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A Guide to Chemical Considerations for the Pre-Clinical Development of Oligonucleotides

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Oligonucleotide therapeutics, a pioneering category of modern medicinal drugs, are at the forefront of utilizing innate mechanisms to modulate gene expression. With 18 oligonucleotide-based FDA-approved medicines currently available for treating various clinical conditions, this field showcases an innovative potential yet to be fully explored. Factors such as purity, formulation, and endotoxin levels profoundly influence the efficacy and safety of these therapeutics. Therefore, a thorough understanding of the chemical factors essential for producing high-quality oligonucleotides for preclinical studies is crucial in their development for further clinical application. This paper serves as a concise guide to these chemical considerations, aiming to inspire and equip researchers with the necessary knowledge to advance in this exciting and innovative field.

Keywords: oligonucleotide synthesis, pre-clinical guidelines, chemical engineering

Introduction

Oligonucleotides are an essential aspect of modern medicine, with 18 approved drugs available to treat various clinical diseases.[1] These drugs are categorized into three main groups: short interfering RNA (siRNA), antisense oligonucleotides (ASOs), and vaccine adjuvants.[1,2] There are two main types of ASOs: gapmers and splice-switching oligonucleotides. Gapmers comprise modified wings with a central DNA core, essential for binding to the target mRNA and facilitating its cleavage by RNase H after forming a DNA–RNA hybrid. On the other hand, splice-switching oligonucleotides are modified with both 2' and phosphate mods throughout. They function by inhibiting or promoting exon inclusion or exclusion in pre-mRNA through steric hindrance.[1,3]

The clinical success of oligonucleotides is primarily due to advanced chemical modifications that improve nuclease resistance, boost accumulation, and prevent recognition by the immune response.[1] These include changes to the phosphate backbone, pentose sugar, and nucleobases,[4,5] with examples such as phosphorothioate (PS), phosphorodiamidate morpholino oligomers (PMOs), 2'-O-methyl RNA, 2'-fluoro-RNA, and 2'-methoxy ethyl (MOE) RNA.[6] The chemical structure of oligonucleotides significantly influences pharmacokinetics/pharmacodynamics, toxicity, and therapeutic duration. All approved oligonucleotides are chemically modified, underscoring the importance of this approach.[1]

Oligonucleotides have now been approved for several tissues, including the liver, muscle, and central nervous system (CNS). The muscle PMOs have been approved to treat Duchenne muscular dystrophy, where they act by skipping specific exons in the dystrophin pre-mRNA to restore the reading frame.[7] For the liver, conjugation of *N*-acetylgalactosamine (GalNac) has been used to target hepatocytes via the asialoglycoprotein receptor.[8–11] Therapeutic use in the CNS is challenging because oligonucleotides do not cross the blood-brain barrier and can be toxic. Local delivery can be achieved via intrathecal injection, after which MOE PS oligonucleotides have been shown to be taken up by most CNS cells.[12] However, their negatively charged phosphates bind to calcium and magnesium ions, causing a signaling imbalance.[13] This can be counterbalanced by formulating the ASO in modified artificial CSF(see below). Nusinersen is one of the most successful oligonucleotide therapeutics and has paved the way for ASO therapeutics development in the CNS. Nusinersen is a fully PS, 2'MOE modified ASO delivered intrathecally, and promotes the inclusion of exon 7 in *SMN2* pre-mRNA to

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increase production of SMN protein in patients with spinal muscular atrophy. Since then, the first gapmer ASO was approved to treat SOD1-related ALS. Another success for the CNS has been the IND approval of Milasen, the first ASO customized for a single person.[14] Mila's story has inspired many researchers to develop customized oligos for single-person diseases.

