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Perspective

Nitrosamine Risk Assessments in Oligonucleotides

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ABSTRACT: The European Medicines Agency, the U.S. Food and Drug Administration, and other regulatory agencies expect that all pharmaceutical products be assessed for the potential presence of *N*-nitrosoamine (nitrosamine) impurities. This white paper addresses general considerations for nitrosamine risk assessments of oligonucleotide products. The authors propose a general risk assessment platform which should facilitate safe, consistent development of new treatments and alignment with regulatory expectations.

KEYWORDS: *oligonucleotide, N-nitrosoamine, nitrosamine, nitrosamine purge, risk assessment, purification, control option 4*

The European Pharma Oligonucleotide Consortium (EPOC)[1] is a collaboration among multiple pharma companies with the aim of sharing chemistry, manufacturing, and controls (CMC) knowledge as well as strategies to enable harmonization of oligonucleotide development and commercialization.

The objective of the consortium is to publish science-based recommendations for the development of oligonucleotide therapeutics in a series of technical and regulatory white papers, drawing on its collective subject matter expertise and complementing that in the literature and guidelines. This public body of prior knowledge endeavors to serve as a reference for industry practice and help establish development principles for oligonucleotides. The consortium aims at being proactive and inclusive, and it anticipates initiating wider discussion on oligonucleotide CMC practice and policy, thus expediting access to these potentially life-changing medicines.

INTRODUCTION

The presence of *N*-nitrosoamines (nitrosamines) in pharmaceutical products gained the attention of health authorities with the discovery of *N*-nitrosodimethylamine (NDMA) in valsartan in 2018. Over the subsequent months, NDMA and other related nitrosamines were discovered in further sartan medications and then in additional product classes and drugs such as ranitidine. In response to this growing public health crisis, health authorities issued guidance requiring marketing authorization holders (MAHs) to perform nitrosamine risk assessments for all synthetic drug products on the market and, if warranted, carry out confirmatory testing and make changes to the product manufacture or control strategy.[2] The requirement was later expanded to include biological products/medicines and marketing applications for new products. In addition, some health authorities have included products in clinical development in the scope of nitrosamine assessments.[3] It is essential for industry to develop robust risk assessment processes and control strategies to ensure that medicines administered to patients are safe.

The potential risk to pharmaceutical products to contain nitrosamines is briefly discussed in ICH Guideline M7 (R1) on Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. The guidance identifies nitrosamines as part of the cohort of concern, compounds which can display extremely high carcinogenic potency and therefore require more stringent control (typically to ng/day levels). Principles for assessing the potential risk of nitrosamine contamination have been prescribed by various health authorities.[2] Pharmaceutical companies and their suppliers have collaborated to develop a common implementation strategy, which has been converted by the European Federation of Pharmaceutical Industries and Associations (EFPIA) working group into the Workflows for Quality Risk Management of Nitrosamine Risks in Medicines.[4] Under the auspices of EFPIA, a general position for the risk assessment of biological products has also been developed.[5] Building on this precedent, this Perspective describes general considerations and principles that can be used to perform nitrosamine risk assessments for oligonucleotide products.

This Perspective considers synthetic production methods for oligonucleotides. Nucleotide products such as mRNA vaccines that are prepared using biological methods are considered to be within the scope of the EFPIA position paper on biological products. Nucleotide products manufactured using biological methods are not considered here, although some of the discussion, for example on purification methods, may be relevant to risk assessments for such products. Chemically synthesized therapeutic oligonucleotides include antisense

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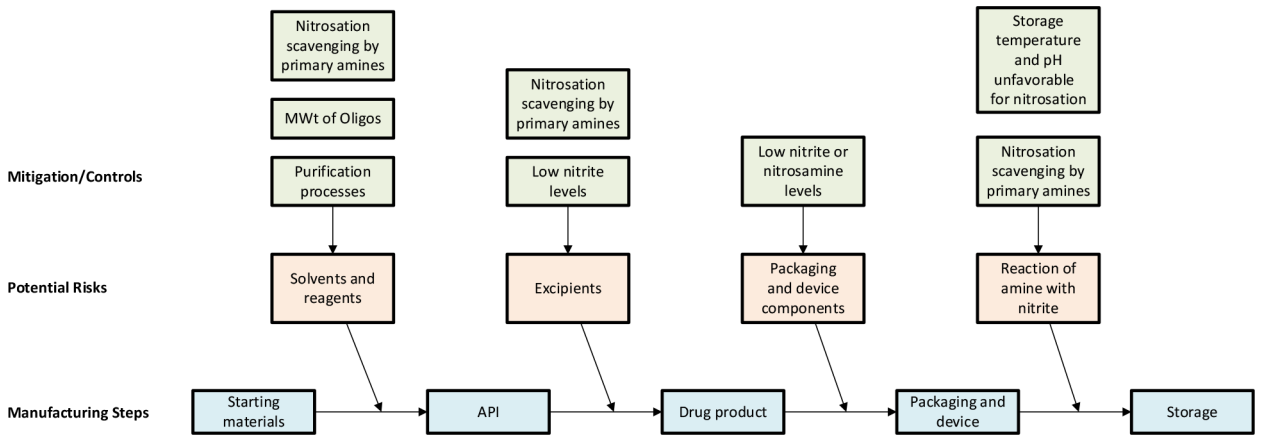

Figure 1. Nitrosamine risks and mitigations for oligonucleotide products.

