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Strategies, Design, and Chemistry in siRNA Delivery Systems

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Abstract

Emerging therapeutics that utilize RNA interference (RNAi) have the potential to treat broad classes of diseases due to their ability to reversibly silence target genes. In August 2018, the FDA approved the first siRNA therapeutic, called ONPATTRO™ (Patisiran), for the treatment of transthyretin-mediated amyloidosis. This was an important milestone for the field of siRNA delivery that opens the door for additional siRNA drugs. Currently, more than 20 small interfering RNA (siRNA)-based therapies are in clinical trials for a wide variety of diseases including cancers, genetic disorders, and viral infections. To maximize therapeutic benefits of siRNA-based drugs, a number of chemical strategies have been applied to address issues associated with efficacy, specificity, and safety. This review focuses on the chemical perspectives behind non-viral siRNA delivery systems, including siRNA synthesis, siRNA conjugates, and nanoparticle delivery using nucleotides, lipids, and polymers. Tracing and understanding the chemical development of strategies to make siRNAs into drugs is important to guide development of additional clinical candidates and enable prolonged success of siRNA therapeutics.

1. Introduction

The presence of endogenous RNA interference pathways in mammalian cells provides a powerful mechanism for the regulation of cellular signaling pathways by enabling precise modulation of gene expression[1]. As one component of the RNAi complex, siRNAs are able to “silence” expression of specific genes with complementary sequences. In contrast to traditional drugs, the sequence of a siRNA therapeutic can be identified based on knowledge

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of the sequence of the messenger RNA (mRNA) that would encode for the protein target. While siRNA has the potential to target essentially any gene, function requires methods to deliver these molecules inside of target cells safely and effectively. The FDA recently approved the first ever siRNA therapeutic, which is a lipid nanoparticle (LNP) called ONPATTRO™ (Patisiran) that encapsulates and delivers siRNA against mutant and wild type transthyretin to treat transthyretin-mediated amyloidosis[2]. We believe that ONPATTRO is the first of many RNAi medicines, but that additional development is required to achieve the broadest clinical application of siRNA. Below we describe how chemistry has played a critical role in improving the efficacy, specificity, and safety of siRNA therapeutics, since the initial description of RNAi in 1998[1],.

In 2006, the Nobel Prize was awarded for the discovery and characterization of RNA interference[1]. RNA interference is mediated by small double-stranded RNA molecules, or siRNAs, which associate with the RNA-Induced Silencing Complex (RISC) inside of cells. After loading, the strands are separated, leaving the anti-sense (or guide) strand to bind complementary mRNA (Fig. 1a)[3–5]. The Argonaute endoribonuclease cleaves the mRNA, which prevents it from being translated to protein[5]. The antisense strand stays bound to RISC, and is able to act catalytically to cleave additional mRNA strands. Although siRNA is generally double-stranded, it can be single-stranded, hairpin, or dumbbell shaped as long as the antisense strand can become loaded into the RNAi machinery[6]. For example, single stranded siRNA is able to activate RNAi in mice, albeit less efficiently than the canonical double stranded forms[7,8].

Delivery of siRNA has been the major challenge to application of siRNA therapeutics in humans.[4,9–12] One key challenge to delivery is the pharmacological properties of siRNA. For example, siRNAs are relatively large (~13 kDa) in molecular weight in comparison to small molecule drugs. They are highly anionic with approximately thirty eight to fifty phosphate groups, which make them difficult to diffuse across cellular membranes. Furthermore, unmodified siRNAs are unstable in the bloodstream and can induce immune responses through interaction with Toll-like receptors[6]. Intravenously administered siRNA must cross the vascular endothelial barrier and then diffuse through the extracellular matrix to function. They must also avoid filtration by kidneys and uptake by non-targeted cells. After cellular internalization, siRNAs need to be released from endosomal compartments, decomplex, and access the RNAi machinery. Throughout this whole process, siRNAs need to have sufficient resistance to nuclease degradation to enable function[13]. Therefore, siRNAs have significant delivery challenges that do not exist for small molecule drugs.[5] Nevertheless, significant progress has been made, and there are currently more than 20 ongoing clinical trials with siRNA-based therapeutics[14–17].

Fig 1b describes a typical process for the development of siRNA therapeutics[15]. First, for a specific biological target, many RNA strands need to be designed and synthesized to identify the most potent sequences along the target mRNA strand. This is generally done both experimentally and computationally[18]. After validation of the *in vitro* potency and specificity, siRNA strands are further stabilized via optimization of chemically modified nucleotides. Finally, a variety of different delivery formulation strategies can be

implemented, as described below. This article will discuss the strategies and design of non-viral siRNA therapeutics from a chemical perspective.

2. Synthesis and chemical modification of siRNA

Chemically modified nucleotides can improve chemical stability and efficacy, increase cell specificity, reduce immunological effects, and decrease off-target effects[6]. siRNA conjugation is one important method to achieve efficacious RNA interference both *in vitro* and *in vivo*. This section will focus on the chemical modification of nucleotides and synthesis of siRNA.

2.1 Nucleotides modification

Nucleotides are the basic building blocks for both DNA and RNA. In general, natural nucleotides are composed of a ribose or 2'-deoxyribose sugar with 1'-nucleobase and 3'-phosphate groups (Fig. 2). Chemical modification of nucleotides dates back to the 1960s[19]. In recent years, the effects of modified nucleotides on siRNA activity have been extensively examined[20,21]. As illustrated in Fig. 2, four major sites have been explored for modification: the 2'-position, phosphate linkage, ribose, and nucleobase[6].

