Critical considerations for developing nucleic acid macromolecule based drug products

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Protein expression therapy using nucleic acid macromolecules (NAMs) as a new paradigm in medicine has recently gained immense therapeutic potential. With the advancement of nonviral delivery it has been possible to target NAMs against cancer, immunodeficiency and infectious diseases. Owing to the complex and fragile structure of NAMs, however, development of a suitable, stable formulation for a reasonable product shelf-life and efficacious delivery is indeed challenging to achieve. This review provides a synopsis of challenges in the formulation and stability of DNA/m-RNA based medicines and probable mitigation strategies including a brief summary of delivery options to the target cells. Nucleic acid based drugs at various stages of ongoing clinical trials are compiled.

Introduction

Since the first clinical trial of nucleic acid (NA) therapy in 1990 for immunodeficiency syndrome, NAs have gained escalated importance in the treatment of complex diseases including cancer and autoimmune diseases [1,2]. In October 2003, the first gene therapy (Gendicine®) was approved in China for treatment of head and neck squamous cell carcinoma. In November 2012, the European market approved the first gene therapy (Glybera®) for the treatment of lipoprotein lipase deficiency disease. Despite being a relatively new paradigm, the field of NA therapy has grown tremendously. Up until 2012, at least 1800 NA-based clinical trials had been completed or been approved worldwide [1]. The vast majority of NA therapy is targeted toward cancer (64.4%), cardiovascular disease (8.4%) and monogenic diseases (8.7%). The remaining 28.5% of NA therapy was targeted toward other indications including neurological, ocular and inflammatory diseases. Table 1 provides a list of NA molecules currently under clinical evaluation.

NAs can be broadly classified based on the size and origin of the molecule. Those that are made synthetically and are about 12–25 base pairs (bp) are typically called oligonucleotides. This includes small interfering (si)RNA, microRNA (miRNA) and antisense oligonucleotides such as

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TABLE 1

Nucleic-acid-based (NAM and short chain NA) therapeutics at various stages of clinical development.

No.	Drug	Clinical Phase	Action	Delivery	Company
Oligonucleotide					
1	Macugen® (pegaptanib)	Approved	Anticancer; VEGF receptor antagonist; angiogenesis inhibitor	PEGyated; ophthalmic	Eyetech/Pfizer
2	HEPLISAV	III	Protein subunit vaccine; prophylactic vaccine; TLR-9 agonist	IM	Dynavax Technologies Corp
3	E-10030	III	PDGF-B ligand inhibitor; PDGF receptor antagonist	PEGylated; opthalmic	Archemix Corp
4	Bevasiranib Sodium	III terminated	Antineovascularisation; VEGF inhibitor	Ophthalmic	Acuity Pharmaceuticals
5	Mycobacterial cell wall–DNA complex	III terminated	Anticancer; apoptosis stimulator	Long-acting; intravesical	Bioniche Life Sciences
6	ARC-1779	II completed	Factor VIII antagonist; coagulation inhibitor	IV	Archemix Corp
7	GRN-163L	II completed	Anticancer; telomerase inhibitor	IV; infusion	Geron Corp
8	REG-1	II completed	Factor IX antagonist; coagulation inhibitor; antidote	IV	Regado Biosciences
9	AGN-211745	II completed	Antineovascularisation; VEGF receptor gene inhibitor; siRNA	Injectable; ophthalmic	Sirna Therapeutics
10	ALN-RSV01	II completed	Viral replication inhibitor; siRNA agent; antiviral	Nasal	Alnylam Pharmaceuticals
11	MIDGE	I/II	Anticancer; immunostimulant		Mologen Holding AG
12	Avrina	II	NF-κB inhibitor; anti-inflammatory	Dermatological	Anesiva
13	Oligonucleotide decoys	II	NF-κB inhibitor; anti-inflammatory	Dermatological	AnGes MG
14	AS-1411	II	Nucleolin inhibitor; apoptosis stimulator; BCL2 gene inhibitor	IV; infusion	Aptamera
15	AVT-01	II	Anti-inflammatory; STAT-1 modulator	Inhalant	Avontec GmbH
16	AVT-02	II	Anti-inflammatory; STAT-1 modulator	Dermatological	Avontec GmbH
17	Agatolimod	II	Anticancer; immunostimulant; adjuvant; TLR-9 agonist	Intratumotal, SC, IV	Coley Pharmaceutical Group
18	IMO-2055	II	Anticancer; antimicrobial; immunomodulator; TLR-9 agonist	Injectable	Idera Pharmaceuticals
19	Li-28	II	Anticancer; immunomodulator	Injectable	Oligovax SA
20	IMO-2125	I completed	Anticancer; immunomodulator; interferon alpha ligand; IL-12 stimulator; TLR-9 agonist; antiviral	SC	Idera Pharmaceuticals
21	CpG-28	I completed	Anticancer; immunostimulant; TLR-9 agonist	Intratumoral	INSERM
22	NU-172	I	Factor IIa antagonist	IV	ARCA Biopharma, Inc
23	AVE-0675	I	Immunomodulator; TLR-9 agonist	Inhalant	Coley Pharmaceutical
24	Oligonucleotide decoys	I	NF-κB inhibitor; cardioprotectant; anti-inflammatory	IV	Osaka University
25	GRO-29A	I	Anticancer; NF-κB inhibitor; I-kappa kinase gamma inhibitor	IV	University of Louisville
Oligonucleotide; virus (recombinant)					
1	BLT-HIV	I	Anticancer; CCR5 gene inhibitor; siRNA agent; TAT gene inhibitor; REV gene inhibitor	IV	Benitec Ltd
Oligonucleotide; antigen					
1	HEPLISAV™	II	Subunit vaccine; therapeutic vaccine; anticancer; adjuvant; TLR-9 agonist	IM	Dynavax Technologies Corp
Oligonucleotide; drug combination					
1	BioThrax® + VaxImmune™	I	Vaccine; immunostimulant; adjuvant; TLR-9 agonist	IM	Emergent BioSolutions
Oligonucleotide; DNA technology					
1	EHT-899	II	Immunomodulator; antiviral	Oral	Enzo Biochem
Oligonucleotide; RNA technology					
1	Ampligen®	I/II	Anticancer; immunostimulant; TLR-3 agonist; 2,5-oligoadenylate synthetase stimulator; antiviral	IV	Hemispherx Biopharma

TABLE 1 (*Continued*)