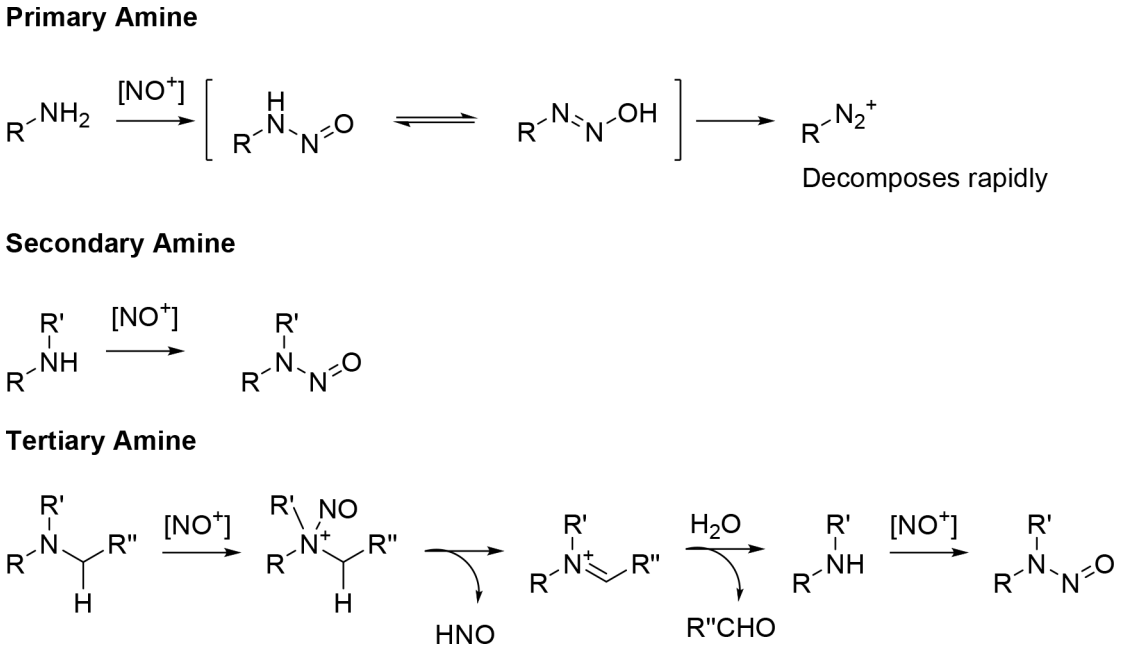

Figure 2. Nitrosation of primary, secondary and tertiary amines.

oligonucleotides, siRNAs, microRNAs, and aptamers. These oligonucleotides are single- or double-stranded, typically 16−30 nucleotides in length, and composed of a combination of natural and modified nucleosides linked via natural phosphate diesters or chemically modified linkages such as phosphorothioate diesters. Many chemically synthesized oligonucleotides are conjugated to targeting ligands, which include galactosamine-*N*-acetate (GalNAc) sugars, poly(ethylene glycol) (PEG) chains, palmitoyl, or polypeptide chains of various types.[6] Recently, hybrid enzymatic/synthetic methods have also been described. While they differ in synthesis and raw material supply chains, downstream processing of chemically and enzymatically synthesized therapeutic oligonucleotide consists of a similar series of chromatography and desalting steps.[7]

In compliance with current regulatory guidance, the proposed risk assessment process considers drug substance production methods (both synthetic and enzymatic), drug product manufacturing (including risk from excipients), and packaging.[2] The general nitrosamines control strategy for oligonucleotides is summarized in Figure 1 and discussed fully in this Perspective.

The formation of a nitrosamine requires exposure of an amine (often called a vulnerable amine and typically a secondary amine) to a nitrosating agent (typically derived from nitrite) under appropriate conditions. The chemistry of nitrosation in aqueous conditions is well-understood and has recently been reviewed in the context of pharmaceutical manufacture by Ashworth et al.[8] Amine nitrosation is also known to occur in nonaqueous solvents.[9] In general terms, secondary amines are nitrosated more rapidly than tertiary amines, which require an additional dealkylation step.[10] Primary amines are nitrosated even more quickly, but the resulting primary nitrosamines rapidly decompose under the nitrosation conditions to unstable diazonium ions without prior CYP P450 activation (Figure 2).[12] Nitrosation of primary amines is therefore not viewed as a patient risk due to the short lifetime of the primary nitrosamines and associated diazonium species produced. The nitrosation of amines is strongly pH-dependent, with the reaction being fastest at lower pH values and very slow at neutral and basic pH values in the absence of other promoters.[8] This understanding of the chemistry of nitrosation underpins much of the following

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discussion and general considerations for the nitrosamine risk assessments of oligonucleotide products.

