective, offering a precise and customizable approach to disease management. Emerging studies underscore the importance of optimizing ASO delivery and efficacy, as these molecules continue to advance as a key component of personalized medicine [3, 4]. The therapeutic potential of ASOs has been demonstrated in various diseases, including spinal muscular atrophy [5], Duchenne muscular dystrophy [6], and certain cancers [7, 8]. The complexity of ASOs, characterized by their size, charge, chemical modifications, and sequence specificity, poses significant challenges for their bioanalytical characterization [9].

The Critical and Emerging Technologies (CET) framework identifies advanced biotechnologies, including ASOs, as pivotal to national security and economic prosperity [10]. The integration of ASO bioanalysis within the CET landscape highlights the dual role of these technologies in advancing healthcare and contributing to broader societal and security objectives [11, 12]. This review aims to provide a comprehensive overview of the bioanalytical techniques, challenges and solutions, regulatory considerations, and future perspectives for ASOs, framed within the context of CETs.

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2. Analytical Techniques in Bioanalysis of ASOs

The successful development and clinical translation of ASOs hinge on the availability of robust bioanalytical methods capable of accurately assessing their pharmacokinetic properties, distribution profiles, and target engagement in complex biological systems. Effective bioanalysis of ASOs involves the accurate quantification and characterization of intact oligonucleotides, their metabolites, and their pharmacokinetic pharmacodynamic, and safety profiles. The following sections detail the primary analytical techniques used in ASO bioanalysis.

2.1. Chromatography-based approaches

Chromatography-based techniques, including high-performance liquid chromatography (HPLC), ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), and capillary electrophoresis (CE), play a pivotal role in the bioanalysis of ASOs [13].

High-performance liquid chromatography (HPLC) is a widely employed technique for the analysis of ASOs in pharmaceutical formulations and biological samples. HPLC separates ASOs based on differences in their physicochemical properties, such as size, charge, and hydrophobicity. Reverse-phase HPLC (RP-HPLC) is commonly used for the separation of ASOs, utilizing a hydrophobic stationary phase and a gradient of organic solvent in the mobile phase. UV or fluorescence detection is often coupled with HPLC for the sensitive quantification of ASOs. Moreover, the advent of ultra-high-performance liquid chromatography (UHPLC) has enabled faster separations and improved sensitivity for ASO analysis [14].

Ion-exchange chromatography (IEC) separates ASOs based on differences in their charge properties [5]. In IEC, ASOs are loaded onto a stationary phase containing ion-exchange resin beads with positively or negatively charged functional groups. ASOs with opposite charges to the resin beads are retained, while neutral ASOs are eluted first. Gradient elution is commonly employed to achieve efficient separation and resolution of ASOs. IEC is particularly useful for purification and analyzing ASO purity and charge variants, which are critical parameters in pharmaceutical development [15].

Size-exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), separates ASOs based on differences in their molecular size [6]. In SEC, ASOs are eluted through a porous stationary phase, with larger ASOs excluded from the pores and eluted first, while smaller ASOs penetrate the pores and elute later. SEC is valuable for assessing ASO size distribution, oligomerization, and aggregation, which can impact their pharmacokinetic properties and therapeutic efficacy. SEC is often coupled with multi-angle light scattering (MALS) or dynamic light scattering (DLS) for accurate determination of ASO molecular weight and size [16, 17].

Capillary electrophoresis (CE) is a powerful technique for the separation and analysis of ASOs based on their electrophoretic mobility [18]. CE separates ASOs in a capillary filled with electrolyte buffer under the influence of an electric field. ASOs migrate through the capillary at different rates based on their size, charge, and conformation. CE offers high-resolution separations, rapid analysis times, and small sample requirements, making it suitable for high-throughput ASO analysis [18]. Moreover, CE can be coupled with various detection methods, including UV absorbance, fluorescence, and mass spectrometry, enhancing its versatility in ASO characterization. When coupled with laser-induced fluorescence (LIF) detection, CE achieves high sensitivity, making it suitable for trace-level analysis [19]. Future advancements in chromatography instrumentation, column technology, and detection methods are expected to further improve the efficiency and accuracy of ASO analysis.