The 2' position is the most common modification site in nucleotides. In 1959, Smith and Dunn isolated 2'-O-methyladenosine from wheat germ and rat liver[22]. The selective synthesis of this compound using diazomethane and 1,2-dimethoxyethane was described by Robins and co-workers[23]. Because chemical modification can potentially inhibit activity, Rana and co-workers performed a chemical modification analysis for siRNA function[21]. Their results indicated that 2'-OHs are not required for siRNA activity and that 2' modification could significantly improve the stability of siRNA and extend their half-life[21]. The 2' position has been modified with a number of residues including 2'-O-methyl, 2'-O-methoxyethyl, and fluoro (Fig. 2). Conformationally constrained nucleotides such as Locked Nucleic Acid (LNA) were developed by Imanishi and Wengel independently[24,25]. Introduction of LNA bases not only improves the stability of siRNA, but can also increase the binding affinity to RNA[20]. Recently, an α-L-tricyclic nucleic acid was developed, which is also highly constrained (Fig. 2)[26].

With regard to the phosphate linkage, chemical synthesis using nucleotide phosphorothioate (including the relevant diastereomers) was the earliest exploration of nucleotide modification[19]. These linkages significantly stabilize the RNA to nuclease degradation such as those found in human serum, but may lead to increased toxicity[20]. Interestingly, DNA phosphorothioation was discovered in 2007 as an endogenous process in bacteria[27]. Recently, Wu and co-workers explored different types of phosphorodithioate on siRNAs and their antitumor activity in mouse models[28]. In addition, a polyamide-based Peptide Nucleic Acid (PNA) was reported by Nielsen and co-workers in 1991[29]. Later, PNA modification was installed at the end of RNA strands[30]. This PNA-siRNA was reported to inhibit the activity of telomerase and introduce cell death of human tumor cells. Boron-containing nucleotide analogues were reported in the early 1990s[31]. A recent study reports that boranophosphate siRNAs can be more potent than native siRNA and phosphorothioate siRNAs[32]. However, PNA and boranophosphate siRNAs have not been investigated as

extensively in animal models as phosphorothioate modified siRNAs, which are in human clinical trials[15].

Modification of the ribose ring has also been investigated (Fig. 2). A morpholino nucleoside was developed in 1989, which has been examined in a number of antisense applications[33]. Morpholino nucleotide oligomers are being examined clinically as a therapy for Duchenne muscular dystrophy[34]. Also of note, Wengel and co-workers developed Unlocked Nucleic Acid (UNA) in 2009[35]. These acyclic UNA based siRNAs were shown to induce silencing *in vitro* and *in vivo*[36]. Moreover, UNA modification was reported to reduce off-target effects and improve biostability in mice. At the same time, hexitol and anitrol based nucleotides were reported, and show potential for use as siRNA modifications[37]. Bramsen and coworkers performed an analysis of chemically modified siRNA for their silencing activity, stability and toxicity in human tumor cells[38]. They report that the sense strand can tolerate diverse chemical modifications, while the antisense strand can be moderately modified under certain conditions. siRNA stabilization does not require the modification of the whole siRNA duplex, but only on a few selected positions. They also reported that UNA modification introduced less toxicity and retained the silencing activity[38]. In 2018, oxabicyclic nucleoside phosphonates were chemically synthesized[39] and might be incorporated into siRNA sequences for gene silencing.

Finally, modification of the nucleobase has been examined to improve the nuclease resistance of siRNA duplexes in serum. For instance, siRNAs containing several 2-thiouracil modified units were thermally stable and showed similar silencing activity compared with the unmodified siRNAs[40]. Other representative examples include 5-bromouracil, 6-diaminopurine, and 2,4-difluorotoluene[21,40–42]. In general, base modifications are not widely applied for siRNA modification, but nevertheless offer an additional opportunity for chemical manipulation.

2.2 Synthesis of siRNA

The synthesis of siRNA is based on research that originated in 1950s[43]. In 1978, Zamecnik and Stephenson reported that a synthetic oligonucleotide complementary to Rous sarcoma virus 35S RNA could inhibit protein expression[44,45]. This work reported that a synthetic antisense oligonucleotide (ASO) could bind to a target mRNA through Watson-Crick base pairing led to substantial research development of ASOs as therapeutics. In 2013, the FDA approved mipomersen, an ASO against apolipoprotein B, for the treatment of homozygous familial hypercholesterolemia[46]. Nusinersen (Spinraza) was later approved by the FDA in 2016 for the indication of spinal muscular atrophy[47]. In general, ASOs do not function *in vivo* through the RNAi pathway and therefore are not considered siRNAs[48]. Nonetheless, research on ASOs provided the basis for much of the chemistry used in siRNA synthesis and chemical modification today.

Solid-phase synthesis is currently the primary approach used to make synthetic RNA[49,50]. As shown in Fig. 2b RNA synthesis is a repetitive chemical cycle in which each nucleotide is added on a solid support. This cycle starts with a deprotection step to remove the protective group on 5’-hydroxyl of the solid support bound nucleotide. The resulting 5’-hydroxyl is then coupled with an activated 3’-phosphorous ester, followed by a capping step

to remove the unreacted nucleotides from the reaction system. The intermediate undergoes another step to oxidize phosphite to phosphorous ester. After the chain assembly, the oligomer is released from the solid support, deprotected, and purified by HPLC. Two types of building blocks were later developed for the efficient synthesis of RNA including 2'-O-TOM and 2'-O-ACE modified nucleotides (Fig. 1c)[49]. Both methods provide a coupling yield of over 99%[49]. The whole process has been successfully automated by utilizing oligonucleotide synthesizers[49].

