



Spherical Nucleic Acid Nanoparticles: Therapeutic Potential

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Abstract

Spherical nucleic acids (SNAs) are highly oriented, well organized, polyvalent structures of nucleic acids conjugated to hollow or solid core nanoparticles. Because they can transfect many tissue and cell types without toxicity, induce minimum immune response, and penetrate various biological barriers (such as the skin, blood–brain barrier, and blood–tumor barrier), they have become versatile tools for the delivery of nucleic acids, drugs, and proteins for various therapeutic purposes. This article describes the unique structures and properties of SNAs and discusses how these properties enable their application in gene regulation, immunomodulation, and drug and protein delivery. It also summarizes current efforts towards clinical translation of SNAs and provides an expert opinion on remaining challenges to be addressed in the path forward to the clinic.

Key Points

Spherical nucleic acids (SNAs) are revolutionizing the fields of diagnostics, gene regulation, immunotherapy, and drug and protein delivery.

This article discusses how the unique structure and properties of SNAs enable them to maximize their effect in various medical applications.

This article also discusses the clinical translation of SNAs and outlines some of the challenges to be conquered in their promising path to the clinic.

genes includes antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and microRNAs (miRNAs) [4]. Many of these have been investigated in clinical trials for the treatment of diseases including cancer, neurological disease, infectious disease, and spinal muscular atrophy, and six nucleic acid-based therapies have been approved by the US Food and Drug Administration (FDA) for clinical use [5]. However, translation and clinical success of nucleic acid therapies has been unsatisfactory due to challenges associated with their delivery.

Systemically injected naked nucleic acids have poor bio-availability and very low cellular uptake, and those that are internalized are at risk of degradation by intracellular enzymes. Moreover, ASOs, siRNAs, and miRNAs must reach the cytosol to interact with their target messenger RNA (mRNA), which is very difficult to achieve [6]. Together, these challenges limit the potential of naked nucleic acids to induce gene regulation. Excitingly, advances in the field of nanomedicine have led to the development of various DNA/RNA nanocarriers such as liposomes, micelles, polymeric nanoparticles, and lipid nanoparticles that have shown promise in pre-clinical and clinical trials, suggesting that nanoparticle carriers may be the solution to the nucleic acid delivery problem [7, 8].

Of the most recently developed nanoparticle-based nucleic acid delivery platforms, spherical nucleic acids (SNAs) have emerged as a promising tool for nucleic acid delivery, enabling their use in various biomedical applications [9, 10]. SNAs consist of densely packed ‘shells’ of radially oriented nucleic acids that are firmly attached to a

1 Introduction

Correcting genetic defects through the delivery of nucleic acids holds great promise for the development of future medicines [1–3]. The types of nucleic acids that can be used to inhibit the transcription and/or translation of overexpressed

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nanoparticle core. The three-dimensional structure of SNAs confers unique physicochemical and biological properties that make them ideal vehicles for nucleic acid delivery. This article discusses the various structures and properties of SNAs and highlights their therapeutic application for gene regulation, immunomodulation, drug delivery, and protein delivery. It also summarizes current clinical translation efforts for SNAs and discusses challenges that must be addressed in the path forward to the clinic.

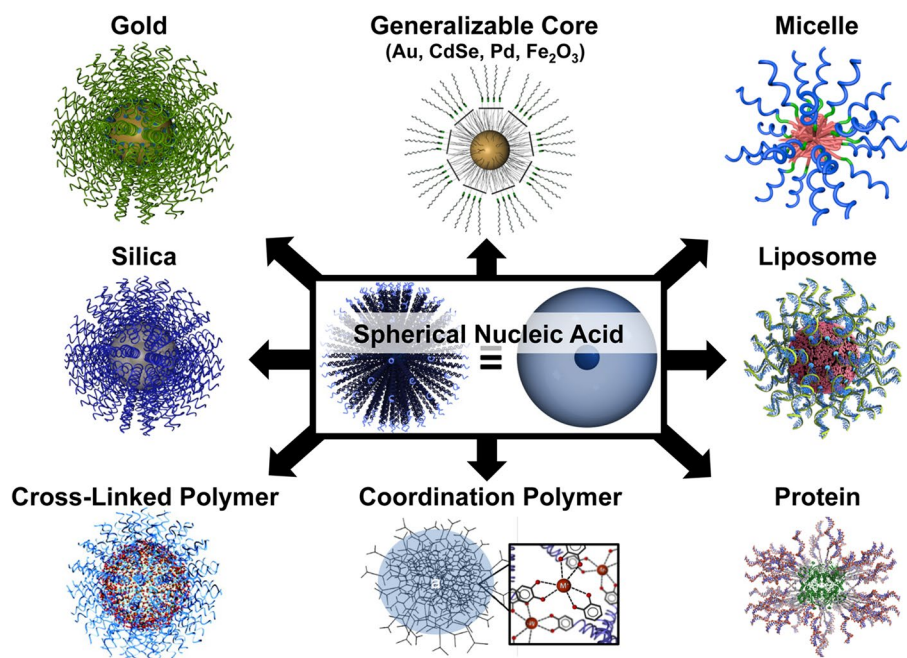
2 Structure and Properties of Spherical Nucleic Acids (SNAs)

SNAs have two components: (1) a densely packed shell of nucleic acids, which are radially oriented around (2) a core nanoparticle, which may be solid or hollow (Fig. 1). The first described SNAs were 13-nm diameter gold spheres coated with non-complementary single-stranded DNA molecules via thiolated linkers, and these SNAs were used to guide nanoparticle assembly into larger ordered structures [11]. Gold nanoparticles were chosen as an ideal SNA core due to their ease of synthesis, tailorable and homogeneous size, simple gold-thiol conjugation chemistry, and unique optical properties. During early studies, SNAs made from DNA surrounding gold nanoparticles were used for diagnostic [12, 13], therapeutic [14], and material assembly applications [15, 16]. Since then, various SNAs have been developed that utilize single- or double-stranded oligonucleotides such as antisense DNA [14, 17, 18], siRNA [19–21], and miRNA [22, 23] for the outer layer, and that

utilize metal-based nanoparticles (gold [11, 24, 25], quantum dots [26], platinum [27], iron oxide [28], silver [29]) or metal-free nanoparticles (hollow silica [17] liposomes [18, 30], micelles [31–35], cross-linked oligodeoxyribonucleotides [36], and proteins [37]) as the core. The various forms of SNAs are shown in Fig. 1. The diversity of the types of SNAs that have been produced shows that their properties are independent of the core, and are dictated by the unique architecture of the nucleic acid shell, making them distinct from linear nucleic acids.