OLIGONUCLEOTIDE-DERIVED NITROSAMINES

Oligonucleotide drug substances and related oligonucleotide impurities do not generally contain nitrosatable aliphatic

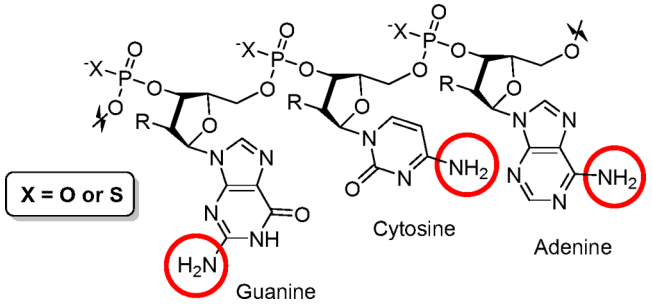

Figure 3. Oligonucleotide primary aromatic amines.

secondary amines but do contain multiple primary aromatic amine groups present in their nucleobases (Figure 3), which are susceptible to nitrosamine formation. The reaction pathway has been described by Caulfield et al. for cytosine and guanine.[11] These primary amines typically form unstable nitrosonium intermediates that rapidly decay to the hydroxyl form and nitrogen. While the reaction has been reported to be relatively slow, the primary aromatic amines in oligonucleotides are present in superstoichiometric amounts relative to aliphatic amine impurities.[10] Therefore, nonbasic primary amine groups on oligonucleotides may be considered as scavengers of nitrosating agents rather than as a risk for the formation of cohort of concern mutagens. Thus, DNA, RNA, and oligonucleotides contain moieties that may provide some inherent structural protection against the formation of mutagenic cohort of concern nitrosamine species.

The predominant mechanism for mutagenicity of nitrosamines requires metabolic activation by oxidation to form an α-hydroxynitrosamine that rapidly rearranges to a diazohydroxide form and ultimately a diazonium species, produced in vivo, that can alkylate DNA (Figure 4).[13] Nucleotide-derived nitrosamines do not contain the requisite α-C−H bond required for this mechanism of activation and as described above decompose rapidly to give the corresponding hydroxy-substituted heterocycles.

In summary, nitrosamine formation from oligonucleotide active pharmaceutical ingredients and their related substances does not present a risk to patients. The focus of the remainder of this Perspective will therefore be on small-molecule nitrosamine impurities. Potential sources of these impurities as well as process steps in which they may be controlled will be discussed in the following sections.

DRUG SUBSTANCE SYNTHESIS

Material Risks (Raw Materials and Starting Materials).

Water. Different water qualities can be used during manufacturing, including potable water, purified water, and water for injection (WFI), which can contain differing levels of nitrite. Nucleic acid active substances are typically formulated into WFI and purified water, which may also be used as solvents during manufacturing, particularly for the later steps in the synthesis. Earlier steps may be carried out in potable water. Recent publications have shown that the level of nitrite, the primary nitrosating agent under acidic aqueous conditions, in potable water rarely exceeds 66 ppb. Since WFI is purified from potable water, typically by distillation or reverse osmosis combined with ultrafiltration or deionization, it has been shown to be depleted in nitrite.[8] Recently, the use of certain ion exchange resins for water purification has been highlighted as a potential source of NDMA when used in combination with water that contains an oxidizing agent, for example water disinfected with chloramine or ozone.[2a] This was limited to certain resins, however, and the measured levels in water were very low (ppt) and present a risk only when large WFI volumes are utilized. For the very unlikely case of nitrosamines being formed due to the use of potable water prior to purification steps, it can be reasonably expected that the nitrosamine impurities would be purged.

The risk of nitrosation due to the presence of nitrite in water is therefore considered to be very low but should be assessed on a case-by-case basis.

Raw Materials. Numerous reagents are used in the production of oligonucleotides. These include various solvents, nucleotide building blocks, ligands, and other ancillary reagents used throughout solid-phase synthesis and downstream processes. While the identity of reagents can vary depending on the type of oligonucleotide being manufactured, many are broadly used. As stated previously, two types of reagents are necessary in combination to produce nitrosamines: secondary or tertiary amines and a nitrosating agent (typically derived from nitrite). Potential sources of those reagents during oligonucleotide synthesis should be identified. While some starting materials for oligonucleotide synthesis are themselves the products of multistep syntheses, for example phosphoramidites or GalNAc ligands, a risk assessment need not include detailed evaluation of early non-GMP steps, as the risk that a given step will influence the starting material quality is rated low when these steps are many chemical transformations and purifications upstream of the drug substance. In addition, the synthetic routes used for the synthesis of contributory raw materials should be risk-assessed for sources of nitrosating agents and secondary or tertiary amines (the primary aromatic amines inherent to oligonucleotide structures are not considered a risk due to their facile reaction and degradation as discussed above).

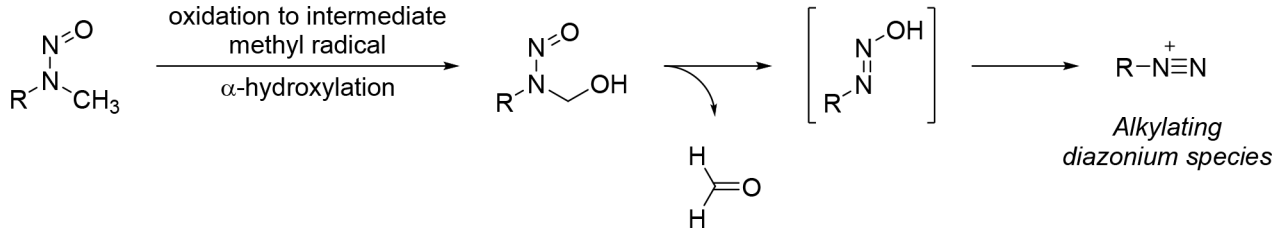

Figure 4. Mechanism of mutagenicity of nitrosamines.