3. siRNA-ligand conjugates

Direct ligand conjugation to siRNA is a promising delivery strategy. Diverse ligands including small molecules, carbohydrates, aptamers, peptides and antibodies have been covalently linked to siRNA in order to improve cellular uptake and target specific cell types (Fig. 3a)[51–55]. An advantage of siRNA conjugates is that they reduce the need for extra delivery materials, and may thereby improve the tolerability and safety profile of the delivery formulation[56]. Because the 5' end of the antisense strand is required for silencing activity, conjugation is typically performed on the sense strand or 3' end of antisense strand[51]. Two synthetic approaches have been applied: parallel synthesis and linear synthesis.

For parallel synthesis (Fig. 3b), siRNA and its relevant conjugate ligand are synthesized in separate synthetic routes and then are conjugated with each other usually through biodegradable bonds[51]. For example, conjugation of membrane permanent peptides (MPPs, **1**) and anti-GFP siRNA was achieved through a disulfide bond using a diamide oxidizing reagent[57]. Rana and co-workers applied a similar strategy to conjugate TAT (**2**), a cell penetrating peptide and siRNA through a succinimidyl 4-[pmaleimidophenyl]butyrate (SMPB) based linker[58]. Condensation of insulin receptor substrate 1 (IRS1, **3**) and siRNA was also achieved through an amide linkage[59]. The above siRNA conjugates were reported to show silencing effects in different human cell lines.

Linear synthesis (functional groups are added sequentially) is also widely used for a variety of chemical conjugations to siRNA (Fig. 3b). In 1989, Letsinger and co-workers reported the synthesis of amide-linked, cholesterol-modified oligonucleotides[60]. In 2004, cholesterol and lipid modified siRNAs were created using a pyrrolidine-based linkage[61]. These lipophilic siRNA conjugates were shown to silence apolipoprotein B through intravenous injection in mice[62], via a lipoprotein-dependent mechanism. Both low-density lipoprotein receptor and scavenger receptor class B type I are required for the uptake of siRNA conjugates by the liver and other tissues.

A similar conjugation strategy was applied to develop hepatocyte-targeted delivery using asialoglycoprotein receptor (ASGPR) targeted ligand, *N*-Acetyl-D-galactosamine (GalNAc)[63]. As shown in Fig. 3c, pyrrolidine derivative **4** and GalNAc derivative **5** were condensed to afford an intermediate **6**[63]. A solid support was installed on the intermediate through a succinic acid linker and gave the substrate **7** that could then be used to generate the siRNA strand (Fig. 3c). Finally, the GalNAc-siRNA strand was synthesized by adding the nucleotide one by one through the solid phase-based approach as discussed above. This

GalNAc-siRNA conjugate is able to significantly silence target gene in hepatocytes via subcutaneous administration at a dose of single digit mg/kg in mice[56]. Results from a clinical trial of this conjugate demonstrate significant reduction of serum transthyretin (TTR) protein for treating TTR mediated amyloidosis[64]. Currently, a series of these GalNAc-siRNA conjugates are in early or late stage of clinical trials[2,17]. Cemdisiran is in phase II clinical investigation for patients with complement-mediated diseases[2,17]. In addition, several promising candidates including Vutrisiran (indication: TTR-mediated amyloidosis), Fitusiran (indication: hemophilia and rare bleeding disorders), Inclisiran (indication: hypercholesterolemia), and Lumasiran (indication: primary hyperoxaluria type 1) are in Phase III clinical trials[2,17]. In addition to the clinical advance, a wide variety of new chemical approaches were reported to optimize the GalNAc-siRNA conjugate. For example, GalNAc can be conjugated on the 2'-position of the ribose[65]. Matsuda *el al* systematically explored the effects of GalNAc on different sites of siRNA strands and identified several potent sequences[65]. Most recently, Parmar and co-workers incorporated (E)-Vinylphosphonate at the 5'- end of the antisense strand, which stabilized the siRNA and improved its potency[66]. These findings provide new insights on next generation siRNA conjugates.

4. Nucleotides derived nanoparticles

Nucleotides have long been utilized as building blocks to assemble a wide variety of nanoparticles[67]. For example, DNA nanostructures, also called DNA origami, have been explored for over 30 years[67]. A number of two-dimensional and three-dimensional nanostructures have been self-assembled through branched DNA motifs and crystalized for characterization and visualization. In 2012, self-assembled DNA-siRNA tetrahedral nanoparticles were developed for siRNA delivery[68]. The 28.6 nm tetrahedron nanoparticles were composed of 186 Watson-Crick base pairs. Each edge is 30 base pairs long and contains a nick in the middle (Fig. 4). This nick is complementary to the overhang of siRNA strands to serve as a siRNA carrier, which was also applied for aptamer-based siRNA delivery[55]. In order to differentiate tumor cells from normal cells, a cancer-targeting ligand, folate, was installed on the nanoparticle surface. The targeted siRNA-DNA origami showed significant silencing in tumor cells at a dose of 2.5 mg/kg (anti-luciferase siRNA) in a mouse xenograft model. It also displayed a longer blood circulation time ($t_{1/2} \approx 24.2$ min) compared with free siRNA ($t_{1/2} \approx 6$ min). Different from the DNA origami, Mirkin and co-workers first reported spherical nucleic acid (SNA) conjugates in 1996, which were made with gold cores and DNA shells[69]. SNA nanostructures are determined by the shape of the cores and the shells can accommodate both single- and double-stranded nucleic acids with sequences of interest. To construct functional SNA, three components were necessary: a particle attachment moiety, a spacer region, and a programmable recognition region[69]. In the past decade, a number of inorganic cores and nucleic acids shells have been investigated for diverse applications including diagnosis, small molecular drug delivery, and DNA and siRNA delivery[69]. By conjugating different siRNA sequences, SNA achieved gene silencing of a variety of biological targets including luciferase, epidermal growth factor receptor (EGFR), and Bcl2Like12[70]. Currently, this platform is in the clinical trial for treating glioblastoma. In addition, RNA nanostructures were also applied to siRNA delivery[71,72]. For