No.	Drug	Clinical Phase	Action	Delivery	Company
Oligonucleotide (conjugated); peptide					
1	AVR-118	II	Viral replication inhibitor; immunomodulator; appetite stimulant; anti-inflammatory; CCR5 antagonist	SC	Advanced Viral Research Corp
2	AVR-123	II	Anticancer; anti-inflammatory; CCR5 chemokine antagonist; vulnerary agent	Aerosol, dermatological	Advanced Viral Research
Protein (conjugated); oligonucleotide (antisense)					
1	AVI-5126	II terminated	MYC gene inhibitor; vasoprotectant	IV	Sarepta Therapeutics
Protein (conjugated); antigen presentation system; oligonucleotide (conjugated)					
1	CYT-004-MelQbG10	II completed	Therapeutic vaccine; anticancer; T lymphocyte stimulator	IV	Cytos Biotechnology AG
Oligonucleotide (conjugated); antigen					
1	Abetimus	III terminated	Immunosuppressant	IV	La Jolla Pharmaceutical Co
2	Tolamba™	II completed	Allergen; vaccine; immunosuppressant; TLR-9 agonist	SC	Dynavax Technologies Corp
Oligonucleotide (antisense)					
1	Vitravene (fomivirsen)	Launched	Viral replication inhibitor	Intravitreal injection	Ionis Pharmaceuticals
2	Alicaforsen	III completed	Anti-inflammatory; ICAM-1 inhibitor; cell adhesion molecule inhibitor	Rectal; oral	Ionis Pharmaceuticals
3	Oblimersen	III	Anticancer chemosensitizer; BCL2 gene inhibitor	IV infusion	Genta
4	Mipomersen	III	Apolipoprotein B100 antagonist; antihypercholesterolemic	SC	Ionis Pharmaceuticals
5	Kappaproct®	III	Nuclear factor kappa B inhibitor; anti-inflammatory	Rectal; oral	InDex Pharmaceuticals
6	AP-12009	III terminated	Anticancer; TGF beta 2 ligand inhibitor;	Intratumoral	Antisense Pharma GmbH
7	Custirsen sodium	II completed	Clusterin inhibitor; anticancer	IV	Ionis Pharmaceuticals
8	LY-2181308	II completed	Survivin protein inhibitor; anticancer; apoptosis stimulator	IV	Ionis Pharmaceuticals
9	SPC-2996	II completed	Anticancer; apoptosis stimulator; BCL2 gene inhibitor	Injectable	Santaris Pharma A/S
10	ASM-8	II completed	Anti-inflammatory; CCR3 chemokine modulator; cytokine receptor common beta chain modulator	Inhalant	Pharmaxis
11	Antisense oligonucleotides	II completed	Stearoyl CoA desaturase-1 inhibitor; hypoglycemic	IV	Ionis Pharmaceuticals
12	Mipomersen	II completed	Apolipoprotein B100 antagonist; antihypercholesterolemic	Capsule	Ionis Pharmaceuticals/ Genzyme
13	AVI-4020	II	Antiviral	IV	Sarepta Therapeutics
14	AVI-4126	II	Anticancer; vasoprotectant	Microparticle; IV	Sarepta Therapeutics
15	AVI-4557	II	CYP450 reductase modulator; drug metabolism modulator	SC; IV	Sarepta Therapeutics
16	HGTV-43	II	Genetically engineered autologous cell therapy; HIV inhibitor	IV; infusion	Enzo Biochem
17	EN-101	II	Acetylcholinesterase inhibitor	Oral	Ester Neurosciences Ltd
18	GS-101	II	Insulin receptor substrate-1 inhibitor; angiogenesis modulator	Ophthalmic	Gene Signal SAS
19	ATL-1102, Antisense Therapeutics/ Teva	II	Integrin alpha-4/beta-1 antagonist; anti-inflammatory; CD49d antagonist	SC	Ionis Pharmaceuticals
20	GTI-2501	II	Anticancer; ribonucleotide reductase inhibitor; metastasis inhibitor	IV	Lorus Therapeutics
21	LOR-2040	II	Anticancer; Ribonucleotide reductase inhibitor	IV; Intravesical	Lorus Therapeutics
22	MG-98	II	Anticancer; DNMT1 gene inhibitor;	IV	MethylGene
23	RX-0201	II	Anticancer; AKT gene modulator	IV	Rexahn Corp
24	AEG-35156	II terminated	Anticancer; apoptosis stimulator; caspase modulator; siRNA agent	IV	Aegera Therapeutics
25	Cenersen	II terminated	Anticancer; p53 gene modulator	IV	Eleos

TABLE 1 (*Continued*)

No.	Drug	Clinical Phase	Action	Delivery	Company
26	Lexgenleucel-T	II	Retrovirus based gene therapy; HIV replication inhibitor; antiviral	IV	VIRxSYS Corp
27	EGFR antisense DNA	I/II	Anticancer; apoptosis stimulator; gene therapy; epidermal growth factor antagonist	Intratumoral	University of Pittsburgh
28	AVI-4126	I completed	Anticancer; MYC gene inhibitor; vasoprotectant	Controlled release; oral	Sarepta Therapeutics
29	EZN-2968	I completed	Anticancer; angiogenesis inhibitor; hypoxia inducible factor-1 alpha inhibitor	Injectable	Enzon Pharmaceuticals Inc.
30	iCo-007	I completed	Raf protein kinase family inhibitor	Long acting; ophthalmic	iCo Therapeutics Inc.
31	IONIS-GCGR$_{RX}$	I completed	Glucagon-like peptide 1 modulator; insulin release stimulator; glucagon receptor modulator; hypoglycemic	SC	Ionis Pharmaceuticals
32	LY-2275796	I completed	Anticancer; EIF protein kinase inhibitor; angiogenesis inhibitor;	IV	Ionis Pharmaceuticals
33	LErafAON	I completed	Radiosensitizer; anticancer; Raf 1 protein kinase inhibitor; anticancer multidrug resistance inhibitor	Liposome; IV	INSYS Therapeutics
34	G-4460	I	Cyclooxygenase 2 inhibitor; anticancer; apoptosis stimulator; MYB gene inhibitor; reverse transcriptase inhibitor; Bcl-2 gene inhibitor	Liposome	Temple University
35	111In antisense oligonucleotide CDK inhibitor imaging (cancer)	I	CDK inhibitor; imaging agent; radioimmunodiagnostic; radiodiagnostic	IV	University of Toronto
36	Veglin	I	Anticancer; VEGF antagonist; angiogenesis inhibitor; metastasis inhibitor	IV	VasGene Therapeutics
Gene therapy, viral					
1	LPL [S447X]	III completed	Lipid metabolism modulator; antiarteriosclerotic; AAV based therapy; lipoprotein lipase stimulator	IM	Amsterdam Molecular Therapeutics BV
2	TG-4010	II/III	Recombinant viral vector vaccine; therapeutic vaccine; anticancer; IL-2 agonist	Subcutaneous	Transgene SA
3	Mydicar	II completed	Sarco-endoplasmic Ca^{2+} ATPase 2 modulator; cardioprotectant; AAV based therapy	IV	Celladon Corp
4	OXB102	II completed	Antiparkinsonian; retrovirus based therapy; dopa decarboxylase stimulator	IV	Oxford BioMedica plc
5	SCID-X	II	Immunomodulator; retrovirus based therapy; genetically engineered autologous cell therapy	IV	Assistance Publique-Hopitaux de Paris
6	CERE-120	II	CNS modulator; antiparkinsonian; AAV based therapy	Implant; injectable	Ceregene
7	Reximmune-C	II	Anticancer; retrovirus based therapy; metastasis inhibitor	IV	Epeius Biotechnologies Corp
8	AAV RPE65	II	RPE gene stimulator; AAV based therapy	Local acting, ophthalmic	Targeted Genetics Corp
9	VMDA-3601	II	VEGF modulator; plasmid based therapy; angiogenesis stimulator	IM	ViroMed
10	AdGVPEDF.11D	I completed	Pigment epithelium-derived factor ligand; AV based therapy; angiogenesis inhibitor	Opthalmic	GenVec
11	NP-2	I completed	Opioid receptor agonist; analgesic; viral vector based therapy	Sustained release; local acting	Diamyd Inc
12	MGMT-transduced hematopoietic cells	I	Chemoprotectant; retrovirus based therapy; hematopoietic stimulant	IV	Case Western Reserve U.
13	KH-901	I	Therapeutic vaccine; anticancer; GM-CSF agonist; AV based therapy	Intratumoral	Chengdu Kanghong Biotechnologies
14	Dystrophin gene therapy	I	AAV based therapy; dystrophin stimulator	Injectable	Genethon

TABLE 1 (*Continued*)

No.	Drug	Clinical Phase	Action	Delivery	Company
15	SCH-721015	I	Anticancer; AV based therapy; interferon alpha 2 ligand modulator	Intravesical	Schering-Plough Corp
Gene therapy, nonviral					
1	EGEN-001	II	Anticancer; IL-12 agonist; immunomodulator; gene therapy	IP	EGEN Inc
2	Gp91phox transgene-encoded MFGS	II	Blood system agent; retrovirus based gene therapy	Infusion	Johann Wolfgang Goethe University
3	VM-202	II	Plasmid based gene therapy; angiogenesis stimulator; hepatocyte growth factor agonist	IM	ViroMed Co Ltd
4	SGT-53	I	p53 gene stimulator	Liposome	Synergene Therapeutics
5	SGT-94	I	Anticancer; retinoblastoma protein modulator; lipid based therapy	Liposome; injectable; infusion	SynerGene Therapeutics
Cell delivery system; cell therapy					
1	NT-501	III completed	CNTF receptor agonist	Ophthalmic implant	Neurotech Pharmaceuticals
2	AC-9401	II	Anticancer; IL-2 agonist; genetically engineered autologous cell therapy	Injectable	AntiCancer treatment
3	GRASPA	II	Anticancer; asparaginase modulator	Injectable	ERYtech Pharma
4	Cell therapy (dopamine producers, Parkinson's)	II	Dopamine receptor agonist; neuroprotectant; antiparkinsonian	Injectable	Titan Pharmaceuticals
5	NsG0202	I	NGF receptor agonist	Injectable	NsGene A/S
Gene-directed enzyme prodrug therapy					
1	Sitimagene ceradenovec	III	Thymidine kinase modulator; anticancer; AV based therapy	Injectable	Ark Therapeutics Group plc
2	CTL-102	II	Anticancer; nitro-oxidoreductase stimulator; AV based therapy	Intratumoral	Cobra Biomanufacturing plc
3	MetXia-P450	II	Anticancer; retrovirus based therapy	Intratumoral	Oxford BioMedica plc
4	Adv-tk/prodrug	II	Anticancer; AV based gene therapy; thymidine kinase stimulator	Intratumoral	Advantagene
5	Ad5-CD/TKrep	I	Anticancer; AV based therapy	Injectable	Harvard Medical School
6	Pro-1	I	Anticancer; lipid based therapy; thymidine kinase stimulator	Liposome; IV	Protiva Biotherapeutics