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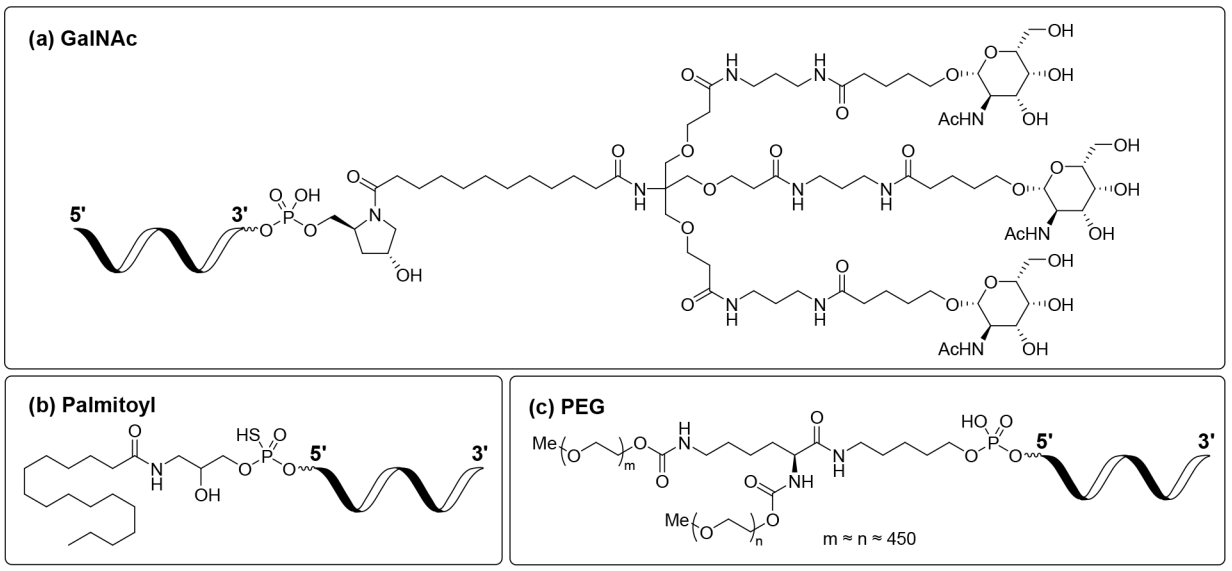

Figure 5. Chemical structures of three common oligonucleotide conjugates.

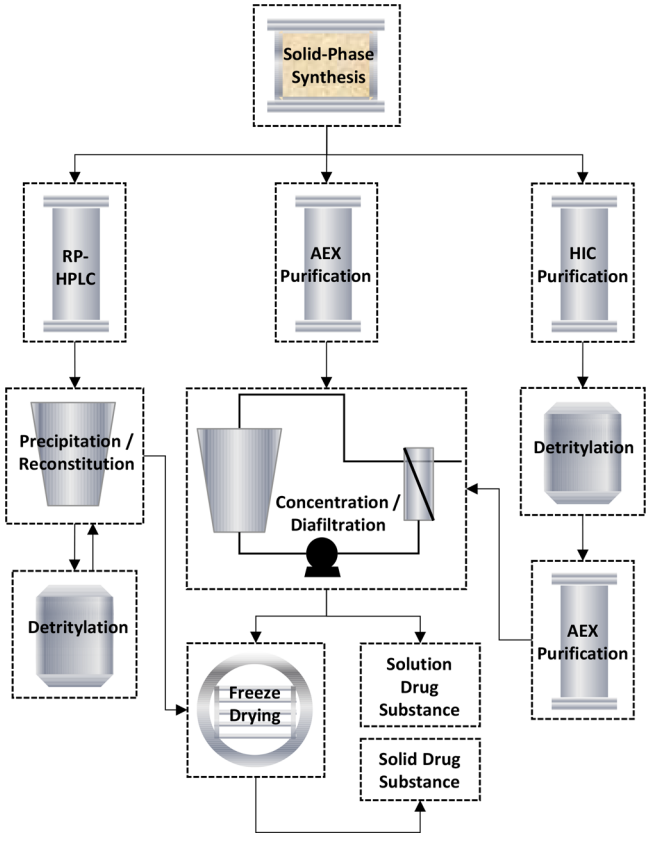

Figure 6. Generalized oligonucleotide solid-phase synthesis process highlighting three common postsynthetic pathways. Left: trityl-on RP-LC purification, solution-phase detritylation, precipitations, and freeze-drying. Middle: AEX purification with concentration/diafiltration, and freeze-drying. Right: HIC purification, solution-phase detritylation, AEX purification, and concentration/diafiltration.

There are three secondary or tertiary amines commonly present at stoichiometric levels during chemical oligonucleotide synthesis: diisopropylamine, diethylamine, and triethylamine. Diisopropylamine is the byproduct of phosphoramidite coupling during oligonucleotide assembly. Diethylamine and triethylamine are often used as mild bases to deprotect the oligonucleotide backbone on the solid support after synthesis prior to cleavage.[14] All have the ability to form nitrosamines upon reaction with nitrites if exposed to acidic conditions and should be considered in the risk assessment. Amines used as reagents may also be contaminated with low levels of nitrosamines.[15]

Oligonucleotide manufacture and coupling to a ligand (if applicable) are typically carried out in organic solvents, which will be free of nitrosating agents, and nitrosating agents are not intentionally added during any stage of oligonucleotide synthesis or purification. However, tetrazole activators that are frequently employed as activators for phosphoramidite coupling may potentially contain trace nitrites as contaminants. Tetrazole and other alkylated analogues are commonly synthesized from sodium azide and the corresponding nitriles, and it is common practice to produce the sodium azide from nitrite salts or esters like ethyl nitrite.[16] Tetrazoles are generally crystalline and purified by recrystallizations, so there is low risk that the nitrite precursor will contaminate the tetrazole reagents. The use of tetrazole activators should still be addressed in the risk assessment, as they are a potential source of nitrite in oligonucleotide products. Given that coupling produces 1 equiv of diisopropylamine, control of nitrite in the used tetrazoles may be critical to avoid formation of *N*-nitrosodiisopropylamine (NDIA).