example, Guo and co-workers constructed multifunctional RNA nanoparticles based on RNA three-way junctions, which showed effective delivery of siRNA targeting survivin[73]. Later on, a diverse set of RNA based nanomaterials were created for gene silencing and other applications[71,74]. In 2012, Hammond and co-workers developed self-assembled RNA interference microsponges via rolling circle replication (RCT), which was applied by viruses to amplify their genes[75]. To achieve this process, they constructed a linear DNA strand encoding the antisense and sense sequences. Also, their ends were partially complementary to the T7 promoter. After hybridization with a T7 promoter, this linear DNA formed a circular DNA with its nick closed by a T4 DNA ligase. During the RNA transcription, T7 RNA polymerase can produce multiple copies of antisense and sense sequences to form hairpin RNA structures. As the concentration of circular DNA increased from 3 nM to 100 nM, RNA products grow from fiber-like structures to sponge-like structures. A single sponge contains approximately five hundred thousand copies of siRNA. After condensation with polyethyleneimine (PEI), the microsponge size reduced from 2 μm to 200 nm[75].

5. Lipid-based delivery systems

Phospholipids are natural components of cell membranes that form lipid bilayers[76]. Liposomes have been developed as drug delivery carriers using a variety of synthetic lipids[76]. They have been widely used to encapsulate small molecule drugs for treating diseases in humans, most notably Doxil for breast cancer and AmBisome for fungal infection[76]. For example, the formulation of Doxil is composed of doxorubicin, methoxypolyethylene glycol 2000 −1,2- distearoyl-sn-glycero-3 phosphoethanolamine (MPEG-DSPE), hydrogenated soy phosphatidylcholine (HSPC), and cholesterol[76]. These previous studies provide important guidance for the development of lipid-based siRNA delivery systems.

5.1 Lipid analogs with cationic head groups and hydrophobic tails

Lipid-based nanoparticles (LPNs), particularly lipids with single or multiple cationic centers (Fig. 5), are highly effective carriers of siRNA[77]. In 2005, Stabilized Nucleic Acid Lipid Particles (SNALPs) for intracellular delivery of siRNA were reported[78]. SNALPs are typically composed of a formulation consisting of an amine-based lipid, cholesterol, a PEG-lipid, as well as helper phospholipids[78]. Early SNALPs were formulated from DlinDMA, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) as a helper lipid, mPEGC-DMA, and cholesterol (DlinDMA:DSPC:Chol:PEG-C-DMA 30:20:48:2 molar percent)[78]. DlinDMA is composed of an ionizable amino head group, a glycerol-based ether linker, and two unsaturated carbon tails (Fig. 5)[78]. Amino lipids are central components of SNALPs, as they play a role in the assembly of the nanoparticles, by binding the siRNA through electrostatic interactions[79]. These amino groups also facilitate endosomal escape, through interaction with endosomal components during acidification[80]. The common structure of this type of lipids includes a cationic head group, a linker, and two long hydrophobic domains (Fig. 5).

In vivo, SNALPs have shown the ability to deliver siRNA to tumor tissues. A phase I clinical trial using DlinDMA based ALN-VSP to target vascular endothelial growth factor (VEGF) and kinesin family member 11 (KIF11 or KSP) demonstrated antitumor activity in patients

with advanced solid tumors (dose >0.7 mg/kg). To improve the delivery efficiency, the ether linker in DlinDMA was replaced by a ketal linker to afford DLin-KC2-DMA[81]. This chemical alteration reduces the transition temperatures of DLin-KC2-DMA and facilitates its ability to form hexagonal structures when it interacts with naturally occurring anionic phospholipids in the endosomal membrane. This process is believed to promote endosomal release[76]. DLin-KC2-DMA showed improved delivery efficiency with an efficacious dose of 0.01 mg/kg for hepatocyte silencing of Factor VII in mice. Recently it was reported that DLin-KC2-DMA SNALPs can transfect leukemia cells *in vivo* as well[82].