2.2. Mass Spectrometry-based approaches

Mass Spectrometry-Based Approaches Mass spectrometry (MS) has emerged as a powerful tool for the characterization and quantification of ASOs due to its high sensitivity, specificity, and ability to provide structural information [20, 21]. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are commonly employed ionization techniques for ASO analysis. MS techniques, such as single-ion monitoring (SIM) and multiple reaction monitoring (MRM), enable precise quantification of ASOs in complex biological samples. Furthermore, the combination of MS with chromatographic separation techniques offers enhanced sensitivity and selectivity for ASO bioanalysis and that is why LC-MS is a cornerstone in ASO bioanalysis [22].

LC-MS enables the separation of ASOs from complex biological matrices and facilitates the identification and quantification of both intact ASOs and their metabolites. Advances in LC-MS/MS, including high-resolution mass spectrometry and tandem mass spectrometry, have enhanced the detection capabilities for low-abundance ASOs, making it indispensable for pharmacokinetic studies [23] . The integration of advanced computing and data processing technologies, as highlighted in the CET list, further augments the utility of LC-MS in handling complex datasets.

Electrospray ionization (ESI) is a widely used ionization technique in MS for ASO analysis. In ESI, ASOs are introduced into the mass spectrometer as ions via a charged aerosol spray, allowing for their detection and characterization. ESI is particularly useful for analyzing ASO purity, charge variants, and modifications, such as phosphorylation and methylation. Moreover, the coupling of ESI with liquid chromatography (LC-ESI-MS) enables the separation and quantification of ASOs in complex biological matrices with high sensitivity and selectivity [24].

Matrix-assisted laser desorption/ionization (MALDI) is another commonly employed ionization technique in MS-based ASO analysis. In MALDI, ASOs are mixed with a matrix material and irradiated with a laser, resulting in the generation of gas-phase ions for mass analysis. MALDI offers advantages such as high throughput, minimal sample preparation, and the ability to analyze ASOs directly from solid-phase supports. MALDI-MS is well-suited for high-throughput screening of ASO libraries and the analysis of ASO binding interactions with target RNA molecules [25, 26].

Tandem mass spectrometry (MS/MS) techniques provide structural information about ASOs and their fragments. MS/MS allows for the characterization of ASO sequences, modifications, and degradation products, facilitating the identification of metabolites and degradation pathways. Moreover, MS/MS-based approaches enable the quantification of ASOs in biological samples with high accuracy and precision [27, 28]. Additionally, MS can be coupled with hybridization-based assays, such molecular beacon assays, for real-time monitoring of ASO-target interactions and quantification [29, 30].

Examining a specific case study can provide valuable insights into the practical application of bioanalytical techniques and the challenges encountered in ASO bioanalysis. Nusinersen, an FDA-approved ASO for spinal muscular atrophy, serves as a prominent example of successful ASO bioanalysis [31, 32]. One of the bioanalytical strategies for nusinersen involved the use of LC-MS/MS for quantification in plasma and cerebrospinal fluid (CSF), coupled with hybridization-based assays for tissue distribution studies. Method validation followed FDA guidelines, ensuring accuracy, precision, and robustness. The comprehensive bioanalytical approach facilitated the understanding of nusinersen's pharmacokinetics and informed dosing regimens [33, 34].

2.3. Hybridization-Based Assays

Hybridization-based assays represent a versatile approach for the detection and quantification of ASOs, leveraging the specific binding affinity between ASOs and complementary nucleic acid probes. These assays encompass a diverse range of methodologies, including hybridization ELISA, hybridization chain reaction (HCR), fluorescence in situ hybridization (FISH), and molecular beacon assays [35-40].