Some of the unique differences between SNAs and linear nucleic acids are highlighted here. First, SNAs have higher affinity constants for complementary nucleic acids than their linear counterparts and they are less susceptible to nuclease degradation due to the high local salt concentration around the nanoparticle core [38]. Second, SNAs can be taken up by almost any cell type (> 50 cell types to date [9]) without the need for auxiliary transfection agents (Fig. 2), and they exhibit > 99% cellular uptake [14] as demonstrated by confocal microscopy. Their uptake is facilitated by binding to scavenger receptors, and they are endocytosed via a lipid-raft-dependent and caveolae-mediated pathway [39]. Finally, SNAs are nontoxic to cells [40], they illicit minimum innate immune response (25-fold less than lipoplexes carrying the same DNA), and they have no effect on blood chemistry in vivo [41]. Together, these unique properties allow SNAs to be utilized as tools for diverse biomedical applications, and the following sections highlight some areas in which SNAs demonstrate substantial potential.

Fig. 1 Schematic showing the various spherical nucleic acid (SNA) structures that have been developed. As a generalizable technology, SNAs consist of a ‘shell’ of nucleic acids surrounding a solid or hollow core nanoparticle. Reproduced with permission from [102]



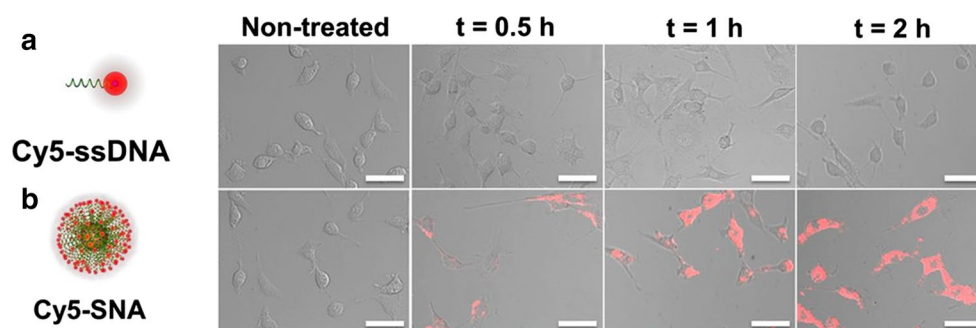


Fig. 2 Oligonucleotide dimensionality dictates cellular endocytosis. **a** Confocal microscopy shows that Cy5-ssDNA cannot readily enter C166 cells. **b** SNAs prepared with the same DNA as utilized in part **a** display substantial cellular uptake within 2 h after incubation. This demonstrates that the 3D architecture of SNAs is critical to their cel-

lular interactions. Reprinted with permission from Choi et al. Mechanism for the endocytosis of spherical nucleic acid nanoparticle conjugates. Copyright 2013. Proc Nat Acad Sci USA [39]. Cy5 cyanine 5, Cy5-ssDNA Cy5-labeled single-stranded DNA, C166 cells mouse endothelial cell line, SNA spherical nucleic acid

3 Therapeutic Application of SNAs

Since SNAs can transfect many tissue and cell types without inducing toxicity, various formulations have been developed to deliver ASOs, miRNAs, siRNAs, chemotherapeutics, and proteins for therapeutic purposes. The following sections describe the diverse applications of SNAs, with an emphasis on their use for treatment of cancer and skin diseases.

3.1 SNAs for Gene Regulation

The first demonstration of using SNAs to regulate gene expression in cells was published one decade ago [14], in which SNAs were made with 13-nm diameter gold nanoparticles coated with ASOs targeting *EGFP* (enhanced green fluorescent protein) mRNA. Upon cell entry, ASOs bind their target mRNA to halt protein translation [14]. Since this initial report, various SNAs have been synthesized to exploit the RNA interference (RNAi) pathway [42]. In RNAi, siRNA or miRNA delivered into cells bind the RNA-induced silencing complex (RISC). The strands unwind, and the strand remaining bound to RISC guides it to targeted mRNA sequences that have perfect (for siRNA) or imperfect (for miRNA) complementarity to initiate their degradation and/or translational repression [42, 43]. A scheme of siRNA-mediated RNAi is provided in Fig. 3 [42]. ASO-based and RNAi-based SNAs have been used to target a variety of genes (e.g., *Bcl2L12* [41], *miR-182* [22], ganglioside *GM3 synthase* [20], epidermal growth factor receptor (*EGFR*) [19, 20], *Malat-1* [30], and others). In vitro gene regulation with SNAs has been summarized in detail elsewhere [10, 30], so the following sections focus on the in vivo use of SNAs for gene regulation.

3.1.1 SNAs for Gene Regulation in Glioblastoma

Glioblastoma is the most malignant form of brain tumor, with a median survival of <2 years [44]. Small-molecule drugs and antibodies that are currently used in the clinic to attack glioblastoma are extremely ineffective due to their limited ability to cross blood–brain barrier or blood–tumor barrier, which leads to the need for high doses that cause systemic toxicity [45–47]. Because SNAs could transfect cells in vitro to regulate gene expression, Mirkin and colleagues investigated whether SNAs could deliver siRNA and miRNA to brain tumors for therapeutic gene regulation [22, 41, 48]. They designed 13-nm gold core SNAs to deliver siRNA targeting *Bcl2L12* [41], an anti-apoptotic gene that is overexpressed in glioblastoma, or *miR-182* [22], a miRNA that is downregulated in glioblastoma and that functions to suppress *Bcl2L12*. Excitingly, both the siRNA- and miRNA-based SNAs could enter glioblastoma tumors in mice following intravenous administration to suppress intratumoral *Bcl2L12* expression, and this slowed tumor growth and prolonged animal survival [22, 41]. Additionally, these SNAs exhibited no apparent toxicity, as evidenced by histopathology of various tissues and analysis of blood chemistry [22, 41]. SNAs have also been developed to suppress the DNA repair protein O6-methylguanine-DNA-methyltransferase (*MGMT*), which is associated with drug resistance in glioblastoma, and these siMGMT-SNAs potentiated the effects of co-administered temozolomide in murine tumor models [48]. Together, these findings demonstrate the immense potential of SNAs as a new treatment for glioblastoma, either alone or in combination with other modalities.