The structure-activity relationship for DLin-KC2-DMA derivatives has been investigated systematically (Fig. 5)[79]. Head groups were substituted by amino groups with different size and ring structures. SAR studies of 56 amino lipids *in vivo* indicate that a dimethyl substitution on the amine head group was preferred to diethyl, diisopropyl, and ringed structures. The linkers ranged from ester, amide, ketal, ether, and carbamate. Efficacy was generally retained for ester, ketal, and carbamate linkers, though the length and functional groups of the linker could significantly affect the activity. For example, both DLin-KC2-DMA and DLin-MC3-DMA displayed significant silencing of FVII in mice[83]. Lipids with amide and ether linkers possessed reduced delivery performance when formulated. The authors show that pKa is an important factor for delivery efficiency and an optimum pKa of the nanoparticles was between 6.2–6.5. Consistent with this report, a recent study investigated the correlation between nanoparticle properties and siRNA delivery efficiency[84]. In this study, pKa was also identified as a key determinant of nanoparticle delivery efficacy[84]. Hydrophobic tails can accommodate diverse functional groups, including unsaturated carbon bonds and small molecules[85]. Maier and coworkers report that an ester bond can be installed in the middle of hydrophobic tails (Fig. 5, L319), which retain delivery efficiency and improve biodegradability[86]. In this collection, DLin-MC3-DMA was reported to be the most potent lipid with siRNA formulations having an ED_{50} around 0.005 mg/kg in mice (Fig. 5)[79]. ONPATTRO™ (Patisiran) formulated from DLin-MC3-DMA was approved by FDA for the treatment of transthyretin-mediated amyloidosis in August 2018[2,87]. A simple substitution reaction was used to prepare the DLin-MC3-DMA (Fig. 5)[85].

In addition to lipids with single cationic center, numerous lipid derivatives and lipid-like materials with multiple cationic centers have been developed[88]. Here, we discuss two representative examples: aminoglycoside and amino acid derivatives. Aminoglycoside-based lipids are composed of an aminoglycosides head, an amide linker, and two unsaturated tails[89] (Fig. 5) Formulated with 1,2-dioleoylsn-glycero-3-phosphoethanolamine (DOPE), lipidic aminoglycoside derivatives are capable of delivering siRNA in various human tumor cell lines[89]. In 2006, an arginine based lipid, AtuFECT01 was reported, which consists of arginine derived head, an amide linker, and two different carbon tails[90]. AtuFECT01 siRNA-lipoplexes were reported to silence in the vasculature of mice via systemic administration. Recently, a similar arginine based liposomal delivery system was developed for hepatic silencing[91]. The lead material was reported to have dose dependent silencing with an ED_{50} of 0.1 mg/kg in mice[92]. Both SNALP and AtuFECT01 based siRNA delivery systems were in clinical trials for treating solid tumors[16].

5.2 Combinatorial and high throughput strategies of lipid-like materials

In 2008, a library of lipid-like molecules, termed lipidoids, was developed for siRNA delivery[93] (Fig. 6). Lipidoids are composed of one or more amine centers and multiple hydrophobic tails. Over 1200 diverse lipidoids were synthesized with a range of functional amines and acrylates or acrylamides through a one-step Michael addition reaction without the need of catalysts or solvents. The amino groups function similarly to the amino head group in the lipid nanoparticles mentioned above, by neutralizing negative charges of siRNA and facilitating cytoplasmic release of siRNA. Lipidoids were formulated using a method similar to SNALPs. In general, lipidoids with amide linkages and more than two alkyl tails (8–12 carbons) had the most activity when formulated with siRNA. One lipidoid termed 98N12–5 (cite figure 6) in particular was investigated for its potential to deliver siRNA[94]. 98N12–5 was capable of silencing both APoB and FVII in mice and non-human primates.

In an effort to improve the delivery efficiency, a library of amino alcohol-based lipidoids was developed using epoxide-based chemistry[95]. A one-step ring-opening reaction between functional amines and epoxides afforded over 100 new lipidoids. The lead material, named C12–200 (Fig. 6), was composed of a piperazine ring and five lipid tails. The ED_{50} of siRNA formulated with C12–200 was reported as low as 0.01 mg/kg for hepatocytes in mice. The potency of this formulation also allows the potential for simultaneous gene silencing. C12–200 formulations containing five different siRNAs at once were shown capable of silencing their target genes in mice. This material was also shown capable of silencing certain immune cells in mice and primates[96]. More recently, several combinatorial libraries have been constructed using structural information gained in these earlier studies[97]. In particular, a lipopetide material termed cKK-E12, (Fig. 6), was developed and show to be the most potent and selective siRNA delivery systems for gene silencing reported thus far for hepatocytes[98]. Meanwhile, Whitehead and co-workers made a series of biodegradable lipidoids such as 503-O13 (Fig. 6)[99]. In 2016, Siegwart *et al* applied a two-step synthetic route, Michael addition and thiol-ene reaction, to prepare degradable dendrimers (Fig. 6), of which 5A2-SC8 induced strong gene silencing effects and extended overall survival of mice with liver cancer[100]. More and more lipid analogs and derivatives are under development, which may facilitate siRNA or other types RNA therapeutics.

6. Polymer-based siRNA delivery

The utility of polymers to function as intracellular delivery systems for nucleic acids, including antisense oligonucleotides (ASOs) and plasmid DNA (pDNA), has been studied for several decades[101,102]. For instance, cationic polymers are able to condense nucleic acids into polyplexes via electrostatic interactions, enhancing cellular uptake and endosome escape. Notably, the physiochemical properties of polymers can be carefully adjusted through bottom-up chemical synthesis. The eventual (nano)structure can also be dictated through chemical synthesis, covering a range of assemblies including block and star shaped copolymers, micelles, dendrimers, solid nanoparticles, polyplexes, polymer-siRNA conjugates, and more. A few polymer-based siRNA delivery systems have shown therapeutic potential in clinical trials[16]. Examples of major classes of polymer-based delivery systems are discussed below.