Fluorescence-based assays utilize fluorescent probes to detect and quantify ASOs based on hybridization events. Fluorescently labeled ASOs or complementary probes are employed to monitor ASO-target interactions in real-time [41, 42]. Fluorescence resonance energy transfer (FRET) assays and molecular beacons are commonly used fluorescence-based techniques for ASO analysis. These assays offer high sensitivity, rapid detection, and the ability to monitor ASO-target binding kinetics. Molecular beacon assays utilize hairpin-shaped oligonucleotide probes with a fluorophore and quencher at opposite ends. In the absence of the target sequence, the molecular beacon adopts a hairpin structure, leading to quenching of the fluorescence signal [43]. Molecular beacon assays enable real-time monitoring of ASO-target interactions with high specificity and single-base resolution.

Hybridization chain reaction (HCR) assays offer a signal amplification strategy for sensitive detection of ASOs. HCR relies on the sequential hybridization of DNA hairpins to generate long, branched DNA polymers in the presence of ASO-target complexes. Fluorescently labeled DNA hairpins serve as amplification probes, leading to signal enhancement proportional to the concentration of ASOs.

Hybridization enzyme-linked immunosorbent assay (ELISA) offers a robust platform for the detection and quantification of ASOs based on their specific hybridization with complementary probes immobilized on ELISA plates. Hybridization ELISA relies on the specific hybridization between ASOs and complementary probes immobilized on the surface of microtiter plates. Upon hybridization, the ASO-probe complexes are detected using enzyme-conjugated secondary antibodies that recognize the hybridized ASO sequences. The enzymatic reaction generates a measurable signal, typically colorimetric or chemiluminescent, which is proportional to the concentration of ASOs in the sample. The design and optimization of hybridization ELISA assays are critical for achieving high sensitivity, specificity, and reproducibility. Factors such as probe design, immobilization chemistry, blocking agents, washing conditions, and detection enzymes must be carefully optimized to minimize background noise and maximize signal-to-noise ratio. Additionally, assay validation parameters, including linearity, accuracy, precision, and stability, should be rigorously assessed to ensure assay robustness and reliability. Hybridization ELISA can employ various detection strategies,

including direct labeling of ASOs with enzymes or fluorescent tags, as well as indirect detection using enzyme-conjugated secondary antibodies or streptavidin-biotin amplification systems. Each detection strategy offers specific advantages and limitations in terms of sensitivity, signal amplification, and compatibility with different sample matrices. Hybridization ELISA has been widely used for the quantitative analysis of ASOs in various biological matrices, including plasma, serum, tissue lysates, and cell culture supernatants. This technique has applications in pharmacokinetic studies, biomarker analysis, therapeutic monitoring, and quality control of ASO-based therapeutics.

2.4. Emerging Techniques

Emerging technologies such as nanopore sequencing and next-generation sequencing (NGS) are beginning to impact ASO bioanalysis [20, 44, 45]. These techniques allow for the direct sequencing of ASOs, providing detailed information on sequence integrity, modifications, and potential off-target effects [46]. Despite their promise, challenges related to data interpretation, throughput, and cost currently limit their routine application in bioanalysis. However, as these technologies mature, they are poised to play a significant role in the future of ASO bioanalysis, aligning with the CET emphasis on advancing biotechnologies. Moreover, the emergence of novel analytical technologies, such as single-molecule sequencing and imaging techniques, holds promise for advancing our understanding of ASO pharmacology and improving drug development processes [47].

3. Challenges and Solutions in ASO Bioanalysis

The bioanalysis of ASOs presents several challenges, including the complexity of biological matrices, the need for high sensitivity and specificity, and the accurate characterization of metabolites. Addressing these challenges requires continuous innovation in analytical techniques, method development, and regulatory strategies.

Matrix Effects and Interferences: Biological matrices such as blood, CSF, plasma, and tissues contain a myriad of endogenous substances that can interfere with ASO detection and quantification [48]. Matrix effects can lead to signal suppression or enhancement, compromising the accuracy of bioanalytical assays. Solutions include the development of robust sample preparation methods, such as solid-phase extraction and liquid-liquid extraction, to minimize matrix interferences. Additionally, the use of internal standards and matrix-matched calibration curves can help correct for matrix-induced variability.