Abbreviations: IV, intravenous; IM, intramuscular; SC, subcutaneous; IP, intraperitoneal; AV, adeno virus; AVV, adeno-associated virus; VEGF, vascular endothelial growth factor; NAM, nucleic acid macromolecule; m-RNA, messeanger ribonucleic acid; DNA, deoxy-ribonucleic acid; LNA, locked nucleic acid; SC, supercoil; OC, open circular; LN, leniar; Tm, midpoint of thermal transition.
This list was compiled from various sources including Pharmacircle.com and clinicaltrials.gov.

locked nucleic acids (LNAs). In general these NAs are used as interference technologies. In this review, for simplicity, NAs that are larger in size (≥200 base pairs), biologically or semibiologically derived and could potentially be considered as prodrugs (expressing proteins of therapeutic interest) are termed nucleic acid macromolecules (NAMs). These molecular modalities include DNA and mRNA, and the schematic representation of expression technology is shown in Fig. 1. Recently, mRNA has emerged as an advantageous macromolecule among NAMs as compared with plasmid DNA [3,4]. These advantages include transient expression, lack of integration to genomic material, no need to cross the nuclear barrier (can be expressed in nondividing cells) and no requirement of additional plasmid backbone or viral packing proteins. However, because the conformation of mRNA is believed to play an important part in the translation to active protein, a lack of understanding of structure–function relationships could lead to biased *in vivo* results. The transient pulse-like delivery of vascular endothelial growth factor (VEGF)-A synthetic chemically modified mRNA (modRNA) was crucial because the sustained delivery of VEGF-A DNA was found to have an adverse effect on vascular function [5].

NAM therapeutics are currently being utilized in three different approaches in oncology: (i) increasing the immune response against tumors [6]; (ii) repairing the defective gene causing the cancer [7]; and (iii) suicide gene strategies [8]. Utilizing NAMs as a modality for cancer therapy has several advantages over protein subunit vaccines including low cost of production, fewer stability issues and no risk of infection as with live attenuated viruses [9,10]. Further, persistence of the NAM code within the host cells leads to prolonged immune response compared with adjuvanted protein subunit vaccines [11]. NAMs can be engineered to include more than one coding sequence including immunomodulating codes such as interleukin (IL)-12 or IL-15 [12]. The immunomodulators or stimulators when co-expressed induce stronger antigen-specific responses and provide enhanced immunity. NAMs can be delivered 'naked', using a viral system or as advanced delivery systems *in vivo*, *in vitro* and *ex vivo*. Although the viral methods, which include retrovirus (RV), adenovirus (AV) and adeno-associated

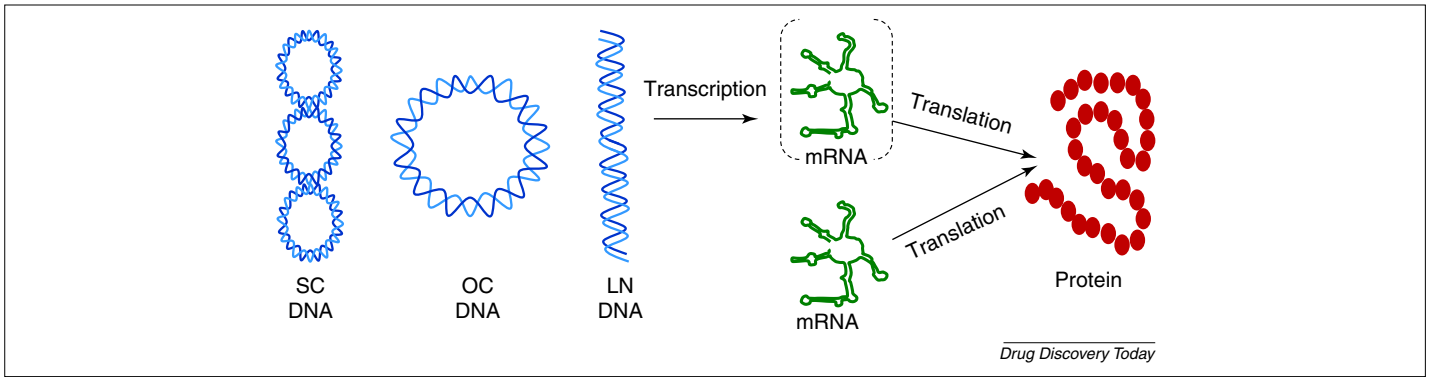

FIGURE 1

Schematic representation of nucleic acid macromolecules (either DNA or mRNA) to express the therapeutic protein of interest. DNA can exist in three different conformational states: supercoiled (SC) – the most compact hypercoiled form; open circle (OC) – the relaxed open circular form; and linear (LN) – the nicked extended DNA. DNA when delivered gets transported into the nucleus where the SC form gets converted into OC and then LN. LN DNA undergoes transcription to form mRNA *in vivo* which then migrates from nucleus to cytoplasm. The transcribed mRNA delivered as a therapeutic modality is translated into the protein of interest in the cytoplasm with the help of cellular machinery.

virus (AAV) delivery systems, are in clinical trials for several diseases they cannot be used for infectious diseases such as HIV or immunocompromised patients (e.g. patients suffering from cancer) because of possible residual viral infection [13]. Nonviral delivery has the potential to overcome many of the limitations associated with viral NAM delivery and has been of much interest in the past few decades. Nonviral NAM delivery includes liposomes, NAM–protein complexes, gene gun delivery, electroporation and intracellular microinjection. Despite the high interest and potential of developing NAMs as therapeutics, NAM delivery suffers from several major drawbacks including a lack of knowledge of formulation and stability and its impact on translation efficiency. The clinical efficacy of NAMs still remains unpredicted in humans because of inherent NAM instability, inability to reach the target site, low transduction and low expression efficacy. The biodistribution and animal PK/PD profiles indicated that only a small portion of NAM dose reached the target when delivered either through intramuscular or transdermal routes [14]. Thus the quest for higher immune responses led to exploration of a variety of formulation excipients to prevent NAM degradation, increased uptake and delivery and improved translation efficiency. Although several reviews cover the challenges and strategies of NAM delivery systems [13,15,16], there is a need for a comprehensive overview of all the challenges and mitigation strategies for naked NAM stability and formulation in nonviral systems. The current review focuses on the formulation challenges encountered during stabilization of naked NAs and mitigation strategies during drug product development. Further, effort has been made to delineate the analytical methods used to characterize NAMs on stability. Because the delivery vehicle is an important element in developing NAM therapeutics, strategies to enhance NAM delivery to the site of action and elicit a therapeutic response have been described where relevant. However, NAM drug substance production either from biologic (i.e. plasmids) or semibiologic (i.e. mRNA) processes are not covered in this review. Although the examples are primarily from DNA, most of the conditions are expected to apply to mRNA as well.

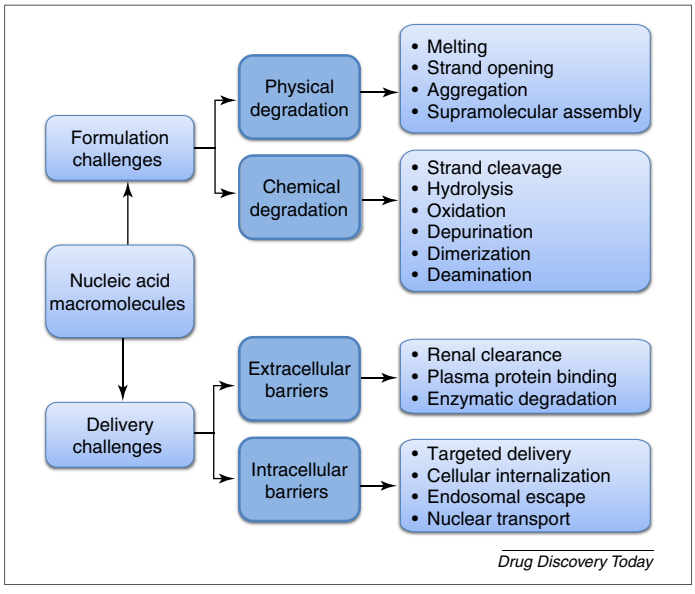

FIGURE 2

Challenges in the formulation and delivery of nucleic acid macromolecules (NAMs). NAM therapeutics need careful considerations regarding formulation as well as their delivery strategy. The inherent physical and chemical instability of DNA or mRNA are categorized under formulation. NAMs undergo several *in vivo* challenges including extracellular and intracellular barriers. Often a delivery system is utilized to ensure that NAMs are efficiently delivered at the site of action to exert therapeutic effect.