An increasing number of therapeutic oligonucleotides are covalently attached (at the 3′ or 5′ end) to targeting or other ligands. Currently, the most common ligands contain GalNAc, palmitoyl, or PEG chains (Figure 5). Many more are under study across the industry, including various types of peptide chains and proteins. In addition to the ligand itself, linkers are employed to make the connection between the oligonucleotide and ligand. Generally, the common ligands and linkers contain primary amines linked via amide bonds. For the ligands shown, the only potential concern would be the hydroxyproline portion of the GalNAc ligand. However, the early introduction of hydroxyproline in the ligand synthesis process,[17] the low risk from formation of nitrosamines from hydroxyproline (*N*-nitroso-4-hydroxyproline has been shown to be noncarcinogenic in rat and mouse[13a]), and subsequent purification during manufacture make this a very

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Table 1. Building Blocks, Reagents, and Materials Commonly Used during the Chemical Synthesis and Purification of Oligonucleotides

chemical type	commonly used examples	notes
solvents	acetonitrile, toluene, pyridine, 3-picoline, tetrahydrofuran, dichloromethane, dimethylformamide (DMF)[a], H_2O	Only DMF is of concern due to its potential dimethylamine impurity.
nucleoside building blocks	phosphoramidites (2′-deoxy, 2′-alkoxy, RNA, 2′-fluoro, locked nucleic acid),[a] morpholinos[a]	Nucleoside phosphoramidites release 1 equiv of diisopropylamine during coupling.
		Small amine impurities from morpholino oligo building blocks are expected to purge. Morpholino oligos contain structural features that need to be considered as sources of small-molecule nitrosamines.
reagents	dichloroacetic acid, trichloroacetic acid, tetrazole,[b] 5-(ethylthio)-1*H*-tetrazole,[b] benzylmercaptotetrazole,[b] 4,5-dicyanoimidazole, *N*-methylimidazole, phenylacetyl disulfide, xanthane hydride, DDTT,[c] iodine, *tert*-butyl hydroperoxide, acetic anhydride, diisobutyl ether, triethylamine (NEt_3),[a] diethylamine ($NHEt_2$),[a] *tert*-butylamine, DBU,[c] ammonia, methylamine	Tetrazole and tetrazole-based activators like ETT and BMT are generally synthesized using sodium azide, and the excess azide may be quenched with a nitrite. Carryover of the nitrite should be considered if these activators are used.
		NEt_3 and $NHEt_2$ are frequently used at the end of synthesis, prior to cleavage, to remove phosphate backbone protecting groups and should be assessed.
solid supports	polystyrene, controlled pore glass	no issues
ligands and linkers	tris-GalNAc, poly(ethylene glycol) (PEG), palmitoyl, aminohexyl linker	There are no issues with the ligands and linkers listed, but others should be risk-assessed
aqueous purification	ammonium sulfate: buffer component (HIC solutions)[b]	Inorganic components need to be assessed for nitrite content, and if nitrite is present, the potential for reaction with vulnerable amines needs to be considered.
	Tris base/Tris hydrochloride: buffer component (HIC/AEX solutions)[a]	Some buffer salts (e.g., phosphates) may act as scavengers (see excipient discussion).
	sodium chloride: buffer component (AEX/UF/DF solutions)[b]	Tris is likely to act as a scavenger (see excipient discussion).
	sodium hydroxide: buffer component (HIC/AEX solutions), detritylation pH adjustment[b]	WFI (see water discussion)
	acetic acid: detritylation pH adjustment[b]	
	sodium phosphate monobasic monohydrate: buffer component (UF/DF solutions)	
	sodium dihydrogen phosphate dihydrate: buffer component (UF/DF diafiltration/rinse solution)	
	sodium phosphate dibasic heptahydrate: buffer component (UF/DF solutions)	
	sodium phosphate dibasic anhydrous: buffer component (UF/DF diafiltration/rinse solution)	
	water for injection (WFI): solvent	

[a]Secondary or tertiary amines or precursors thereof. [b]Should be assessed for nitrite content. [c]DDTT = (*Z*)-*N*,*N*-dimethyl-*N*′-(3-thioxo-3*H*-1,2,4-dithiazol-5-yl)formimidamide; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