3.1.2 SNAs for Gene Regulation of Skin Disorders

There is growing interest in using SNAs and other gene regulatory agents to combat diseases where the mode of

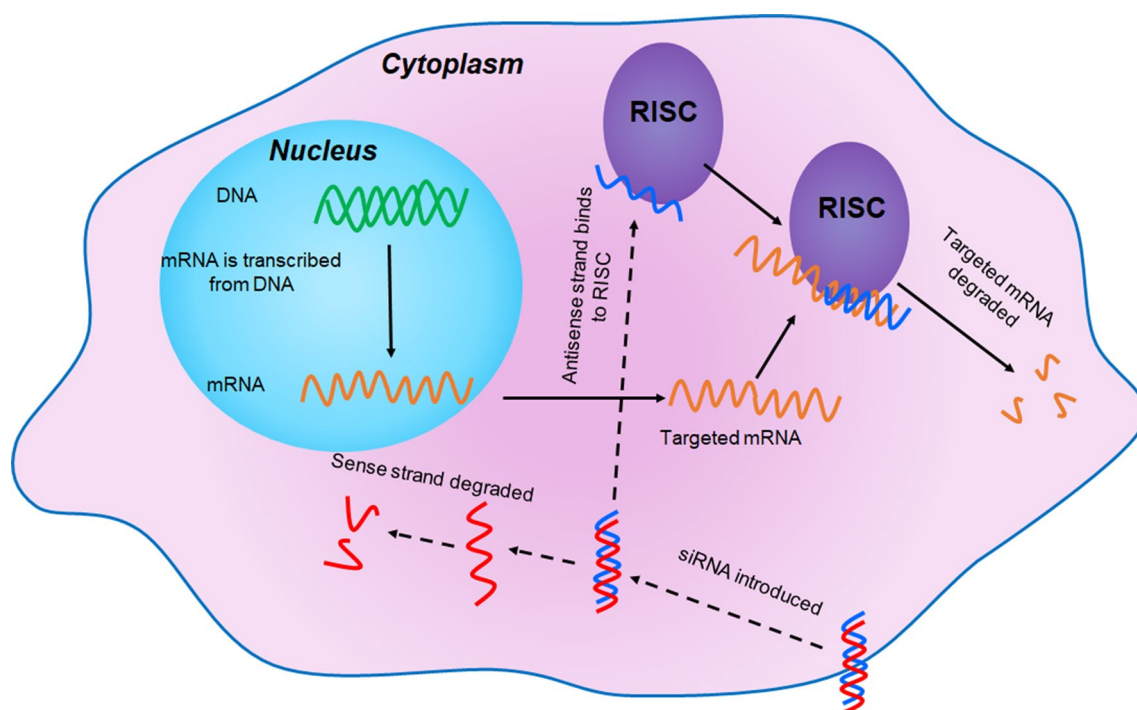


Fig. 3 Scheme depicting RNA interference therapy. Ordinarily, DNA is transcribed into mRNA, which is then translated into protein. In RNA interference therapy, siRNA (shown here) or miRNA is delivered into cells complexes with the RISC and guides it to complementary mRNA molecules in the cytoplasm, which are subsequently

degraded. Reprinted with permission from Kreuzberger et al. Nanoparticle-mediated gene regulation as a novel strategy for cancer therapy. Copyright 2017. Delaware J Public Health [42]. mRNA Messenger RNA, miRNA microRNA, RISC RNA-induced silencing complex, siRNA small interfering RNA

administration is topical, rather than systemic. Topical delivery is ideal for skin disorders due to the accessibility and minimal risk of systemic toxicity. However, intradermal delivery of naked oligonucleotides is nearly impossible without disrupting the thick epidermal barrier. To demonstrate that SNAs could penetrate skin to regulate gene expression, Zheng et al. designed siRNA-based gold-cored SNAs to inhibit *EGFR* [19]. These SNAs penetrated almost 100% of keratinocytes *in vitro* to silence *EGFR* expression, and were > 100-fold more potent than commercial transfection agents at delivering siRNA into mouse skin and human skin equivalents (Fig. 4a) [19]. Based on these findings, SNAs are now being developed for various skin disorders, including diabetic wound healing and psoriasis [20, 49, 50].

With respect to diabetic wound healing, Randeria et al. showed that topically applied siRNA-based SNAs could efficiently downregulate ganglioside-monosialic acid 3 synthase (*GM3S*), a gene that is overexpressed in diabetic mice and responsible for insulin resistance and impeded wound healing [20]. As shown in Fig. 4b, topically applied *GM3S* SNAs completely healed wounds in diet-induced obese mice within 12 days [20].

Regarding psoriasis, liposomal SNAs (L-SNAs) coated with ASO oligodeoxynucleotides (ODNs) targeting tumor necrosis factor- α (TNF- α) were developed to suppress the

effect of cytokines and chemokines that lead to activation of psoriasis [51]. Cyanine 5-labeled L-SNAs were internalized by normal human epidermal keratinocytes (NHEKs) within 15 min and successfully penetrated human abdominoplasty and psoriatic skin within 24 h (Fig. 5a, b). Moreover, TNF- α L-SNAs totally reversed the psoriasis phenotype clinically, histologically, and transcriptionally in an imiquimod-induced psoriasis-like mouse model (Fig. 5c, d), suggesting the potential for future translation of L-SNAs to treat human psoriasis [51]. Overall, the findings regarding the use of SNAs for diabetic wound healing and psoriasis confirm that SNAs have substantial promise to enable personalized gene therapy via topical delivery.

3.2 SNAs for Immunomodulation

In addition to being used for gene regulation, nucleic acids (and SNAs) can also be used for immunomodulation. Modulating immunity via delivery of immunostimulatory or immunoregulatory nucleic acids is a very attractive approach to treat various disorders [52, 53]. For example, pathogen-associated molecular patterns such as CpG ODNs (short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides) and Poly I:C (polyinosinic-polycytidylic acid, a synthetic analog