Factors effecting NAM stability

In general, naked NAMs in solution are not stable enough for long-term storage at room temperature compared with oligonucleotide drugs. They degrade rapidly depending on the solution composition and storage conditions. Many NAM delivery systems such as liposomes, polymeric particles or transfection vectors do not improve the solution stability significantly. In addition, NAMs could have unique stability issues, similar to large molecule proteins (Fig. 2). Long-term storage or prolonged exposure of NAMs to high temperature causes chemical degradation including oxidation, depurination, depyrimidination, deamination and hydrolysis. Other oxidative damage includes formation of covalent intrastrand purine dimers [17]. Depurination often leads to strand breakage via β-elimination. Conversion from the supercoiled (SC) to open circular (OC) form is a sign of such an event, although

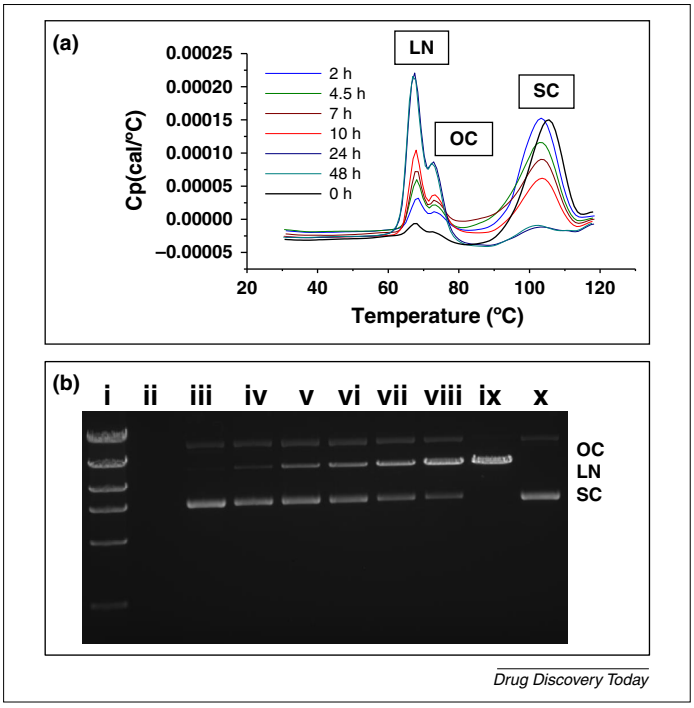

FIGURE 3

Metal-catalyzed oxidative degradation of plasmid DNA. DNA samples were incubated with ferric chloride and were analyzed at different time points. DNA undergoes degradation from supercoiled into open circle and linear forms with increasing incubation time. **(a)** Thermal scans using differential scanning calorimetry and **(b)** quantitative agarose gel electrophoresis with the sample legends: (i) nucleic acid molecular weight markers, (ii) buffer, (iii) 2 h, (iv) 4.5 h, (v) 7 h, (vi) 10 h, (vii) 24 h, (viii) 48 h, (ix) 72 h and (x) 0 h incubation of DNA with metal ions.

formation of apurinic/apyrimidinic (AP) sites does not necessarily alter the SC content of DNA [18]. The following section briefly summarizes the stability-influencing factors for naked NAMs and NAMs in delivery systems.

Temperature

Temperature is a key stability-influencing factor. It can affect physical and chemical stabilities of NAMs. At elevated temperatures, the sulfur substitutions on the phosphate backbone (phosphorothioates) could easily be exchanged back to dissolved oxygen, making the molecule more susceptible to nuclease attack. Rapid temperature increase often causes DNA to unfold or melt – a transition from helix to coil and increase in UV optimal density (hyperchromicity) as high as 40% [19]. NAMs typically have two defined thermal stabilities: intrinsic and extrinsic. Extrinsic stability is defined by the amount of SC content. The melting temperature (T_m) of SC DNAs is generally higher than the OC or linear form, because the SC form requires more energy to unwind itself (Fig. 3). Plasmid DNA pMB290 (5.2 kb) has two T_m in solution, first in the range of 60–70 °C for linear or OC and the second at 109 °C for SC transition [20]. The intrinsic stability of NAMs by contrast depends upon the G–C bp content, which defines the intrinsic stability of NAMs. The T_m of NAMs empirically follows Eq. (1):

$$T_m = 176 - (2.6 - X_{GC})(36 - 7.04 \log C_S) \quad (1)$$

where X_{GC} is the molar fraction of G–C bps in NAMs and C_S is the molar concentration of a monovalent, inert electrolyte such as NaCl [21]. However, there are no studies directly correlating the SC with the GC content in the primary structure.

NAMs undergo various temperature-dependent chemical degradations that can be predicted or examined using secondary structures. The loss of SC to OC for naked DNA (caused solely by depurination and β-elimination) was predicted to be >80% in 40 weeks at 30 °C at pH 7.4, whereas it was less than 5% at 5 °C for the same period [22]. The activation energy of depurination was found to be 31 kcal/mol [19] and 24.5 kcal/mol for the chain breakage at AP sites (the chain breakage was mainly the result of β-elimination) in double-stranded (ds)DNA [23]. DNA β-elimination leads to the formation of the degradation product 3′-terminal trans-α, β-unsaturated aldose, which further forms a *cis*-isomer and its hydrated products upon heating at pH 7.0 via δ-elimination [24]. Although the relative rates of contribution of these degradation reactions can depend on the solution conditions, there are some general rules. The degradation rate constants were estimated to be 3.0×10^{-11}/s for depurination and 5.4×10^{-7}/s for β-elimination at 37 °C at pH 7.4, whereas the rate constant of cytosine deamination was 7×10^{-13}/s at 37 °C at pH 7.4, too slow to be a major factor [22]. In comparison, 0.069 AP sites per DNA molecule (6.6 kb) were formed after 2 years at 30 °C [18]. Depending on the extent and location of chemical degradation, formation of an AP site might not often lead to immediate loss of genetic information. Several depurination events in *Bacillus subtilis* plasmid DNA are necessary to cause one inactivating hit in a transforming DNA molecule [19].

Mechanical shear

The effect of shear stress on NAM stability has been studied extensively. Shear stress can cause changes in the tertiary structure (i.e. the SC content) of the plasmids which in turn can affect the translation efficacy of the NAMs once delivered into the cells. The SC form of NAMs is less susceptible to nuclease attack and is also efficient in the sustained formation of the linear form and transcription to mRNA. Larger the molecular weight of DNA, more sensitivity to shear-stress induced degradation. Double-stranded λ-DNA (48 kb) easily degrades into two single-stranded molecules in water under mechanical turbulence [25]. Generally, smaller particles experience less tensional force compared with larger particles in a given shear field. It was considered that a plasmid smaller than 5 kb would not be very sensitive to shear-induced degradation as compared with longer DNA [26]. It was calculated that reduction of plasmid DNA from 100 nm to 25 nm via condensation would reduce the effective force on the particle by about 16-fold. The linear or OC forms are more susceptible to degradation compared with the SC form, because they have larger hydrodynamic sizes. The SC form of a plasmid DNA (5.7 kb) in TE buffer was stable during mesh nebulization but the OC form degraded to a certain degree as observed by agarose gel electrophoresis (AGE) [27]. NAM degradation is also dependent on the amount and the type of shear stress. When plasmid DNAs (6 and 20 kb) were passed through capillary tubes (ID = 0.18 mm and 0.25 mm, respectively) the loss of SC content was proportional to the number of passes through the capillary tubes, the degradation rate of plasmids highly correlated with the average elongation strain rate or the pressure drop at

the entrance region [28]. The results also indicated that laminar shear stress (within the capillary tube) does not play a significant part in DNA degradation. Nebulization is a common process for inhalation therapies and can generate different amounts of shear depending on the nebulization processes, such as jet, ultrasonic and mesh nebulization. Jet nebulization caused loss of SC by 40%, 95% and 87% for cosmid DNA of 5 kb, 9.8 kb and 37 kb, respectively, in 10 mM Tris–HCl buffer at pH 8.5 (the linear form was not detectable) [29]. Although shear is the main cause for DNA damage during nebulization, the potential pH change during nebulization (increased air exchange) could be an additional cause for DNA damage. Further nebulization with MicroAIR® mesh appeared to be milder than jet or ultrasonic nebulization of DNA (5.7 kb) in TE buffer [27]. Similarly nebulization of polycation-complexed calf thymus DNA in PBS led to pronounced agglomeration [30].