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Table 2. Predicted Purge Factors for Process Steps Used in the Manufacture of Oligonucleotide Drug Substance

unit operation	purge factor	justification
solid-phase synthesis	10 per each two column volumes (CVs) of wash	Solid-phase synthesis utilizes frequent solvent rinses to wash reagents and byproducts out of the column. These washes are reported to purge soluble small molecules at a rate of 10-fold for every two CVs of wash.[22]
chromatography (RP-HPLC, AEX, HIC)	up to 1000 per chromatography operation	Uncharged small molecules tend to have significantly lower affinity and elute before the oligonucleotide product in all chromatography modes commonly employed. A purge factor of up to 1000 may be applied per chromatography operation depending on the relative retention behavior of the impurity and the product.[22]
precipitation	10	Oligonucleotides can be purified and desalted by precipitation from alcohol/water mixtures. A small molecule impurity that is freely soluble in the solvent mixture can be assigned a purge value of 10.[23]
diafiltration	10 per each five diavolumes	Small molecules that can freely pass through the diafiltration membrane while the oligonucleotide product is retained and that are soluble in the diafiltration solvent system may be assigned a purge factor of 10 per each five diavolumes utilized.[22]

low concern. Other ligands used should be risk-assessed according to the same principles as other therapeutics. In general terms it can be factored in when doing a risk assessment that there is much more depletion of ligand small-molecule impurities when the solid-state coupling takes place at the 3′ end (washings during oligo synthesis; see purge discussion below) compared to coupling of the ligand at the 5′ end.

In summary, the majority of raw materials used in oligonucleotide synthesis are not expected to present a risk of nitrosamine formation for the reasons discussed above. Where risk is identified in a raw material, additional risk assessment or mitigation may need to be carried out.

Chemical Synthesis of Oligonucleotides. Synthetic antisense oligonucleotides (ASOs) or siRNAs are generally prepared by solid-phase synthesis techniques using a modern version of the original phosphoramidite approach.[18] After synthesis, the oligonucleotides are cleaved from the solid support, chromatographically purified, desalted, in some cases further purified in solution, and then isolated as a solid drug substance or directly as a drug product solution.[19] Some processes utilize late-stage protecting group removal (like detritylation) and can feature conjugation to different ligands.

Oligonucleotides are typically manufactured using a platform solid-phase synthesis (SPS) process, which utilizes a computer-controlled synthesizer and typically consists of sequential cycles of four steps each:[7,20] (1) deprotection of the 4,4′-dimethoxytrityl (DMT) alcohol protecting group from the solid support or the growing oligonucleotide chain, (2) phosphoramidite coupling, (3) oxidation/thiolation of the resulting P(III) phosphite linkage to a P(V) species, and optionally (4) acyl capping. Once the oligonucleotide of the desired length has been assembled on the solid support, it is commonly treated with a secondary or tertiary amine to deprotect the backbone and then with an aqueous amine or other base to effect cleavage from the support and deprotection of the base protecting groups. Platform SPS typically incorporates washing cycles between the sequential steps to remove excess reactants, impurities, and byproducts from the growing oligonucleotide on the solid support. No nitrosating agents are intentionally added during these steps where a secondary or tertiary amine (e.g., triethylamine) may be used.

Alternative Syntheses of Oligonucleotides. In addition to the solid phase/chemical synthesis of oligonucleotide drug substances, other manufacturing methods such as enzymatic methods and liquid-phase synthesis have been reported. Such methods are out of the scope of this Perspective, although the rationale discussed here may be applied to the risk assessment of these complementary and innovative methods where equivalent unit operations and materials are used.[7,20]

Chromatographic Purification and Downstream Processing. Purification is a critical step to ensure product quality following all oligonucleotide manufacturing processes. Three different approaches are typically employed in large-scale purification of chemically synthesized oligonucleotides (Figure 6):

(1) Reversed-phase liquid chromatography (RP-LC), where water and methanol with added sodium acetate are used as mobile phases. The purified DMT-protected substrate is then deprotected by acetic acid followed by sodium hydroxide neutralization. The final oligonucleotide is precipitated in ethanol followed by lyophilization.

(2) In the traditional anion exchange chromatography (AEX) approach, the crude product is purified, followed by ultrafiltration/diafiltration (UF/DF) and potential lyophilization.

(3) Recently, a fully aqueous purification process was established that employs hydrophobic interaction chromatography (HIC), a solution-phase detritylation, and AEX.[21] The AEX eluate is then concentrated, buffer-exchanged via a UF/DF step, and adjusted to the final concentration for drug product manufacturing. Like RP-LC and traditional AEX purification, reagents typically used in the HIC/AEX process (Table 1) do not include secondary or tertiary amines. Phosphates and Tris (as applicable) can act as scavengers (see excipients discussion) and eliminate the risk of nitrite potentially present in inorganic components of eluents. Hence, the risk of formation of nitrosamine impurities can be excluded.

As noted above in the water purification discussion, any risk of contamination from leachable substances in the processing equipment, especially single-use, should be investigated.

Purge Factors for Oligonucleotide Synthesis. A recent paper from EPOC discusses the purge of impurities during oligonucleotide synthesis with particular focus on process-related impurities (PRIs). These include small-molecule impurities such as starting materials, reagents, solvents, and process byproducts (e.g., protecting group derivatives).[22] The results and discussion in this Perspective are also relevant to the purge of small nitrosamine and vulnerable amine impurities that may be similarly introduced during the synthesis of oligonucleotides.

Due to the large molecular mass difference between oligonucleotide drug substances and the small-molecule impurities, these chromatography steps are typically several fold more effective at depleting small-molecule entities than the crystallization steps often used in small-molecule drug substance syntheses to purge impurities. The evaluation indicates that there is a low risk of nitrosamine impurities in raw materials and processes involved in the oligonucleotide purification. Furthermore, the purification processes provide a good opportunity

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to remove any nitrosamine and vulnerable amine impurities in the system.