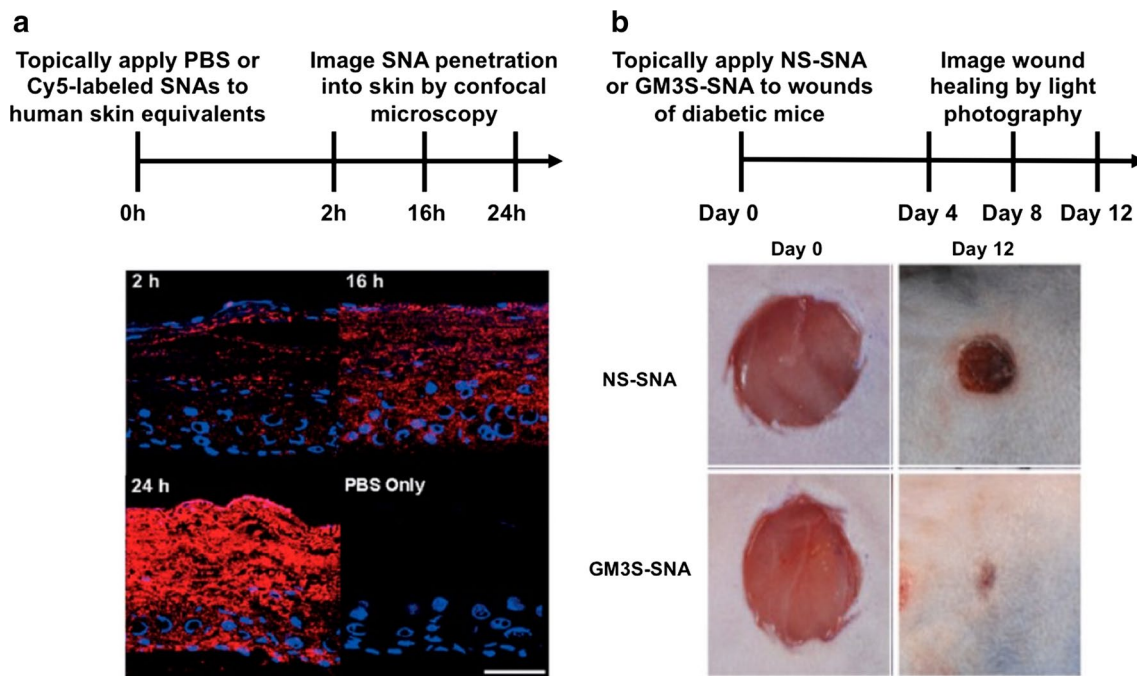


Fig. 4 Topically applied SNAs can penetrate skin to regulate target genes and improve wound healing. **a** Cy5-labeled SNAs (red) are present throughout the stratum corneum and nucleated epidermis of human skin equivalents (EpiDerm; MatTek) following a single application. Blue, Hoechst 33343-stained nuclei. Scale bar = 50 μ m. Reprinted with permission from Zheng et al. Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. Copyright 2012. Proc Nat Acad Sci USA [19]. **b** Representative images of wounds in diabetic obese mice treated with NS-

SNA or GM3S-SNA. Topical application of GM3S-SNAs resulted in more rapid wound healing. Reprinted with permission from Randeria et al. siRNA-based spherical nucleic acids reverse impaired wound healing in diabetic mice by ganglioside GM3 synthase knockdown. Copyright 2015. Proc Nat Acad Sci USA [20]. Cy5 cyanine 5, NS-SNA nonsense SNA, GM3S ganglioside GM3 synthase, GM3S-SNA SNAs targeting GM3S, NS-SNA nonsense SNA, PBS phosphate buffer saline, SNA spherical nucleic acid

of double-stranded RNA) can bind to endosomal toll-like receptors (TLR-9, TLR-7/8, and TLR-3) to promote systemic immune response and are useful as vaccines and cancer immunotherapies [54–57]. Conversely, immunoregulatory nucleic acids that antagonize toll-like receptors have been utilized in the treatment of psoriasis, lupus, and arthritis [52, 58, 59]. Due to their unique 3D orientation, ODNs attached to SNAs have increased binding affinity for their target, which results in improved immunomodulation. Below, the types of SNAs that have been designed for immunostimulatory and immunoregulatory purposes are described.

The immunostimulatory role of CpG ODNs has initiated widespread investigation of their use as vaccine adjuvants and cancer immunotherapies [56, 57, 60]. Various nanoparticles have been designed to deliver CpG ODNs to induce Type 1 and Type 2 helper cell immune response in various diseases [61–67]. In this direction, Radovic-Moreno et al. developed immunostimulatory SNAs (IS-SNAs) by coating gold or liposomal nanoparticles with CpG ODNs. These IS-SNAs induced higher production of pro-inflammatory cytokines by murine macrophages and human peripheral blood mononuclear cells than their soluble counterparts [68]. Additionally, the

immunostimulatory activity of the SNAs was independent of core material. It was also shown that the unique 3D structure of the IS-SNAs was critical to their enhanced function (Fig. 6a). The team designed immunostimulatory liposomal SNAs (IS L-SNAs) that had CpG 7909 ODNs with phosphodiester backbones presented on their surface ('external CpG 7909-po L-SNAs'), and compared them to liposomes that encapsulated the CpG-7909-po ODNs ('internal CpG 7909-po Liposomes'). The external CpG 7909-po L-SNAs were approximately 3-fold more potent than the internal CpG 7909-po liposomes when incubated overnight with Ramos-Blue cells (Fig. 6a). To measure whether this improvement was simply due to greater uptake of the L-SNAs, they prepared liposomes with internal CpG 7909-po ODNs that were also coated with immunologically inactive d-A₂₀ all-phosphodiester ODNs ('external A20-po L-SNAs/Internal CpG 7909-po') to give them an SNA structure, and tested their activity in the same cell line. They found no difference in activity for these internal CpG/external d-A₂₀ L-SNAs as compared with the internal CpG 7909-po liposomes (Fig. 6a). This suggests that the external orientation of immunostimulatory ODNs on SNAs provides increased availability for