Freezing and drying

Freezing can potentially damage NAMs, depending on the solution composition, glass transition temperature and freezing rate [31]. Freezing can induce structural and conformational changes in the NAM leading to decreased solution stability. It was reported that the molecular size of the DNA could significantly impact its sensitivity to repeated freeze–thaw cycles. DNA larger than 100 kb is more susceptible to freeze–thaw. In addition, DNA concentration has a role with lower concentrations being more sensitive to freeze–thaw [32]. Many excipients could potentially stabilize NAMs during the freezing process. Sugars such as glucose or sucrose in a ratio to DNA of approximately 1000 (by weight) could provide complete protection during freezing but other crystallizing sugars such as mannitol are not as effective [31]. Transition metal impurities in sugars generate reactive oxygen species (ROS) especially during the drying step of lyophilization which could lead to strand breakage in NAMs [33]. SC content and transfection rates decreased as the levels of metal-induced ROS increased. Diethylene triamine pentaacetic acid (DTPA) and bathophenanthroline disulfonate (BPS) attenuated ROS generation and preserved DNA integrity. During lyophilization some of the water molecules in the grooves of the DNA double helix that are structurally essential could be removed if the product is over dried. The over-dried DNA (under phosphorous pentoxide) cannot retain a double-helical conformation, making the bases more vulnerable to damage [34].

Presence of metal ions

Metal contamination is often found to play a crucial part in the destabilization of NAMs. Trace amounts of metals are often present in many raw materials and can also get introduced from processing equipment during routine operations. Human DNA can aggregate in the presence of Mn^{2+} ions [35]. At a ratio of [Mn]/[P] = 46.3, incubation of such a mixture at pH 7.5 at 70 °C for 1 h led to formation of dAMP, dCMP, dGMP and TMP in the supernatant. Other transition metals that catalyze DNA degradation to dNMP included Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+}. Alkali earth element metal ions including Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}, however, did not induce significant degradation. Divalent cations catalyze hydrolysis of phosphodiester and *N*-glycosyl (base–sugar) bonds in DNA [34]. Among all the metals, Cu^{2+} and Fe^{2+} ions seem to be stronger degradation catalysts. Incubation of plasmid DNA pGPXβ9 at room temperature for 2 days in a solution containing 50 μM $CuCl_2$, 1000 μM catechol and 2 mM phosphate buffer at pH 6.8 fragmented the DNA completely [36]. Cu(II) and catechol degraded DNA via the intermediate formation of a DNA-copper-hydroperoxo complex, possibly owing to the high binding affinity of copper to DNA. The stability constant was $\log K = 9.3$ for Cu(I)DNA [no complex stability constants available for Cu(II)DNA]. Neither Chelex® cation exchange nor dialysis were successful in removing copper; however, the iron content was reduced from 55 to 15 μg/l by Chelex® treatment [37].

Metal ions also often accelerate excipient-mediated degradation of DNA. This is especially the case if the excipient is an antioxidant. Cu(II) was shown to accelerate fragmentation of calf thymus DNA in the presence of cysteine or ascorbic acid upon incubation at 37 °C at pH 7.4 for 60 min [38]. Copper(II) and iron(III) accelerated the quercetin-induced oxidative damage on DNA in the presence of ascorbic acid or curcumin [39]. Ferrous ions accelerated the degradation (strand breaks) of calf thymus DNA in the presence of bleomycin, dithiothreitol and molecular oxygen [40]. Hence, metal chelators such as EDTA or DTPA are included in most NAM formulations to sequester metal ions essential for activity of nucleases.

ROS and irradiation

ROS can easily oxidize NAMs, leading to strand breakage. The reaction is often mediated through generation of free radicals and uncontrolled chain reactions. Free-radical-induced oxidation was considered a major degradative process for NAMs in pharmaceutical formulations [18]. Formation of 8-hydroxyguanine catalyzed by hydroxyl radicals was considered a major mutagenic base lesion in living cells [34]. There are several ROS-induced strand breakages of DNA, including $\cdot ABTS^{+}$, $\cdot O^{2-}$, ·OH, H_2O_2 and HOCl [41]. Incubation of SC phage DNA at 37 °C for 90 min in a solution containing 50 mM H_2O_2, 50 nM ferrous iron ($FeSO_4$), 10 mM NaCl and 1 mM phosphate buffer at pH 7.5 caused significant nicks in the SC [42]. It was demonstrated that H_2O_2 induces formation of modified bases, mainly 8-oxodGua and FapyGua (ring-opened aldehyde derivative) in the presence of Cu^{+}, Cu^{2+}, Cu^{2+}–phenanthroline complex in a concentration-dependent manner [37]. Irradiation of NAM is equally damaging, because this process generates hydroxyl radicals [37]. Exposure of DNA to γ-radiation led to formation of oxidized bases (base lesions), mainly 8-oxo-7,8-dihydroguanine in aqueous solution and the guanine degradation products were produced in much higher yields than lesions of the other bases. Polyamines have been reported to protect DNA against strand breakage induced by radiation. It was found that exposure of calf thymus DNA at 0.1 mg/ml to γ-rays in aerated aqueous solution at doses from 0 to 50 Gy led to proportional formation of modified bases.

Formulation stabilization of NAM

Owing to the enormous size and conformations of NAMs, formulation development of NAM therapeutics dealing with physical, conformational and chemical instability is very challenging. Strand breakage and integration to host cell DNA is the biggest concern similar to immunogenicity concerns in protein aggregates. However, with careful consideration of the nature of the NAM and its surrounding environment, proper choice of excipients could lead to a successful formulation addressing long-term

storage stability, efficient cellular uptake and/or delivery to the nucleus. The following sections briefly discuss formulation approaches that have been utilized to stabilize DNA in aqueous or solid state and under various processing conditions including freeze–thawing and drying.

pH

Solution pH has a strong effect on the stability of NAMs. The degradation mechanism of DNA typically includes a two-step process: depurination and β-elimination, which are acid- and base-catalyzed, respectively [18]. Generally NAMs are mostly stable under a weakly basic environment. For example, a 6.6 kb plasmid DNA, formulated in salt containing bis-Tris–propane buffers pH 6–9, is most stable at pH 9 in terms of retaining SC content as analyzed by agarose gel electrophoresis [18]. The same DNA when formulated at the same pH of ~7 in PBS buffer had significantly improved stability compared with its counterpart, which suggests that buffer type can impact DNA stability significantly. Depurination of *B. subtilis* plasmid DNA was much slower at pH 7.4 than at pH 5.0 [19]. It was predicted that the loss of SC to OC for naked DNA was >80% in 40 weeks at 30 °C at pH 7.4, whereas less than 20% would be lost at pH 8.5 [22].

The solution pH often controls the rate of hydrolysis of NAMs. Fragmentation of plasmid DNA pSG100 was found to increase gradually when the solution pH dropped from 7.0 to 6.5, 6.0 and 5.8 [43]. Phosphodiester and *N*-glycosyl (base–sugar) bonds in NAM are susceptible to hydrolysis, particularly in the presence of divalent cations [34]. It was found that 50% of the 260 μg/ml human DNA (281 bp) in solution containing 100 mM NaCl and 10 mM Mn^{2+} ions at pH 7.5 was degraded into dNMPs when incubated at 70 °C for 1 h but minimal degradation was found at pH 7.0 [35].

In addition to strand breaks, other degradation reactions can also occur under acidic or basic conditions. NAM base residues are susceptible to hydrolytic deamination with cytosine being the main target [34]. Deamination of cytosine was detected in a variety of aqueous buffers of pH <6.0 upon incubation at 95 °C [44]. At acidic pH (e.g. 5), sodium bisulfite can cause deamination of a cytosine residue in single stranded (ss)DNA, and the deaminated bisulfite adduct is converted into uracil through elimination of bisulfite at alkaline pH (>13), whereas the pyrimidine sulfite adduct is converted into a basic site via an *N*-glycoside bond cleavage [45]. Incubation of calf thymus DNA with 0.5 M perchloric acid or 0.4 M trichloroacetic acid results in oxidation and formation of methylglyoxal [46]. Acid treatment of calf thymus DNA can lead to depurination [24]. The solution pH certainly influences the size and charge of condensed plasmid DNA complex during condensation [47].