6.1 siRNA-polymer bioconjugates

Direct conjugation of siRNA to polymers offers an attractive avenue to improve stability, pharmacokinetics, cellular uptake, and delivery. Polymers with a history of medical use, such as poly(ethylene glycol) (PEG) and poly(lactic-*co*-glycolic acid) (PLGA) have been conjugated to siRNA to facilitate delivery[103,104]. PEG conjugated to siRNA via an acid-labile linker was reported to facilitate gene silencing in hepatoma cells in vitro[103] and in a tumor model[104]. Kataoka and co-workers pioneered the use of polyion complexes (PICs) for drug delivery, and used this system to graft siRNA through disulfide linkages to a polymer to improve the physicochemical properties and transfection efficacy[105]. The hydrophobic nature of PLGA within siRNA-PLGA conjugates was employed to form self-assembled micelles[106]. PLGA has also been used for local delivery of siRNA via the depot approach. Local drug eluter (LODER) has been developed by Silenseed[107] as a potential approach to treat pancreatic cancer targeting mutant G12D KRAS. Following injection into an inoperable tumor, PLGA degrades and mediates sustained release of siRNA. This system is currently the approach in a recruiting Phase 2 clinical trial. Saltzman and coworkers reported using PLGA-spermidine nanoparticles gene silencing in the vaginal lumen and uterine horns of mice following topical delivery[108]. The development of Controlled Radical Polymerization (CRP) has made a major impact on polymer science, and these techniques have also been used to form bioconjugates[109], star polymers, nanogels[110], and other delivery materials for siRNA[111]. Lastly, in situ radical polymerization of siRNA enabled formation of siRNA nanoparticles and intracellular delivery[112].

One example of a polymeric siRNA delivery system in clinical trials is the Dynamic PolyConjugates system, which enabled >60% silencing of apoB in hepatocytes *in vivo* at a dose of about 2.5 mg/kg (50 μg siRNA and 800 μg polymer per mouse)[113] Key components of this design included an amphipathic poly(vinyl ether) with acid-labile maleamate bonds that cleave inside of the endosome, PEG chains for shielding, and *N*-acetylgalactosamine ligands to target hepatocytes. Similarly, a poly(butyl amino vinyl ether) (PBAVE) polymer was designed with side chains of alkyl and amino groups to mediate cellular uptake and endosomal release, and GalNac groups for hepatocyte targeting. This polymer was conjugated to siRNA, providing a single-component system with multiple functionalities for distinct purposes. The Dynamic PolyConjugate design has shown promising results or the treatment of Hepatitis B[114]. Newer generation PolyConjugates, that improve on the initial design have been reported to fully silence liver genes (0.2 mg/kg in non-human primates), producing a 7 week effect.[115] A co-injection strategy using PBAVE and cholesterol-siRNA is a clinical candidate for the treatment of hepatitis B (HBV).[116] Furthermore, the original PBAVE polymer has been alternatively modified with a melittin-like peptide to endow similar reversibly masked endosomolytic properties and entered a Phase I clinical trial.[117,118]

Merck & Co. have reported a similar design that utilizes a cationic, amphilphilic poly(vinyl ether) copolymer backbone with pendant carboxy dimethylmaleic (CDM) pH masking groups, liver targeting *N*-acetylgalactosamine (NAG), and immunostealth (PEG) groups[119]. siRNA against ApoB was covalently attached, and the polymer conjugates showed effective single digit mg/kg silencing in the liver. A biodegradable design based on polypeptide[120]

and poly(amido amine)[121] conjugates further expanded the *in vivo* activity and tolerability of the siRNA conjugates.

6.2 Polymeric complexes

One of the initial requirements of polymeric carriers is the ability to complex siRNA. This has most frequently been achieved using electrostatic interactions between positively charged groups on polymer chains and the negatively charged phosphates in siRNA molecules. Tertiary amines are particularly well suited for siRNA delivery because they can be charged at low pH during self-assembly with siRNA, neutral at extracellular pH, and positively charged after endocytosis to enable endosomal release. This process may involve charge mixing with endosomal lipids, pH buffering / proton sponge effects, among other mechanisms to facilitate escape of siRNA into the cytoplasm[122–124]. Although efforts have been made to use other interaction parameters, such as intercalation and physical entrapment, these efforts have yielded less efficacious carriers[125]. In addition to functional groups that mediate siRNA binding, a second fundamental feature of efficacious polymers for siRNA delivery is hydrophobicity. It is understood that hydrophobic interactions can further stabilize siRNA-polymer nanoparticles above the nanoparticle pKa. This can be accomplished by inclusion of hydrophobic molecules (e.g. cholesterol) or by chemical modification of polymers with hydrophobic domains (e.g. alkyl chains). Thus, an optimal balance between pKa (6.0 – 6.5) and hydrophobicity is implicated in effective materials for siRNA delivery[126–131].

(Linear (LPEI) and branched (BPEI) PEIs have been extensively investigated for pDNA and siRNA delivery. In general, BPEI provides greater complexation ability with siRNA due to the flexible structure, an increased number of charges per volume, and more folding options. [132] Modification of PEI with neutral or anionic moieties has been shown to reduce cytotoxic effects, sometimes without loss of the endosomal rupture abilities. In particular, hydrophobic modifications have been shown to improve siRNA delivery[133,134]. For example, lipid derived BPEI can efficiently deliver siRNA to endothelial cells and silence multiple endothelial genes in mice[135,136]. Moreover, the formulation showed selective silencing in endothelial cells. In addition, branched polyamidoamine dendrimers (PAMAM) have been investigated for siRNA delivery[137–140]. Interestingly, by tuning the formulation, PAMAM-based delivery systems can deliver siRNA to endothelial cells, hepatocytes, or tumor cells. [141] In addition, formulated with small molecule amines or lipids, non-cationic polymers such as PLGA are capable of delivery siRNA[108,142]. PAMAM-RNA complexes have also been incorporated into degradable polymer scaffolds to mediate controlled local release and sustained gene silencing to increase survival of mice bearing aggressive triple-negative cancer[143].