Sensitivity and Specificity: Achieving high sensitivity and specificity in ASO bioanalysis is critical, particularly for low-dose therapeutics and in the presence of complex biological matrices. Enhancing assay sensitivity can be achieved through the optimization of detection techniques, such as increasing the resolution and sensitivity of mass spectrometers. Specificity can be improved by utilizing highly selective ligands and optimizing hybridization conditions in sequence-specific assays. The integration of multiple analytical techniques can also enhance both sensitivity and specificity, providing a more comprehensive analysis of ASOs.

Metabolite Identification and Quantification: Accurate identification and quantification of ASO metabolites are essential for understanding their pharmacokinetics and potential toxicological effects. Metabolite profiling requires the use of high-resolution mass spectrometry and advanced data analysis algorithms to detect and characterize metabolites [49]. Developing comprehensive metabolite libraries and employing in silico prediction tools can aid in the identification process. Additionally, establishing standardized protocols for metabolite characterization will ensure consistency and reliability across studies. This requirement aligns with the CET focus on responsible development and safety of emerging biotechnologies [50].

Regulatory Compliance and Standardization: Navigating the regulatory landscape for ASO bioanalysis involves adhering to diverse guidelines and standards set by different regulatory bodies [51]. Ensuring compliance requires a thorough understanding of regulatory requirements and the implementation of standardized bioanalytical methods. Developing harmonized guidelines and fostering international collaboration can streamline the regulatory approval process, facilitating the global advancement of ASO therapeutics. Standardization efforts, including the adoption of best practices and quality control measures, are essential for maintaining consistency and reliability in ASO bioanalysis [52].

Cost and Accessibility: The high cost of advanced analytical instruments and the specialized expertise required for ASO bioanalysis can limit accessibility, particularly for smaller laboratories and research institutions [53]. Addressing cost barriers involves developing cost-effective bioanalytical methods and leveraging shared resources, such as centralized core facilities and collaborative networks. Additionally, advancements in automation and high-throughput technologies can reduce labor costs and increase assay efficiency, making ASO bioanalysis more accessible.

4. Regulatory Considerations

The regulatory landscape for ASOs is evolving in tandem with advancements in bioanalytical techniques and the broader CET framework. Regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), have established specific guidelines tailored to the bioanalysis of oligonucleotide therapeutics [54, 55]. Key regulatory considerations include method validation, metabolite characterization, and immunogenicity assessment.

Bioanalytical Method Development and Validation: A validated method is crucial for the approval of a drug because it ensures the reliability, accuracy, and reproducibility of the data generated during drug development. Regulatory agencies require validated methods to confirm that the drug meets the necessary safety, efficacy, and quality standards. Without validation, even the most innovative research may be deemed unreliable, as the data cannot be consistently reproduced or trusted. This lack of reliability can lead to significant setbacks in the approval process, potentially delaying or preventing a promising drug from reaching the market. Therefore, method validation is fundamental in translating great research into a successful, approved therapeutic.

Developing robust bioanalytical methods for ASOs involves optimizing parameters such as selectivity, range, stability, sensitivity, accuracy, precision, and reproducibility [56]. Method development must account for the unique properties of ASOs, including their chemical modifications and the complexity of biological matrices [57]. Validation of these methods requires adherence to regulatory guidelines to ensure reliability and reproducibility across different laboratories and studies [58].

Immunogenicity Assessment: Immunogenicity is a critical concern in large molecule therapeutics such as proteins, nucleic acids, ASO, monoclonal antibodies, etc., as the development of anti-drug antibodies (ADAs) can affect the drug's efficacy and safety profile. Regulatory guidelines emphasize the need for robust assays to detect and characterize ADAs. These assays must be highly sensitive and specific, capable of identifying ADAs at low concentrations without cross-reacting with endogenous antibodies [59, 60].

Regulatory Harmonization: Harmonizing regulatory guidelines across different regions is crucial for the global advancement of ASO therapeutics. International collaboration among regulatory bodies facilitates the standardization of bioanalytical methods, promoting consistency and reliability in ASO bioanalysis [61].