Buffer

Buffers, as key solution excipients, have a significant effect on DNA stability. Tris–HCl and phosphate are commonly used buffers in DNA solutions. Their stabilization effect has been reported in several studies. It was demonstrated that 5 mM Tris–HCl significantly reduced γ-ray-induced base modification (10 times lower), presumably because of the hydroxyl radical scavenging properties of Tris–HCl [37]. Nicking of SC phage fd DNA (fd RF DNA) upon incubation at 37 °C was inhibited by addition of 1 mM Tris–HCl (pH 7.5) or 0.1 mM EDTA in a solution containing 50 mM H_2O_2, 50 nM (or 100 nM) ferrous iron ($FeSO_4$), 10 mM NaCl and 1 mM phosphate buffer at pH 7.5 [42]. Formation of single-stranded from double-stranded λ-DNA (48 kb) reduced significantly in buffer as compared with water [25]. Other buffering agents might not have such a protective effect. Using calf thymus DNA as a model DNA, Lynch *et al.* demonstrated that nebulization of polycation–DNA complexes in water and glucose solution with a vibrating plate nebulizer did not indicate any pronounced agglomeration as occurred in phosphate buffer saline [30].

Electrolytes

Based on Eq. (1), where X_{GC} is the molar fraction of G–C bps in DNA and C_S is the molar concentration of a monovalent, inert electrolyte such as NaCl [21], increasing ionic strength should generally lead to increased stability of DNA. Indeed, many reports demonstrated such relationships. Higher ionic strength was found to inhibit NAM depurination [19]. Incubation of SC phage DNA at 37 °C for 90 min in a solution containing 50 mM H_2O_2, 50 nM (or 100 nM) ferrous iron ($FeSO_4$), 10 mM NaCl and 1 mM phosphate buffer at pH 7.5 caused significant nicking, increasing the concentration of NaCl from 50 to 500 mM, dramatically reducing the rate of the reaction [42]. Several salts inhibited fragmentation of plasmid DNA pGPXβ9 during incubation at room temperature for 2 days in a solution containing 50 μM $CuCl_2$, 1000 μM catechol and 2 mM phosphate buffer at pH 6.8 [36].

However, such an inhibitory effect of high ionic strength is not always the case. In fact, the opposite trend was observed in several studies. The rate of formation of dNMPs in human DNA (281 bp) in the presence of Mn^{2+} at pH 7.5 upon incubation at 70 °C for 1 h increased after addition of 100 mM NaCl or other monovalent salts [35]. In the presence of bleomycin, Fe(II), dithiothreitol and molecular oxygen the rate constants for oxidation-induced strand breaks in calf thymus DNA for the fast and slow reaction phases increased with increasing ionic strength from 15 mM to 215 mM NaCl [40].

It is obvious that these solutions contain metal ions and the empirical rule might not apply well to metal-catalyzed reactions, because salts not only change the DNA environment but also interact potentially with the intermediate of DNA degradation (e.g. DNA–copper-hydroperoxo complex) [36]. Similarly, tetramethyl ammonium chloride at 1 or 1.5 M increased the T_m of calf thymus DNA by several degrees in a solution containing 1 mM Tris–HCl, 10 mM NaCl, 0.2 mM EDTA, pH 7.2 [21]. Salt could neutralize charges in the DNA backbone without causing condensation and shorten the distance per coil and decrease the effective diameter of DNA [26].

Antioxidants and free radical scavengers

Oxidation of NAMs is another major degradation pathway that often leads to strand breaks. It is intuitive that use of any antioxidants would inhibit such degradation. The use of such antioxidants, however, often accelerates NAM degradation. For example, in the presence of copper(II) ions, cysteine (an antioxidant) actually induced degradation (oxidation) of deoxyribose and fragmentation of calf thymus DNA when incubated at 37 °C at pH 7.4 [38]. Ascorbic acid, another reducing antioxidant, exerted an even greater effect than cysteine. The damaging effect of cysteine

can be prevented by catalase or antioxidant thiourea. Similarly, ascorbic acid and curcumin stimulated metal-catalyzed quercetin-induced oxidative damage on DNA [39]. These agents are apparently pro-oxidants and antioxidants. Such a complex effect was also reported in a different study. Although deferoxamine significantly inhibited ROS formation during lyophilization (drying step) of trehalose-containing lipid–DNA formulations in the presence of different levels of transition metals, it promoted double strand breakage at the same time [33]. By contrast, nonreducing free radical scavengers, such as ethanol or methionine, could potentially prevent or mitigate damage of DNA plasmid from free-radical-induced oxidation.

Chelating agents

Addition of chelating agents can often inhibit metal-catalyzed degradation of NAMs but not always. Addition of DTPA enhanced the stability of DNA (6.6 kb) in the presence of Fe^{3+}, Cu^{2+} or Cu^{+} but decreased its stability in the presence of Fe^{2+}, whereas EDTA increased its stability in the presence of Fe^{2+}, Cu^{2+} or Cu^{+} but slightly decreased the stability in the presence of Fe^{3+} [18]. Similarly, although DTPA and BPS were able to inhibit formation of ROS during lyophilization (drying step) of trehalose-containing lipid–DNA plasmid formulations with different levels of transition metals, EDTA significantly enhanced the generation of ROS [33]. It was demonstrated that, through combining EDTA with a free radical scavenger such as ethanol, DNA stability could be significantly improved [18].

Alkylating agents

Treating DNA with an alkylating agent, for example duocarmycin A, leads to formation of a metastable covalent adduct at *N*3 of adenine ($T_{1/2}$ of 7.5 h at 37 °C) and, upon heating at 90 °C for 5 h at pH 7.0, the adduct forms a basic-site-containing oligomer with release of duocarmycine adenine adduct [24]. Further heating at 90 °C for 30 h led to strand breakage as a result of β-elimination with the formation of trans-α, β-unsaturated aldose. Also the depurination rate greatly increased *in vivo* after treatment of cells with alkylating agents (formation of methyl adenine) [19].

Other excipients

Formulation excipients play a significant part in NAM stability, because they can change the environmental properties of NAM and can interact directly with NAMs. The enzymatic degradation (by DNase I) of plasmid DNA pSG100 reduced by one order of magnitude in the presence of 0.05% maltol or 1 mM putrescine and was completely inhibited in ≥0.2% maltol, ≥0.01% octyl gallate or ≥0.5 mM spermine [43]. Condensing agents generally stabilize NAMs, primarily because they cause compaction of NAM structure and thereby reduce accessibility to harmful stresses or agents and nucleases. Jet nebulization caused loss of SC by 40%, 95% and 87% for cosmid DNAs of 5 kb, 9.8 kb and 37 kb, respectively, in 10 mM Tris–HCl buffer at pH 8.5 and complexation of DNA with cationic agents like polyethyleneimine or poly-L-lysine was sufficient to overcome this damage [29]. Similarly, the protective effect of polyamine complexing agents, including linear polyethyleneimine, branched polyethyleneimine, polyarginine and polylysine, was also reported [30]. Other polycationic ligands or polymers such as chitosan and protamine sulfate also help protect NAMs through a condensation mechanism and also act as delivery vehicles. Spermine, spermidine and putrescine at concentrations greater than 0.1 mM were able to protect against the degradation of bases (formation of 8-oxo-7,8-dihydroguanine and 5-hydroxycytosine) in calf thymus DNA upon exposure to γ-rays in aerated aqueous solution [48].

Some excipients have been found to destabilize DNA. Amino acids such as lysine, histidine and arginine at 0.05 M accelerated chain breakage of DNA by 7-, 6- and 3-fold at 70 °C in a pH 7.4 solution, respectively [23]. Putrescine, a diamine, had a much stronger effect: the chain breakage increased 25-fold at apurinic sites. The negative effect of these zwitterions was supported by another study where several zwitterionic osmolytes, namely glycine, sarcosine (*N*-methylglycine), *N*,*N*-dimethylglycine and betaine (*N*,*N*,*N*-trimethylglycine), reduced the T_m of calf thymus DNA by more than 10 °C at high concentrations in a solution containing 1 mM Tris–HCl, 10 mM NaCl, 0.2 mM EDTA, pH 7.2 [21].