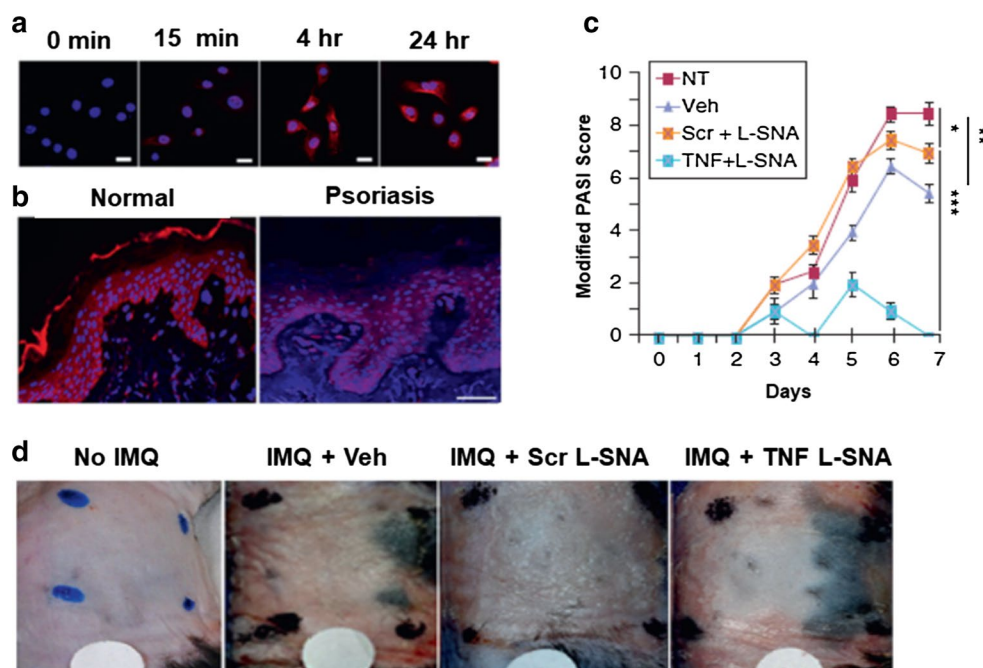


Fig. 5 Topically applied SNAs for treatment of psoriasis. **a** Time-dependent uptake of Cy5-labeled liposomal SNAs in NHEK cells. Nuclei are blue (DAPI). Scale bars = 20 μ m. **b** Cy5-L-SNAs diluted to 30 μ M in Aquaphor/PBS (1:1) and applied to normal or psoriatic human skin explants in lifted cultures are visible throughout the samples after 24 h incubation. Scale bars = 50 μ m. **c**, **d** Psoriatic skin samples exposed to no treatment, vehicle, L-SNAs containing scrambled RNA (Scr + L-SNA), or L-SNAs containing TNF- α -targeted RNA (TNF + L-SNA) were assessed clinically by modified PASI

score. All data shown in **c** are with imiquimod treatment, with modified PASI scores for untreated mice all equal to 0. Reprinted with permission from Lewandowski et al. Topically delivered tumor necrosis factor- α -targeted gene regulation for psoriasis. Copyright 2017. J Investigative Dermatol [51]. Cy5 cyanine 5, IMQ imiquimod, L-SNAs liposomal SNAs, NHEK cells normal human epidermal keratinocyte cells, NT no treatment, PASI Psoriasis Area Severity Index, PBS phosphate buffer saline, Scr scrambled RNA, SNA spherical nucleic acid, TNF- α tumor necrosis factor- α , Veh vehicle

them to interact with their targeted TLR-9 receptors within endosomes, which ultimately improves their intracellular activity.

Moving in vivo, Radovic-Moreno et al. demonstrated that IS-SNAs increased interleukin (IL)-12 and interferon (IFN)- γ production ~10-fold in mice, confirming the particles have systemic immunostimulatory capabilities. No difference was found between SNAs that delivered CpGs with phosphodiester or phosphorothioate backbones. Moreover, when the model antigen ovalbumin was attached to IS-SNAs, robust humoral and cellular immune responses were induced in mice, as evaluated by IgG2a titers and induction of IFN- γ producing T cells. Impressively, Radovic-Moreno et al. showed that this enhanced immune response could translate into improved anti-tumor efficacy [68]. Specifically, they showed that mice bearing EG.7 OVA tumors that were treated with antigen-loaded IS-SNAs experienced a profound and durable tumor growth remission (Fig. 6b), which doubled survival rates [68]. Overall, this shows that IS-SNAs have a substantial advantage compared with their linear counterpart.

While Radovic-Moreno et al. demonstrated the potential utility of IS-SNAs based on gold or liposomal cores, Banga

et al. showed that cross-linked micellar IS-SNAs could be made from the FDA-approved thermosensitive block co-polymer pluronic F127 [36]. TLR-9 agonist CpG ODNs with lipid tails were intercalated into the hydrophobic regions of Pluronic F127 micelles, and subsequently chemically cross-linked to form a stable structure. Micellar SNAs were made with CpG 1826 or CpG 7909 ODNs, demonstrating the versatility of the synthesis, and these IS-SNAs showed enhanced activity in HEK and Ramos-Blue cells, respectively, compared with linear CpG ODNs. By using FDA-approved precursors to synthesize SNAs, this work brought SNA technology one step closer to clinical translation [36].

Finally, immunoregulatory SNAs (IR-SNAs) have also been designed by conjugating CpG 4084F TLR9 antagonist sequences to gold nanoparticles to evaluate their effect in fibrotic mice. Administration of the IR-SNAs to nonalcoholic steatohepatitis (NASH) mice led to a 40–51% reduction in fibrosis relative to controls when the animals' livers were evaluated by histology and scored for fibrosis and NASH [68]. This demonstrates the potential of this technology to be utilized for either activation or suppression of the immune system. Overall, the results to date indicate that one could attach virtually any ODN sequence to SNAs to