Table 2 contains theoretical purge factors for various processing steps used in the manufacture of oligonucleotide drug substance.

In summary, the extensive purification processes used in oligonucleotide manufacture are expected to efficiently purge any small-molecule nitrosamines and vulnerable amines that may be present in the drug substance.

Drug Substance Container Closure. Oligonucleotide drug substances are often isolated as lyophiles but may also be kept in aqueous solution.[19] The lyophilized drug substance may be stored in plastic containers. Solution APIs could be stored in plastic bottles or other suitable containers. For lyophiles, reactions in a solid state are typically very slow, and therefore, extraction or leaching of nitrosamines or precursors from the container is unlikely. For any contact material employed for solution API, storage material compatibility as well as extractable and leachable (E&L) studies should be performed. For any form of drug substance (lyophilized or in solution), it is recommended to check suppliers' declarations for the risk of the presence of nitrosamines or relevant vulnerable amine precursors in the packaging material that could leach into the oligonucleotide drug substance. If this reveals a risk, an E&L study specific to the compound of concern should be performed using analytical evaluation thresholds (AETs) that reflect the Acceptable Intake of the corresponding nitrosamine. In summary, risks from packaging materials should be considered when conducting the drug substance risk assessment.

DRUG PRODUCT MANUFACTURING AND PACKAGING

Oligonucleotide formulations can commonly be divided into aqueous solutions (ASs) and lipid nanoparticles (LNPs). The risk for nitrosamines can be introduced or controlled through the formulation components, the primary packaging and device, and the process and storage conditions. In most cases, oligonucleotide medicines are formulated using excipients that are not susceptible to nitrosation (e.g., acetate, citrate, phosphate-buffered saline) and present no intrinsic risk of nitrosamine impurity formation, as they do not contain vulnerable amine structural fragments. However, excipients can contain nitrite impurities that enable nitrosamine formation in the presence of vulnerable amines. In addition to the discussion of excipients below, the following aspects of drug product manufacturing are considered. It is important to note that several of the excipients used in drug product manufacture are known to inhibit the formation of nitrosamines. If these excipients (citrate, etc.) are used, the risk of nitrosamine formation can be considered negligible.[24] Additional details on the risk factors stemming from the excipients, drug product manufacture and storage, and primary packaging are provided below.

Excipients. Excipients typically used in standard aqueous oligonucleotide solutions for injection are listed in Table 3. This table is not exhaustive but should cover the majority of common excipients used in oligonucleotide formulations.

In-house testing by a number of companies and consultation of a cross-industry excipient database[25] have shown that the nitrite content in relevant excipients (specifically sodium citrate, sodium chloride, sodium carbonate, monobasic sodium hydrogen phosphate, citric acid, sucrose) is less than 1 ppm. For most of these materials, the testing has shown the level of nitrite to be

Table 3. Typical Excipients in Solutions for Injection

function of excipient class	discussion	potential to introduce nitrosating agents
solvent/water	Oligonucleotide drugs are typically sterile solutions for parenteral injection and therefore use WFI.	The risk of nitrosation due to the presence of nitrite in water is considered to be very low but should be assessed on a case-by-case basis (see the discussion of WFI above).
tonicity agents	Tonicity agents are included to match the formulation tonicity with that of the administration site. This is usually sodium chloride or a saccharide-derivative.	Typically, there is a low risk[a] of introduction of nitrite or vulnerable amines
buffers	Buffers are used to control the pH of the formulation and ensure good stability while minimizing injection discomfort. These can be weak acids or bases or buffer systems consisting of e.g., acetic acid and Tris, sodium dihydrogen phosphate, sodium acetate, or sodium citrate.	Typically, there is a low risk[a] of introducing nitrite or vulnerable amines. Citrate and Tris may act as scavengers. Phosphate buffers have been reported to scavenge sodium nitrite at pH 6.4.
pH-modifiers/adjusters	Sodium hydroxide and hydrochloric acid are the most commonly used strong base and acid.	Typically, there is low risk[a] of introducing nitrite or vulnerable amines
anticoagulant	Inorganic divalent salts such as calcium chloride and surfactants such as polysorbates, poloxamers, and oligosaccharides (sucrose) are used to prevent coagulation.	low risk[a] of introducing nitrite
cryoprotectants	oligosaccharides, polyols	low risk[a] of introducing nitrite
antioxidants	e.g., vitamin E	low risk[a] of introducing nitrite
LNP components	solid lipids (e.g., phospholipids, cholesterol, cationic lipids, shielding lipids), emulsifier(s)/surfactant(s), cosurfactantd (optional), solvents/cosolvents (optional)	The cationic lipids can include secondary or tertiary amines as well as quaternary ammonium compounds. Their potential to be transformed into nitrosamines needs to be assessed on a case-by-case basis.
gases	nitrogen	Typically, there is low risk[a] of introducing NO_x or vulnerable amines

[a]Low risk refers to materials that contain <5 ppm nitrite or vulnerable amine.