Block copolymers of PEG-*b*-PLA and PEG-*b*-PLGA have been used to create core-shell hybrid nanoparticles, often with inclusion of cationic lipids to enable siRNA encapsulation and release. For example, Yang et al.[144,145] utilized PEG-*b*-PLA/BHEM-Chol nanoparticles to silence polo-like kinase 1 (Plk1) to inhibit tumor growth in mice. PLGA-based block copolymer systems have also been developed by Farokhzad and coworkers[146,147] for systemic delivery of siRNA against various targets, including demonstration of efficacy in a

prostate cancer model. Linear poly(β-amino esters) (PBAEs) have also been extensively explored for the *in vivo* delivery of nucleic acids, including siRNA[148–153].

The use of well-defined polymer architecture and precise cross-linking chemistry are also useful strategies for siRNA delivery. Wagner and co-workers showed that the incorporation of two or three cysteine cross-links into poly(amido amines) was not necessary for pDNA delivery, but was absolutely critical to achieve siRNA delivery[154,155]. It has also been shown that delivery via block[156] and core-shell[127] type architectures helps to increase electrostatic interactions by physically concentrating the cationic charges. Dimethylamine and piperazine groups, cross-linked into the cores of well-defined polymers with PEG shells, provided enhanced complexation and delivery as compared to more than 100 other amine-based cross-linkers screened in a 1,536-member combinatorial library[127]. Triblock copolymers of poly(LPEI-*b*-(propylene glycol)-*b*-LPEI) nicely elucidated the effect of polymer composition and architecture on delivery. Whereas $LPEI_{50}$-*b*-PPG_{36}-*b*-$LPEI_{50}$ showed poor efficacy, decreasing the LPEI block length and increasing the hydrophobic block to $LPEI_{14}$-*b*-PPG-*b*-$LPEI_{14}$ greatly improved delivery[157]. The use of endosome destabilizing agents is an additional way to enhance block copolymer-mediated delivery[158]. Overall, tertiary amines and alkyl chains have been identified as key functional groups for effective siRNA delivery across a variety of siRNA delivery materials including lipids and polymers.[93,126–130,159]

6.3 Biopolymers

Natural materials have also been used for siRNA delivery. Of note, Davis and co-workers demonstrated that cyclodextrin-based self-assembling polymeric nanoparticles can facilitate siRNA delivery, including in humans[160,161]. For instance, a cyclodextrin-containing polymer (CDP), a PEG stabilization agent, and human transferrin have been used in self-assembled nanoparticles where transferrin acts as a targeting ligand for transferrin receptors that are frequently overexpressed on cancer cells. The self-assembled four component formulation was delivered IV to patients with solid cancers in a Phase 1b clinical trial. Following siRRM2 nanoparticle treatment, mRNA levels (M2 subunit of ribonucleotide reductase (RRM2)) and the protein (RRM2) was reduced. Significant silencing has been measured in humans at doses of 18–30 mg m^{-2} and 2.5–5.0 mg/kg in mice[160,161]. Chitosan is another natural cationic polymer that has been used for siRNA delivery. It has been shown that higher MW chitosan provides better complexation and stability, whereas lower MW chitosan with specific degrees of deacetylation offer better intracellular release[162,163]. Huang and co-workers showed that a liposomeprotamine-hyaluronic acid (LPH) nanoparticle formulation could enable silencing of CD47 in tumor tissues after IV administration[164]. This siRNA-mediated silencing of CD47 inhibited the growth of tumors in multiple models, including melanoma and lung metastasis.

Proteins have also been utilized for siRNA delivery. Lieberman, Song, and co-corkers designed a protamine-HIV-1 envelope antibody fusion protein that could deliver siRNA selectively to cells expressing the HIV-1 envelope[165]. This targeting approach was subsequently applied $Her2^+$ breast cancer models using siRNAs complexed with a Her2-ScFv-protamine peptide fusion protein[166]. Additionally, Liu and coworkers demonstrated

that supercharged green fluorescent protein (GFP) with a net theoretical charge of +36 could form a siRNA-protein complex and enable delivery to a variety of cell lines[167]. Synthetic amino acid-based polymers are also attractive for delivery. A derivative of polyglutamic acid that incorporated a cell-penetrating helical structure was used for siRNA delivery and shown to cause pore formation in cell membranes, thereby enhancing delivery[168].

7. Outlook

In the past two decades, significant advances have been made in the development of siRNA therapeutics for treating diverse diseases. Chemists are capable of synthesizing siRNA with modified nucleotides to achieve high efficacy, high stability, and high specificity. However, in order to maximize the advantages of siRNA therapeutics in humans, there are still formidable challenges for the delivery systems. To address the issues associated with potency, selectivity, and safety, many strategies have been applied to develop new delivery materials.