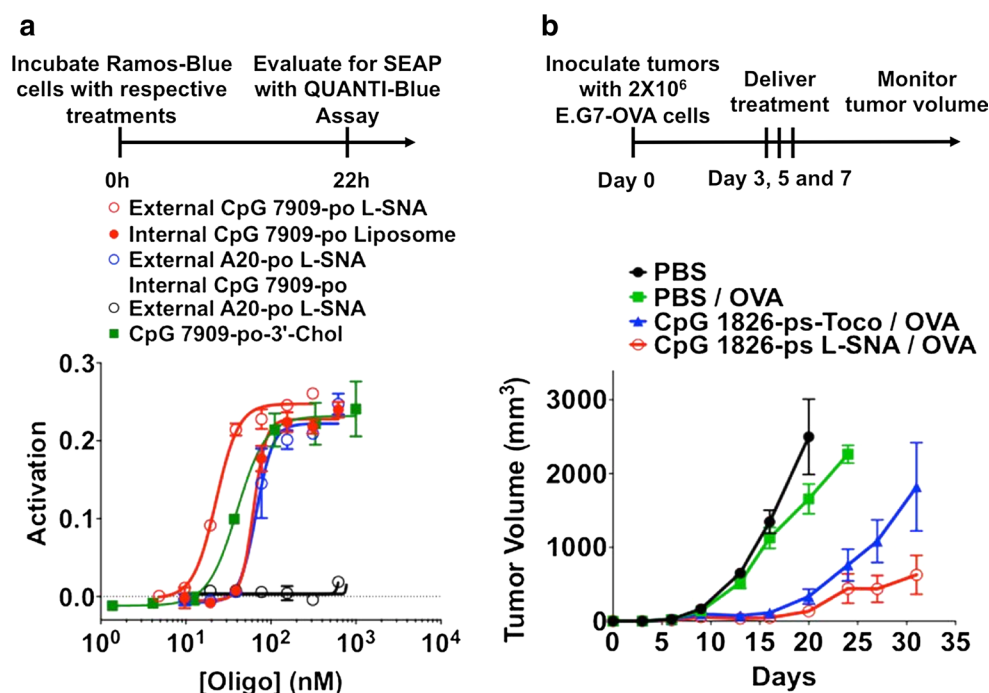


Fig. 6 IS-SNAs demonstrate increased potency in vitro and reduce tumor growth in mice bearing lymphoma tumors. **a** Significance of the orientation of CpG ODNs in IS L-SNA function. Comparison of IS L-SNAs made with CpG 7909 TLR9 agonists on their exterior to liposomes made with CpG 7909 on their interior, with or without an exterior coating of d-A₂₀ all-PO ODNs. The external presentation of CpG 7909 ODNs in L-SNAs is critical to their superior performance. **b** Tumor growth curve of mice bearing subcutaneous E.G7-OVA lymphoma tumors treated with either liposomal IS-SNAs containing tocopherol-modified CpG ODNs and OVA antigens, or with freely

delivered OVA alone or in combination with CpGs. The experimental design is shown above the figure. Reprinted with permission from Radovic-Moreno et al. Immunomodulatory spherical nucleic acids. Copyright 2015. Proc Nat Acad Sci USA [68]. CpG ODNs short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides, E.G7-OVA mouse lymphoma cell line engineered to overexpress ovalbumin protein, IS-SNA immunostimulatory SNA, L-SNA liposomal SNA, ODN oligodeoxynucleotides, OVA ovalbumin, PBS phosphate buffer saline, PO phosphodiester, SEAP secreted alkaline phosphatase inducible by NF-κB, SNA spherical nucleic acid

modulate immune response, suggesting SNAs have great potential as immunostimulatory or immunoregulatory agents for the treatment of various diseases.

3.3 SNAs as Vehicles for Drug and Protein Delivery

3.3.1 SNAs for Drug Delivery

Chemotherapeutics such as cisplatin, carboplatin, paclitaxel, and doxorubicin are widely used for cancer treatment [69, 70]. To overcome the poor solubility and toxicity of these drugs, various carriers have been developed. Additionally, researchers have created pro-drugs, which remain inert until they are metabolized within the body to form an active compound. Although pro-drugs are safer to use than standard chemotherapies, they still require an effective carrier. SNAs are attractive vehicles for pro-drug delivery because they have the unique ability to enter cells without provoking a significant immune response. Dhar et al. demonstrated this by synthesizing Pt(IV) pro-drug SNAs that had Pt(IV) pro-drugs attached to DNA oligonucleotides

containing terminal dodecyl amine groups via amide linkages. The Pt(IV) pro-drug SNAs co-localized with microtubules upon entering HeLa cervical cancer cells and were more potent than soluble cisplatin against four different cancer cell lines [71]. Likewise, Zhang et al. synthesized paclitaxel pro-drug SNAs by attaching a carboxylic acid derivative of paclitaxel to terminal amine groups of DNA oligonucleotides [72]. Covalently attaching hydrophobic paclitaxel to SNAs increased its solubility by ~50-fold, and paclitaxel-SNAs were more toxic than either free paclitaxel or DNA-paclitaxel duplexes against the multi-drug-resistant human uterine sarcoma cell line MES-SA/Dx-5 [35].

In contrast with the above approaches, where drugs were loaded on SNAs' exterior, an alternative strategy is to load drugs in SNAs' interior and exploit the nucleic acid shell for its transfection capabilities. For example, Banga et al. attached oligonucleotides to the surface of polymeric nanoparticles (PNPs) that were loaded with doxorubicin via EDC chemistry (Fig. 7a). The doxorubicin-loaded SNAs were stable in biological media, could penetrate SKOV3 cancer