5. Future Perspectives

The future of ASO bioanalysis could be linked to advancements in CETs, particularly in areas such as biotechnology, data processing, and regulatory science. The following sections explore key future directions that can shape the field.

Integration with Critical and Emerging Technologies: The integration of ASO bioanalysis with other CETs, such as artificial intelligence (AI), quantum computing, and advanced materials, will drive significant advancements in the field. AI and machine learning algorithms can enhance data analysis and interpretation, streamlining the identification of ASO metabolites and improving assay precision. Quantum computing holds the potential to revolutionize data processing capabilities, enabling the handling of vast and complex datasets generated in ASO bioanalysis [62, 63]. The development of more sensitive and specific detection techniques will significantly enhance ASO bioanalysis. Technologies such as NGS and nanopore sequencing are expected to become more accessible and integrated into routine bioanalytical workflows [64]. These advancements will enable the detection of ASOs at lower concentrations and in more complex biological matrices, thereby improving the accuracy of pharmacokinetic and pharmacodynamic studies. The alignment of these technologies with the CET list underscores their strategic importance in maintaining U.S. leadership in biotechnology [65].

Personalized Medicine: As ASO therapeutics become more personalized, bioanalytical methods must evolve to accommodate individual patient needs [4, 66]. This shift towards personalized medicine requires the development of assays that can handle the unique properties of individual ASOs and their specific targets. Personalized bioanalysis will involve the integration of advanced computational tools and machine learning algorithms to tailor assays to individual therapeutic profiles. This approach aligns with the CET emphasis on leveraging advanced technologies to deliver tangible societal benefits.

Collaborative Research and Development: Collaborative research and development efforts between academia, industry, and government agencies will drive innovation in ASO bioanalysis [67]. Collaborative initiatives can facilitate the

sharing of knowledge, resources, and technological advancements, accelerating the development of novel bioanalytical techniques. Public-private partnerships and interagency collaborations, as encouraged by the CET framework, will be instrumental in overcoming technical challenges and advancing the field of ASO bioanalysis. These collaborations will foster a multidisciplinary approach, integrating expertise from various fields to enhance the robustness and versatility of ASO bioanalytical methods.

Sustainability and Green Chemistry in Bioanalysis: Sustainability and the principles of green chemistry are becoming increasingly important in the development of bioanalytical methods [68]. Reducing the environmental impact of bioanalytical processes involves minimizing the use of hazardous solvents, optimizing resource efficiency, and implementing waste reduction strategies. Incorporating green chemistry principles into ASO bioanalysis aligns with the CET focus on sustainable and responsible technological advancements. Future bioanalytical methods will need to balance analytical performance with environmental sustainability, fostering the development of eco-friendly and efficient bioanalytical practices.

Enhanced Data Security and Privacy: With the increasing reliance on advanced computational tools and data-intensive techniques in ASO bioanalysis, ensuring data security and privacy is paramount [69, 70]. Implementing robust data encryption, secure data storage solutions, and access control mechanisms will protect sensitive bioanalytical data from unauthorized access and breaches. Compliance with data privacy regulations, such as the General Data Protection Regulation (GDPR), is essential for maintaining the confidentiality and integrity of patient information [71]. The CET framework emphasizes the importance of data privacy and cybersecurity technologies, highlighting the need for secure bioanalytical workflows that safeguard both patient information and proprietary analytical methods. Enhanced data security measures will be critical in maintaining the integrity and confidentiality of bioanalytical data in the era of digital transformation.

6. Conclusion

The bioanalysis of antisense oligonucleotides is a rapidly advancing field, driven by the growing therapeutic potential of these molecules and their integration within the broader framework of critical and emerging technologies. Advanced analytical techniques, stringent regulatory considerations, and strategic alignment with CETs are essential for the accurate characterization and quantification of ASOs. Future advancements in detection technologies, personalized medicine, and regulatory harmonization will further enhance the capabilities and impact of ASO bioanalysis. Continued innovation, collaboration, and adherence to high standards will be crucial in overcoming the challenges associated with ASO bioanalysis and realizing the full potential of these groundbreaking therapies.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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