Analytical techniques for NAM stability studies

For NAMs to be commercialized as drugs, they must be stable for extended periods of time typically at 2–8 °C. With regard to stability, NAMs have some very distinct advantages over protein-based pharmaceuticals, which are susceptible to loss of biological activity caused by small changes in their tertiary or quaternary structure. Biophysical techniques such as circular dichroism, FTIR, fluorescence and isothermal titration calorimetry are used as characterization tools to understand the conformational changes and excipient interactions. Aside from precipitation or aggregation with or without the involvement of higher order structures, NAMs usually require a chemical modification for an irreversible loss of biological activity or translation into misfolded or inactivated protein. The chemical integrity of NAM is usually monitored in all structural stability studies. Table 2 provides a summary of analytical methods used to determine NAM drug substance or product stability during discovery through commercialization. The following sections detail the characterization of NAMs and their degraded products.

General stability of NAMs

One of the major degradation pathways for NAMs in aqueous solution is the process of depurination. Depurination is acid-catalyzed and is initiated by protonation of the purine base at *N*-7 that leads to the cleavage of the *N*-glycosidic bond to produce an AP site (base free sugar). The AP site contains a chemically altered aldehyde form of sugar, susceptible to β-elimination, leading to cleavage of the phosphodiester backbone. In addition, the aldehyde could react with amino groups on NAM bases of another NAM molecule to produce concatamers. Monitoring strand breakage could be the most convenient and sensitive way to quantitate the plasmid damage by depurination, β-elimination and oxidation.

Strand breakage in the NAM backbone often converts SC plasmid DNA to OC and linear (L) forms, which can typically be monitored using AGE. In recent years, however, many laboratories have preferred anion exchange chromatography (AEC) for the analysis of clinical grade plasmid material [49]. AEC exploits the electrostatic interaction between the negatively charged phosphate group in the DNA backbone and the positively charged functional group in the stationary phase. The overall binding is

TABLE 2

Analytical tools to characterize NAM-based drug substance and drug products.

Parameters	Analytical tools	Purpose in drug development
Primary structure	X-ray crystallography, nuclear magnetic resonance (NMR), atomic force microscopy (AFM), sequencing, restriction map	Drug substance release and characterization
Size	Agarose gel electrophoresis (AGE), dynamic light scattering (DLS)	Drug substance characterization and stability
Aggregation	DLS	Drug product characterization
Purity/quantity	UV–Vis spectroscopy, fluorescence, quantitative agarose gel electrophoresis (Q-AGE)	Drug substance/drug product release
Secondary structure	Circular dichroism (CD), fourier transform infrared (FTIR)	Drug substance characterization
Strand breakage	AGE, anion exchange chromatography (AEC), capillary electrophoresis (CE)	Drug product stability
Depurination	Spectrophotometry (labelled tagged), CE	Drug product stability
Oxidation	HPLC, gas chromatography/mass spectrometry (GC/MS)	Drug product stability
Deamination	GC/MS	Drug product stability
Melting temperature	Differential scanning calorimetry (DSC)	Drug product characterization
Excipient interaction	Isothermal titration calorimetry (ITC)	Drug product characterization

dependent on the local attractions (different isoforms have different local charge densities) generated by opposite charges in local proximity. Using a suitable salt gradient, this interaction is manipulated for effective resolution and quantitation of different topological isoforms. Generally, for AEC-based analysis of DNA, method development requires optimizing the key parameters on a case-by-case basis. To this end, several groups have evaluated different anion exchange resins, mobile phase components, flow rate, pH, column temperature and gradient slopes [50].

Capillary gel electrophoresis represents another attractive alternative to AGE for analysis of plasmid isoforms such as open circle, linear and SCs [51,52], and a representative graph is shown in Fig. 3b. This high-throughput approach offers high separation efficiency, sensitivity and reproducibility. Before analysis, NAM is stained with YOYO and hydrodynamically injected into the coated capillary. The separated bands are detected by laser-induced fluorescence (LIF) with excitation at 488 nm and emission at 520 nm. Excellent linearity was achieved over a wide concentration range (0.06–4 μg/ml).

Analysis of the AP site

Often the rate of β-elimination is not sufficient to cleave the AP sites as quickly as they are introduced by depurination. This leads to accumulation of AP sites in the NAM over time. Moreover, excipients in NAM formulations can alter the rate of depurination and β-elimination in an unexpected way. Quantitation of the number of accumulated AP sites per plasmid could therefore be performed during storage in the final formulation, and at relevant storage temperatures. A number of methods have been developed for the assay of AP sites [19,22]. One method is based on the alkylation of the aldehyde group at the AP site sugar with a biotin-labelled tag. Following alkylation, the biotin tag is treated with a horseradish-peroxidase–advidin complex and detected spectrophotometrically. This assay can detect approximately one AP site per 10^4 bps.

Capillary electrophoresis with laser-induced fluorescence (CE-LIF) has also been investigated for ultrasensitive analysis of abasic site formation in NAMs [53]. The abasic sites are tagged with a fluorescent aldehyde-reactive probe. The NAM is precipitated with ethanol, and then analyzed by CE-LIF. The fluorescently tagged NAM peaks are directly proportional to the amount of *N*-7 methyl guanines. The CE-LIF method has a detection limit of 1.2 basic sites per 100 000 bases or *ca*. 20 attomoles of abasic sites.

Analysis of NAM oxidation

Free-radical-induced oxidation is a common mode of chemical degradation of NAMs in aqueous solution. This mechanism should be suspected if the stability of the NAM is worse than predicted. In many cases, it seems likely that only very small quantities of radicals will be produced in pharmaceutical preparations of NAM, and a very sensitive assay is required to detect damage products in the NAM.

Two of the most commonly identified markers of oxidative NAM damage are thymine glycol and 8-hydroxyguanine. Methods used to quantitate these and other damaged bases in NAM include HPLC with electrochemical or fluorescence detection, gas chromatography/mass spectrometry (GC/MS) and immunological assays [52,53]. The sensitivity of these methods is similar, allowing the detection of a damaged base if present at one molecule per 10^5–10^6 bases. One GC/MS method (using electron capture negative ionization detection) for quantitating thymine glycol requires as little as 4 μg of DNA and can detect one molecule of thymidine glycol per 3×10^8 normal bases. MS/MS is also useful to monitor the desulfurization of the phosphorothioate backbone with a molecular weight difference of 18 Da for the replacement of sulfur by oxygen.

Repair endonucleases are also useful for detecting oxidized bases in NAMs [54]. A number of enzymes have been identified that cleave the NAM backbone at 8-hydroxyguanine and thymine glycol and at several other sites of hydroxyl radical damage. The basis of these assays is the relaxation of SC plasmid produced by cleavage of the plasmid at the site of the damaged base. The use of several enzymes with different specificities is potentially a useful method to identify the types of oxidized bases produced under different conditions.

Analysis of deamination

Deamination of cytosine might not be a major factor to cause NAM damage in pharmaceutical formulations, because the rate of cytosine deamination is far too slow ($k = 7 \times 10^{-13}$/s, pH 7.4, 37 °C) [55]. However, even brief periods of heating to high temperatures (which could occur during some cell lysis procedures) could cause significant deamination of cytosine. In addition, nucleophiles like bisulfite can induce cytosine deamination. Therefore, assays should be performed to estimate the amount of cytosine deamination that has occurred in the NAM before formulation and during storage. A very sensitive GC/MS method [56] using negative chemical ionization has been developed that can detect one uracil molecule per 3×10^7 normal bases.

Characterization of cation–NAM complexes

Complexes between NAM and various cationic complexing agents are promising vehicles to deliver genetic information into cells for gene therapy or vaccines. The positively charged compounds that have been used so far include basic polypeptides, polyethylenimine, amino dendrimers, various synthetic copolymers and cationic lipids (CLs). The CLs containing vectors are, however, the most thoroughly investigated. When NAMs and CLs are combined, regular and irregular particle-like structures are formed with a size range of 50–200 nm depending on process parameters, but the resultant structures display significant size heterogeneity. The actual composition of lipoplex formulation is more difficult to define than expected. Although only two macromolecular components can be present, they can be present either as pure species or as complexes of unknown composition. Considering that complex formation is due to electrostatic interaction between the negatively charged NAM and cationic agents, aggregation and/or agglomeration can become problematic. NAMs based on the solution conditions have a tendency to form supramolecular assemblies through intramolecular and intermolecular hydrogen bonding of complementary sequences. Apart from increased viscosity this also plays a significant part in particle heterogeneity when condensed with cationic ligands.