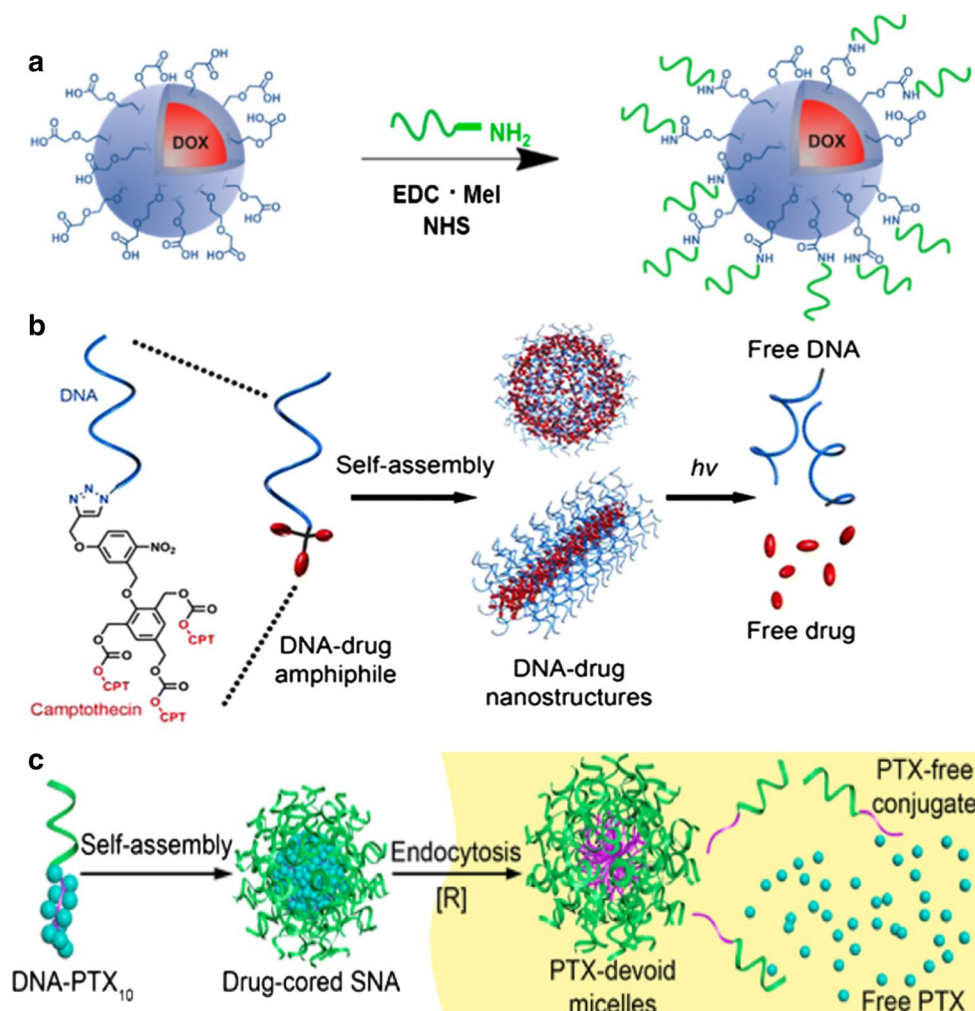


Fig. 7 Scheme of various nanostructures that utilize the nucleic acid 'shell' of SNA architectures to facilitate cell uptake and drug delivery. **a** Drugs such as doxorubicin (DOX) can be loaded within polymer nanoparticles that are then coated with amine-terminated oligonucleotides via EDS-NHS chemistry. Reprinted with permission from Banga et al. Drug-loaded polymeric spherical nucleic acids: enhancing colloidal stability and cellular uptake of polymeric nanoparticles through DNA surface-functionalization. Copyright 2017. American Chemical Society [73]. **b** Camptothecin (CPT) can be conjugated to DNA via azide bonds and these DNA-drug amphiphiles will self-

assemble into DNA-drug nanostructures. Reprinted with permission from Tan et al. Light-triggered, self-immolative nucleic acid-drug nanostructures. Copyright 2015. American Chemical Society [74]. **c** DNA-PTX micelles can be produced by conjugating PTX to DNA via disulfide linker. Reprinted with permission from Tan et al. blurring the role of oligonucleotides: spherical nucleic acids as a drug delivery vehicle. Copyright 2016. American Chemical Society [35]. CPT camptothecin, DOX doxorubicin, PTX paclitaxel, SNA spherical nucleic acid

cells, and were more cytotoxic against these cells than free doxorubicin [73].

Similarly, Tan et al. prepared light-activated self-immolative micellar nanostructures with SNA-like features for intracellular delivery of the hydrophobic drug camptothecin [74]. DNA strands were attached to camptothecin via self-immolative photolabile linkers, which enabled the triggered release of camptothecin upon light activation (Fig. 7b). The DNA-camptothecin nanostructures exhibited enhanced stability against DNaseI and similar cell killing activity as free camptothecin in SK-BR-3 breast cancer cells. Further, to enable multifaceted therapy, Tan et al. designed

DNA-paclitaxel micellar nanoparticles in which the nucleic acid component acted as both a therapeutic payload for intracellular gene regulation and as the drug delivery vehicle [35]. Paclitaxel was covalently conjugated to antisense DNA targeting *Bcl2* (an anti-apoptotic gene), and these amphiphilic nucleic acid drug conjugates (NADC) self-assembled into nanoparticles that are structurally analogous to SNAs (Fig. 7c). These self-immolative SNAs successfully delivered paclitaxel into SKOV-3 ovarian cancer cells and reduced Bcl-2 expression by 70% [35]. This study provided the proof-of-concept data to develop carrier-free delivery systems that utilize NADCs for combination therapy [35].

Although further studies need to be done to understand the in vivo biodistribution and pharmacokinetics of drug-SNA nanoconjugates, the strategies presented in these works show that SNAs are promising tools for intracellular drug delivery, and this promise can be extended to the delivery of other modalities such as antibodies, aptamers, peptides, and proteins, as described in the following section.

3.3.2 SNAs for Protein Delivery

Intracellular protein delivery has widespread biomedical applications [75–77], but proteins are very challenging to deliver intracellularly due to their large size, charged surface, low stability in biological fluids, and degradation during systemic circulation [78]. Many strategies for intracellular protein delivery have been examined, including covalent [79–81] or non-covalent conjugation to polymers and cell penetrating peptides [82, 83], or delivery via nanocarriers such as liposomes [84], micelles [85, 86], and lipid nanoparticles [87, 88]. Brodin and colleagues hypothesized that the unique features of SNAs could be exploited to enable superior intracellular protein delivery. Specifically, they hypothesized that proteins could serve as the core of SNAs and the dense shell of nucleic acids surrounding these proteins could promote their cellular uptake. To demonstrate this, the model protein β -galactoside (β -gal) was conjugated to ~25 DNA strands and these protein SNAs (Pro-SNAs) were evaluated for their stability and intracellular catalytic activity. The β -gal Pro-SNAs showed ~20- to 280-fold higher uptake in multiple mammalian cell lines compared with freely delivered β -gal. Moreover, Pro-SNAs retained their secondary structure and catalyzed the hydrolysis of β -glycosidic linkages, whereas equal concentrations of free β -gal showed little to no catalytic activity [37]. Future work will need to validate whether this strategy can be extended to any therapeutic protein, and whether it is also robust in vivo.

4 Clinical Translation of SNAs

Building upon the papers that have pre-clinically validated SNAs as potent entities for gene regulation [22, 41], immunomodulation [51, 68], and drug/protein delivery [18, 36, 37], Exicure, Inc., a clinical stage biotechnology company that was founded by David Giljohann and Chad Mirkin, is driving forward the clinical translation of SNAs [89]. Since SNAs have the unique ability to penetrate many tissue and cell types, Exicure is developing proprietary SNA platforms for a wide range of diseases with unmet medical needs. Their lead candidates for specific targets are shown in Table 1, which also includes SNA structures being translated by other sponsors.

Four types of SNAs have reached the clinical stage. The first, NU-0129, which delivers siRNA targeting *Bcl2L12*, is currently being evaluated for its safety in a phase I clinical trial in glioblastoma patients in the US [90]. The second, AST-005, targets TNF- α with antisense oligonucleotides, and is applied in a gel formulation to psoriatic lesions. AST-005's safety, tolerability, and effect on disease-related biomarkers were recently evaluated in a phase I clinical trial in 15 patients with chronic plaque psoriasis. The results showed that AST-005 met the safety and tolerability requirements, and excitingly, the highest dose resulted in a statistically significant decrease in TNF- α mRNA expression in psoriatic lesions [89, 91]. This suggests that SNA-based therapeutics that can be applied locally, such as to the skin, eyes, lung, and gastrointestinal tract, will have a very promising path forward to the clinic.