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Table 4. Summary of Oligonucleotide Nitrosamine Risk

operation	risks	controls	overall risk
raw materials	impurities: amines	specifications	low
	nitrosating agents	purging in downstream processing	
synthesis	amine reagents used	SPS washing of reagents	low
	amine impurities and byproducts	avoidance of nitrosating agents	
purification		high purge capability	low
excipients	impurities	inhibition potential	low
formulation	impurities in excipients	pH of drug product solution	low
		specifications	
		use of inhibitors	
packaging	extractables and leachables	supplier's declarations, data generation in E&L studies, specifications	low (when appropriate controls are in place)
	presence of nitrosamines or vulnerable amines in rubber stoppers		
storage	see formulation and packaging (leachables over shelf life)	pH of drug product solution	low (when appropriate controls are in place)
		leachables data, specifications, use of inhibitors	
overall process			low

less than 0.5 ppm. With the exception of Tris, the typical excipients do not contain a nitrogen moiety, and the use of amines in the excipient manufacture is unlikely. Tris is a tertiary-alkyl-substituted primary amine and is expected to act as an effective scavenger in the presence of nitrosating agents.

Lipid nanoparticles (LNPs) used in some formulations can contain a number of functional excipients in addition to the API: solid lipids (e.g., phospholipids, cholesterol, cationic lipids, shielding lipids), emulsifier(s)/surfactant(s), cosurfactants (optional), and solvents/cosolvents (optional).[26] For formulations used for oligonucleotide drugs, this may be a combination of phospholipids, cholesterol, PEG-lipids, and ionizable cationic lipids.[27] The cationic lipids can include secondary or tertiary amines as well as quaternary ammonium compounds (e.g., phosphatidylcholine) with pK_a values typically below 10. These amines can be sensitive to nitrosamine formation when exposed to nitrosating conditions. The sensitivity of tertiary amines and quaternary ammonium compounds for nitrosamine formation depends on the substituents and requires case-by-case evaluation, particularly when the amine is at the end of the lipid chain. While tertiary and quaternary amines can be nitrosated, this requires additional chemical steps, and the greatest risk is likely the presence of secondary amine contaminants that can react with nitrite from other excipients.[28] Therefore, the risk assessment needs to take into account the likelihood that nitrites are present in LNP formulations. However, considering the molecular weight of the amines, it is unlikely that nitrosamines potentially formed from these amines can be oxidized by cytochrome P450 (CYP) and form a mutagenic/carcinogenic species. In cellular systems this oxidation mostly occurs enzymatically by CYP isoenzymes. Large molecules with a nitrosamine group would be sterically unfavorable substrates for CYP binding and activation, in which the N−N bond needs to be in proximity to the heme group of CYP.[29] The heme group is embedded within CYP, and access can take place only through channels, which restricts the size of oxidation substrates. The mutagenicity of most nitrosamines has been shown to decrease significantly as the size exceeds 12 to 14 carbon atoms.[30] The relatively high molecular weight of LNP components therefore makes it very unlikely that the predominant mechanism for activation of nitrosamines will take place with such large substrates.

Liquid Compounding/Filling. Compounding and filling typically take place at room temperature under aqueous conditions. As injectable medicines, oligonucleotides are formulated in WFI (see the discussion above).

Primary Packaging and Device (PP&D). Oligonucleotide products are typically stored in impermeable glass or low-permeability resin containers with elastomer stoppers. Compatibility of the drug product with the container closure system is typically established through E&L studies. The risk of contamination with nitrosamines or nitrosamine precursors should be assessed based on supplier statements and/or data. Based on that information, if a potential risk is identified, it can be determined whether a nitrosamine-specific E&L study is needed under product-relevant conditions, applying AETs using established nitrosamine acceptable intakes.

Sterilization. The mechanism of sterilization for packaging components should also be taken into account, with particular attention to any use of nitrogen dioxide to sterilize packaging components prior to filling and assembly or as a terminal sterilization/surface sterilization procedure (as applicable) after filling and stoppering of the primary container. Nitrogen dioxide is a nitrosating agent and can react with vulnerable amines. This requires a product-specific evaluation of the impact of NO_x sterilization on the oligonucleotide drug product components if there is a risk that nitrosatable amines are present.

Drug Product Storage Conditions. From a stability perspective, oligonucleotide formulations containing phosphate buffers can be considered safe, as they contain an inherent inhibitor against nitrosamine formation. Phosphate buffers have been reported to scavenge sodium nitrite at pH 6.4 through the formation of a five-membered-ring intermediate from dihydrogen phosphate and dinitrogen trioxide, which has weaker nitrosation ability than N_2O_3.[31] In addition, the pH of oligonucleotide formulations is typically near neutral, which is above the optimum pH for nitrosation with nitrite.[8]

In summary, product pH and the presence of nitrosation scavenging groups within the formulation lead to the conclusion that there is minimal risk of nitrosamine formation during drug product storage.

CONCLUSIONS

The overall conclusions for the risks and controls during different operations related to oligonucleotide production are summarized in Table 4. The risk for formation of nitrosamines in the final drug product is low, but the contributions from components should be verified by evaluating their synthesis,

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Org. Process Res. Dev. XXXX, XXX, XXX−XXX

applying relevant controls, consulting suppliers, and/or generating relevant data.

These general considerations can be used to support the risk assessment of oligonucleotide products and can also inform decisions during the development of new oligonucleotide products. However, this Perspective does not preclude a thorough product-specific risk assessment.

The authors hope that this application of platform thinking to nitrosamine risk assessment for oligonucleotide products will ensure high-quality and safe products for patients, facilitate rapid development of new treatments, and support constructive discussions with health authorities.

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Notes

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