Gel electrophoresis is used to determine the positive:negative charge ratio that minimizes the unbound NAMs [52,53]. Because of the relative charge and size differences between lipoplexes and NAMs, electrophoresis produces widely separated bands. The gel retardation assay shows that with increasing charge ratios the amount of unbound NAM decreases and the quantity of lipoplexes retarded near the top of the gel increases. Complete retention of NAM often does not occur until an excess of positive lipid charge to negative charge exists. The stability of the lipid–NAM complexes has also been characterized with gel electrophoresis. Complexes that have undergone increasing levels of degradation produce bands corresponding to more unbound NAM.

Differential scanning calorimetry (DSC) has been extensively used to characterize the stability of cationic lipid–NAM complexes [57]. It is possible to measure the distinct T_m of the lipid, plasmid and lipoplexes in a mixture and also the associated heat capacity changes. In the case of complexes that contain cationic lipids that undergo phase transitions, a sharp transition is typically seen. When the bilayer of the lipid is stabilized by the DNA, this transition is usually shifted to a higher temperature. Different lipoplexes have varied thermal stability.

Advanced formulation consideration to enhance NAM delivery

Formulation excipients stabilize NAM for the long term during product storage and transportation. However, poor *in vivo* (serum) stability results in poor pharmacokinetics meaning that advanced formulation techniques are required to transport NAM to the active site. To achieve this successfully, NAMs need to be efficiently transported to the target cells, transfect the cells, express the encoded antigen and initiate pharmacological and/or immune responses. In the case of NAM-based vaccines it is important to use the delivery system that can efficiently transport and present it to the antigen-presenting cells (APCs). NAMs have been shown to be effective in eliciting pharmaceutical and immune responses from different delivery routes such as intramuscular [2,58,59], intradermal [60–64], intraperitoneal [65–68], intravenous [66,69–72], oral [72–75], intranasal [76–80], pulmonary [81–85] and transdermal [64,86–88] delivery. The following section discusses briefly the advanced stability parameters to be considered during the *in vivo* transport of NAMs from the site of injection to the site of action.

Systemic transportation

The *in vivo* stability of NAM delivery complexes is dependent on the chemical stability of the therapeutic NAM and the physical stability of the delivery systems. Nucleases that exist in the extracellular space have been shown to degrade NAM rapidly in spite of several modifications made to the structure [89]. Fortunately, this degradation can be circumvented by either condensing the NAM with polycations or by complexing with polymers that bind to but do not condense the NAM [90,91]. Generally a minimum positive:negative charge ratio (charge phase diagram:cationic excipients to NAM phosphate backbone) needs to be maintained to achieve the stabilization effect.

Another hurdle to overcome is the physical instability of NAM delivery complexes. Aggregation of NAM complexes is often observed in cationic lipids or polymer systems when prepared near charge neutrality. Increased ionic strength in the biological environment can also have significant effects on the physical properties of the complexes [92]. An emerging trend in recent studies is the use of cross-linking to achieve the balance between the stability of polyplexes and lipoplexes in the blood and the controlled release of NAM in the cytosol. It was reported that covalent cross-linking of the reactive amino groups of NAM polyplexes effectively prevents salt-induced aggregation as well as dextran-sulfate-mediated displacement of NAM. To prevent aggregation and interaction with serum components, polyethylene glycol (PEG) was conjugated to poly-L-lysine to stabilize the surfaces sterically [93].

Cellular uptake

A gene must first gain access to the cells where it can be expressed using intracellular machinery. The size and charges of lipoplexes and polyplexes can influence extravasation into tissues after delivery. Because most organs have low capillary permeability, large particles cannot transverse through endothelial cells or extravagate from the vasculature into the interstitial space. The efficiency of endocytosis has been proposed as an important rate-limiting process for cationic liposomes in the transfection of Chinese hamster ovary (CHO) cells [94]. Cellular uptake of NAM has been

demonstrated to be a significant barrier to transfection *in vivo* [95]. Correlation between cellular uptake and gene expression efficiency is further strengthened by a recent study in which the conjugation of a cationic PEG lipid to stabilized plasmid–lipid particles was shown to result in a 50-fold increase in the uptake of the particles by baby hamster kidney cells, which resulted in 106-fold enhancement in transgene expression [96]. Most NAM complexes are formulated to possess a net positive charge because it leads to higher levels of gene expression.

Endosomal escape

Following endocytosis, the intracellular vesicles fuse with endosomal compartments. An important strategy to improve the gene expression level of a gene delivery system is to facilitate endosomal escape. The failure of nonviral-based NAMs to escape from the early endosomes could result in their trafficking to the late endosomes and lysosomes where NAMs would be degraded [92]. By contrast, viral vectors readily escape from the endosomes by exploiting the low pH to trigger a cascade of events that finally enable their exit [97]. Using this mechanism incorporation of viral, fusogenic peptides can aid in the endosomal escape of nonviral-based NAM delivery and improve the gene expression. Polycations that are capable of buffering the endosomal pH and cause lysis of endosomes have also led to enhanced gene expression in cultured cells [97,98]. In a recent study, PEG-detachable polyplex micelle NAM delivery was developed [99]. Micelles that have detachable PEG which is sensitive to an intracellular reducing environment showed significantly higher gene transfection efficiency than a micelle without disulfide bonds in spite of similar levels of cellular uptake. The improved transfection efficiency was attributed to the effective endosomal escape caused by PEG detachment in the endosome. Another strategy using cationic liposomes incorporating fusogenic peptides from glycoprotein H of herpes simplex virus was able to improve endosomal escape of cationic liposome–NAM complexes and achieve up to a 30-fold increase in gene expression in human cell lines [100].

Nuclear transport

Nuclear transport is not a barrier for mRNA but for DNA-based NAMs. The ultimate goal of DNA delivery is the delivery of exogenous (re-engineered) DNA to the cell nucleus where transgenes can undergo transcription. After vesicular escape, the DNA must traffic to and enter the cell nucleus. The nuclear membrane presents a major barrier of DNA entry. Strategies are in development to enable DNA translocation through the nuclear pore complex (NPC). Some nuclear localization signals have been reported to be incorporated to DNA delivery vehicles and several of them have led to significantly improved delivery efficiency. A classical strategy is to attach a nuclear localization signal (NLS) peptide to plasmid DNA [101], or alternatively conjugate to DNA-condensing agents [102]. The most promising report so far was on a double stranded linear capped CMV luciferase–NLS gene containing a single NLS peptide [103]. Up to 1000-fold improvement in transfection was observed in dividing HeLa cells and 10–30-fold enhancements in macrophages, neurons and hepatocytes. Other strategies including hexon-mediated nuclear import [104] and simple polycationic-polymer-mediated nuclear transport have been reported [105]. Sequence specificity of nuclear import has also been employed as a strategy for improving DNA nuclear transportation [106,107].

Concluding remarks

In the past decade, immense amounts of interest and data have been generated in the field of NAM-based therapeutics and we are closer to getting an answer to the question: NAM based medicines – when will they come to the drug store? The ease of NAM manufacturing, relatively fewer molecular liabilities compared with proteins, the ability to hit the targets that are otherwise not accessible for protein drugs, providing tissue specificity and long-acting ability are the major driving forces to develop NAM-based therapeutics. Our enhanced knowledge of the role of immune systems in the regulation of cancer and other immune-deficient disorders has pushed the development of NAM-based vaccines to unprecedented advances. In 2005, the first DNA vaccine against West Nile virus was approved by the FDA for horses but it is still in clinical trials for human use [108]. Although still in clinical phases, NAMs might soon become a major therapeutic class for treatment of various diseases in humans. With such an urgent need, there arises a need of knowledge of formulation science and delivery. Deeper understanding of the physical and chemical behavior of NAMs and the effect of various formulation excipients will lead to the development of a successful drug product. Lessons learned from macromolecule formulations, such as those for proteins and oligonucleotides, can be used to avoid potential formulation failures. As discussed, a major and unique challenge faced by NAM therapeutics is the need for a suitable delivery system and ability to make direct correlation of the active protein expression to therapeutic doses. A promising delivery system is the nonviral delivery system, which needs to be considered during DNA formulation development. With the accumulation of knowledge of complex delivery systems, such as liposomes, polymers, targeting moieties including cell-penetrating peptides and monoclonal antibody conjugates, and nuclear localization signals, clinical translation of NAMs is improving and NAMs could soon be broadly available for treatment of a wide range of diseases.

Conflicts of interest

The authors declare that they have no actual or potential competing financial or personal interests that might be perceived to impact the discussion reported in the current article.

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