Beyond AST-005, Exicure also has several other therapeutic candidates in development, and the company has raised over US\$75 million to pursue translation of SNA technology. An application to conduct phase I clinical trials for XCUR17, which uses ASOs to target interleukin-17

Table 1 Clinical pipeline for SNA-based therapeutics [89]

Therapeutic candidate	Target	Indication	Development stage	Sponsor
NU-0129 siRNA SNA	<i>Bcl-2</i> -like protein 12	Glioblastoma	Phase I	Northwestern University
AST-005 antisense SNA	TNF	Mild to moderate psoriasis	Phase I	Exicure; partnership with Purdue Pharma
XCUR17 antisense SNA	IL-17RA	Mild to moderate psoriasis	Phase I	Exicure
Unnamed	IL-4RA	Mild to moderate psoriasis	Lead optimization	Exicure
Unnamed	Undisclosed target	Epidermolysis bullosa simplex	Lead optimization	Exicure
AST-008 CpG SNA	TLR9	Solid tumors	Phase I	Exicure
Novel TLR9 agonist	TLR9	Solid tumors	Optimization in non- human primates	Exicure

siRNA small interfering RNA, SNA spherical nucleic acid, TNF tumor necrosis factor

receptor- α , was submitted in collaboration with a medical regulatory body in Germany, and this trial will examine the SNAs' safety and tolerability in patients with psoriasis microplaques [92]. Exicure is also translating AST-008, a TLR9 agonist SNA, for immuno-oncology applications in combination with immune checkpoint agents. A phase I clinical trial is being conducted (NCT03086278) [93] to evaluate its safety, tolerability, pharmacokinetics, and pharmacodynamics in healthy volunteers after subcutaneous administration [92]. Overall, the clinical translation of SNAs is occurring at an extraordinary pace, and this is likely to continue given the positive pre-clinical data that has been reported for various SNA platforms.

5 Conclusion and Future Outlook

While exciting preclinical and early clinical trial data encourages the continued development of SNAs as therapeutics, maximizing the efficiency of their delivery to disease sites remains a challenge that, if addressed, will greatly improve their therapeutic ratio. SNAs are not alone; a recent analysis of nanoparticle delivery to tumors revealed that < 1% of administered nanoparticles accumulate within tumors [94]. This underscores the need for next-generation SNAs to overcome biological delivery barriers and maximize SNA accumulation within the targeted tissue. Future work should seek to (1) control the biodistribution of systemically delivered SNAs, (2) improve the intracellular trafficking of SNAs to maximize cytosolic or nuclear delivery as desired, and (3) prevent premature oligonucleotide degradation during delivery. Towards these goals, identifying the physicochemical properties of SNAs that dictate their interactions with complex biological environments will maximize their clinical utility.

Upon exposure to biological systems, proteins in the local environment rapidly coat nanomaterials. This protein corona influences the fate of nanomaterials *in vivo*, including interactions with cells, clearance by macrophages, immunogenicity, and biodistribution. Indeed, SNAs incubated in human serum develop a corona containing proteins involved in blood coagulation, lipid transport, molecular transport, and immune response [95]. Studies to unravel the factors that influence corona formation identified oligonucleotide sequence as a key player; G-rich sequences produce dense coronas with high immune protein content, while T-rich sequences produce relatively sparse coronas. Consequently, G-rich SNAs undergo greater uptake by macrophages and accumulate more heavily in the liver and spleen than T-rich SNAs [96]. Given the importance of SNA composition in dictating biological interactions, future research should consider opportunities to control the protein corona to regulate the *in vivo* fate of SNAs. The most widely investigated

approach to control protein corona formation has been passivating SNAs with poly(ethylene glycol) (PEG). PEGylated SNAs exhibit increased circulation time but decreased cellular uptake relative to SNAs without PEG [97]. Continued research seeking to control protein corona content by modulating the surface of SNAs could enable better delivery to disease sites and improve the therapeutic efficacy of SNAs.

Improving the intracellular trafficking of SNAs is a second challenge to address moving forward. Studies elucidating the mechanisms by which cells internalize SNAs found that SNAs engage scavenger receptors on cell surfaces to trigger endocytosis and subsequently accumulate within late endosomes [39, 98]. Maximizing endo-/lyso-somal escape is a bottleneck that must be overcome to recognize the full clinical potential of SNAs. Future research incorporating materials such as cationic polymers and cell penetrating peptides into SNA design may improve their cytosolic delivery, but in utilizing these materials delivery efficacy must be balanced with cytotoxicity [99].

A final challenge to address is ensuring the nucleic acid cargo is stable until it arrives within the cytosol of the targeted cells. One study found that nucleic acids disassemble from gold SNAs within 16 h of cellular entry and are subsequently exported from the cell [98]. The disassembly was attributed to endonuclease activity near the gold surface [100], which was surprising, since steric hindrance is greatest at this location and endonuclease activity is prevalent at a different location for free siRNA. Chemically modifying the siRNA at the cleavage site with a 2'-O-Methyl group increased the half-life of the siRNA on SNAs to ~20 min compared with ~2 min for unmodified SNAs, but since intravenously injected SNAs can remain in circulation for ~24 h [41], further increasing the half-life is desirable. A follow-up study revealed that siRNA sequence, spacer length and content, loading density, and PEG backfill size all contribute to the interactions of siRNA-SNAs and nucleases [101]. These studies demonstrated the importance of achieving a high density of nucleic acids and stabilizing agents on the particle surface to increase steric hindrance against nucleases and prevent nucleic acid degradation.

Although there are some challenges that need to be overcome, SNAs have a promising path forward towards the clinic. One of the major benefits of SNAs is that their high biocompatibility and modular design enables them to deliver multiple components into single cells, improving therapeutic response. For example, this flexibility would be of tremendous value in improving vaccines and immunotherapies. SNAs could be designed to deliver multiple antigenic proteins in combination with immunostimulatory ODNs to antigen presenting cells (APCs) to induce a robust immune response against diseases such as flu, dengue, and more. Moreover, drug delivery via liposomal SNAs or micellar SNAs could be potentiated by designing

the SNAs to co-deliver various adjuvants, small molecule immune potentiators (SMIPs), and/or antigenic proteins. One could also envision delivering immunostimulatory nucleic acids or STING (stimulator of interferon genes) agonists with SNAs in combination with current immunotherapies to modulate the tumor microenvironment and identify novel and potent treatment combinations. Overall, advancements in nucleic acid sequencing and synthesis have made it easier to identify target genes associated with disease progression and synthesize oligonucleotides that can regulate these genes. Combining these efforts with advances in SNA technology will enable more effective and personalized treatments for many types of human diseases to become reality.

Compliance with ethical standards

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Conflict of interest CHK, JRM and ESD have no conflicts of interest to declare.

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