The production of oligonucleotides involves multiple stages: synthesis, deprotection, purification, desalting, and quality control.[15] The synthesis is highly automated and conducted on a solid support with stages including deblocking, coupling, oxidation, and capping.[15,16] The synthesis scale is inversely proportional to the number of wells on a plate. For a 96-well synthesis, a 1000 nmol scale synthesis can be performed. However, for a 192-well synthesis, the scale is 500 nmol. For a 384-well synthesis, it would be a maximum of 100 nmol. Although the process is generally efficient, it inevitably generates impurities, some of which are removed during synthesis. Impurities can arise from failed couplings, sulfurization, and incomplete deblocking. The deprotection stage may introduce other impurities, such as desulfurization and incomplete removal of protecting groups.[17,18] We refer the interested reader to Capaldi *et al.*'s 2017 publication for more detail.[19] Purification is tailored to eliminate synthesis and deprotection by-products, with particular attention to toxic salts. Following purification, rigorous quality control, including high-resolution liquid chromatography-mass spectrometry, is essential to confirm the oligonucleotides' identity (correct mass) and purity.[17]

This paper aims to guide researchers, including those without a background in chemistry, to successfully use chemically modified oligonucleotides by understanding critical chemical factors for the pre-clinical development of therapeutics for CNS.

In Vitro (Cell Culture) Evaluation of Oligonucleotide Efficacy

The level of final purity of an oligonucleotide and whether HPLC purification is required is determined by its final use. For *in vitro* cell culture experiments, HPLC purification is usually unnecessary. Ethanol precipitation and/or ultrafiltration is essential to remove impurities from synthesis and deprotection and exchange the oligonucleotide's counterion to a non-toxic form, i.e., sodium. Further desalting or buffer exchange may be necessary. UV purity of the full-length peak should be >70%, with the final purity depending upon the final application. The identity of the oligonucleotide determined by HRMS should be within ±1 Da of the expected mass (Fig. 1B).

Note: When comparing two oligonucleotides in experiments, they must follow the same post-synthesis process and have similar final purities.

In Vivo (Animal Studies) Evaluation of Oligonucleotide Efficacy

It is crucial to consider the side-products that result from the oligonucleotide preparative process since they can significantly affect both efficacy and toxicity. Therefore, to evaluate oligonucleotides in animal models, it is essential to have strict criteria for purity, which should be at least 85%. If the initial purity of the oligonucleotide is above the threshold, HPLC purification is unnecessary. If it is below the threshold, HPLC purification is required. Desalting or buffer exchange is necessary to remove toxic salts and have non-toxic counterions, such as sodium. The oligonucleotide should be formulated into proper buffer solutions, such as PBS.

Endotoxins are components from the outer membrane of Gram-negative bacteria that cause strong immune responses in humans and animals.[17] Detecting and removing endotoxin is necessary when performing pre-clinical evaluations *in vivo*. The FDA has strict guidelines on therapeutics' endotoxin concentration (EU/mL), defined by the route of administration, patient weight, and injection volume.[20,21] The endotoxin limit can be measured from the equation El = K/M, where *K* is the endotoxin units (EU)/kg for a given route. For routes that the drug enters the CSF (ICV and IT), this is 0.2 EU/kg,

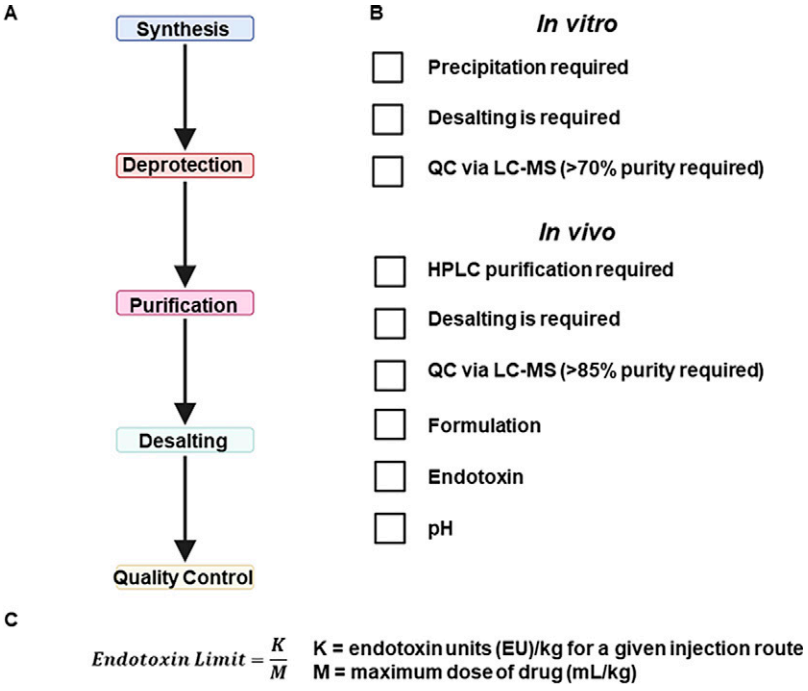

FIG. 1. Checklist of chemical considerations for the pre-clinical development of oligonucleotides. **(A)** The oligonucleotide synthesis pipeline: synthesis, deprotection, purification, desalting and quality control. **(B)** Checklist for *in vitro* and *in vivo* considerations when using oligonucleotides in pre-clinical studies. **(C)** Endotoxin limit calculation. Where *K* is endotoxin units (EU)/kg for a given injection route, and *M* is the maximum dose of the drug (mL/kg).

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and for the other routes, it is 5 EU/kg. *M* represents the maximum dose of the drug in mL/kg.

Oligonucleotides are acidic due to their phosphate backbones, affecting their tolerability *in vivo*. Therefore, it is essential to buffer the oligonucleotide to physiological pH ($\sim$7.4). However, the final pH value will depend on other factors, including the delivery method.[17]

Injections into the Central Nervous System

When injected into the CNS, oligonucleotides can cause toxicity because they are highly negatively charged and can sequester calcium and magnesium ions, disrupting the polarization of the neurons.[13] To prevent this, oligonucleotides must be pre-formulated with calcium and magnesium using modified artificial CSF solutions before injection into the CNS.[13,22]

An example of artificial cerebrospinal fluid (aCSF) that effectively reduces acute toxicity between antisense oligonucleotides (ASOs) and small siRNA includes the following composition: 137 mM NaCl, 5 mM KCl, and 20 mM D-+-glucose in an 8 mM HEPES buffer. It is essential to maintain a final ratio of 14 (Ca^{2+}): 2 (Mg^{2+}) for the divalent cations (calcium and magnesium).[22]

Conclusion

Several chemical factors, such as purity, formulation, and endotoxin levels, must be considered when buying or creating new oligonucleotides for medical purposes. This paper provides detailed information on these factors, and the central figure presents a checklist that researchers can use in their pre-clinical development.

Author Disclosure Statement

A.A.R. discloses being employed by LUMC that has patents on exon skipping technology, some of which has been licensed to BioMarin and subsequently sublicensed to Sarepta. As coinventor of some of these patents, A.A.R. was entitled to a share of royalties. A.A.R. further discloses being *ad hoc* consultant for PTC Therapeutics, Sarepta Therapeutics, Regenxbio, Dyne Therapeutics, Lilly, BioMarin Pharmaceuticals Inc., Eisai, Entrada, Takeda, Splicesense, Galapagos, Sapreme, Italfarmaco and Astra Zeneca. In the past 5 years *ad hoc* consulting has occurred for: Alpha Anomeric. A.A.R. also reports being a member of the scientific advisory boards of Eisai, Hybridize Therapeutics, Silence Therapeutics, Sarepta Therapeutics, Sapreme and Mitorx. SAB memberships in the past 5 years: ProQR. Remuneration for consulting and advising activities is paid to LUMC. In the past 5 years, LUMC also received speaker honoraria from PTC Therapeutics, Alnylam Netherlands, Italfarmaco and Pfizer and funding for contract research from Sapreme, Eisai, Galapagos, Synaffix and Alpha Anomeric. Project funding is received from Sarepta Therapeutics and Entrada via unrestricted grants.

W.v.R.M. discloses being employed by LUMC, which has patents on exon skipping approaches for neurological disorders. In the past, some of these patents have been licensed to ProQR Therapeutics. As coinventor on these patents, W.v.R.M. is entitled to a share of milestone payments and royalties. W.v.R.M. further discloses being *ad hoc* consultant for Accure Therapeutics and Herbert Smith Freehills. Remuneration for these activities is paid to the LUMC. LUMC also received funding for contract research from UniQure and Amylon Therapeutics.

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