Chemists often design delivery systems considering elements, bonds, and functional groups. The key chemical properties of successful carriers are largely consistent between lipid and polymer-based systems. These include hydrophobic modifications, tertiary amines, and the ability to interact with short siRNA strands via multiple types of bonding interactions. Currently, lipid- and lipidoid-based siRNA materials are highly effective delivery systems. The key components of lipids and lipidoids are cationic/ionizable groups, functional linkers, and lipid tails. Extensive SAR studies have provided design criteria, as described above. Moreover, formulation methods are also very important to the efficacy *in vivo*. Direct conjugation of small molecule ligands or polymers to siRNA offers the advantage of a being single component delivery system with defined composition. For example, GalNAc-siRNA conjugates not only provide an approach for ligand based cell internalization without the need of cationic materials, but also target hepatocytes specifically. In addition, synthetic and natural materials also offer ways to tune the degradation and responsiveness of the delivery system. For efficient siRNA delivery, cationic materials that include additional stabilizing interactions (e.g. hydrophobic modifications) and utilize architecture (e.g. well defined polymer morphology, cross-linking) are the most promising. Outside of the classical drug delivery systems, there are also new strategies to develop siRNA therapeutics. For instance, DNA origami, RNA nanoparticles, SNA were utilized to deliver siRNA to tumor cells and other forms of RNA can activate RNAi. Overall, the major challenges for siRNA therapeutics are to increase the efficacy, enhance cell-tissue specificity, and improve the safety.

Future efforts may include 1) delivery using new targeting ligands and chemical probes that specifically bind to surface markers on diseased cell populations, 2) increasing efficacy (particularly with regard to understanding how much siRNA enters the cytoplasm and how to better facilitate that process[169,170]), 3) widening of therapeutic window using materials with low toxicity, 4) design of materials with defined degradation products that can be metabolized (important when siRNA-based drugs will be repeatedly dosed), 5) simplification of the formulation procedure, and 6) delivery to organs other than the liver. Chemists have the ability to build materials on the molecular level and are well suited to

meet these challenges to make siRNA therapeutics successful in the clinic for a broad range of diseases. We hope that ONPATTRO is the first of many future RNAi therapeutics to effectively treat human disease.

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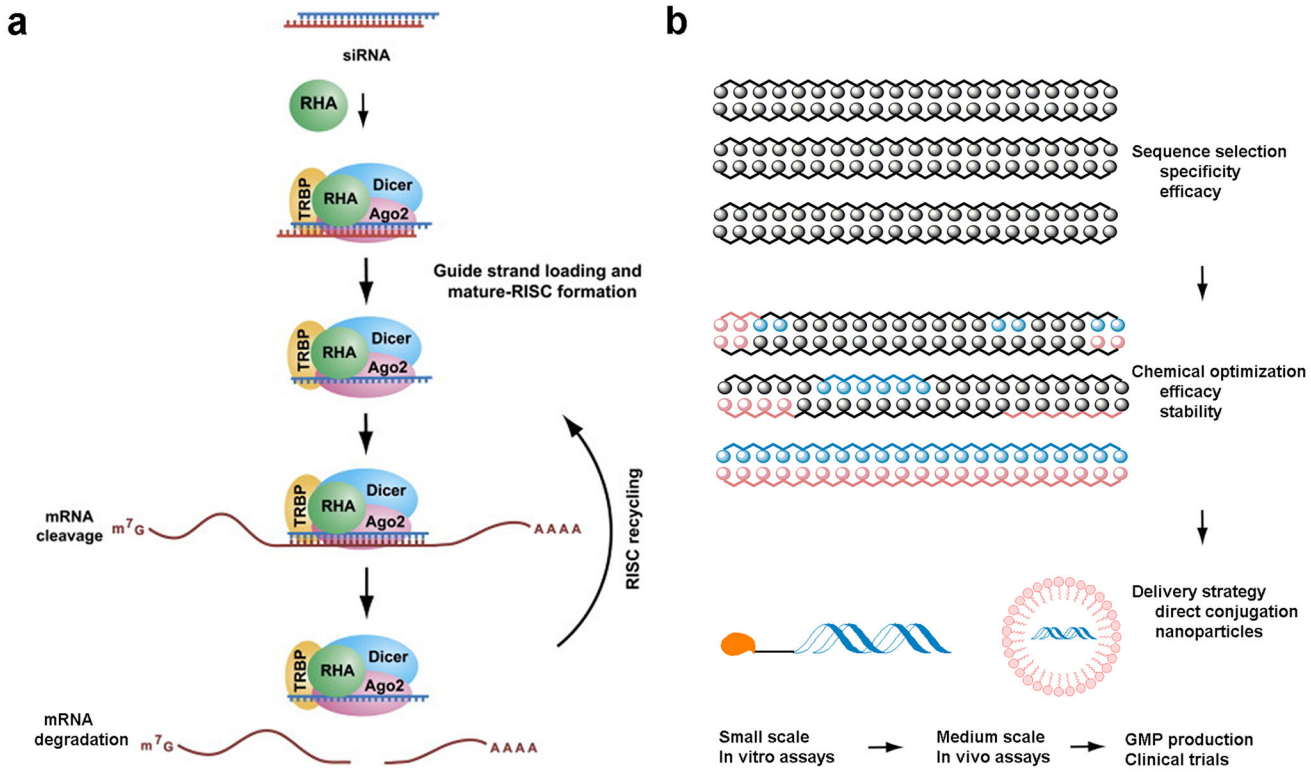


Fig. 1. Mechanism of RNA interference and strategies for siRNA synthesis and delivery.

a. An illustration of RNA interference process[3]. **b.** Strategies and procedures for siRNA development and delivery.

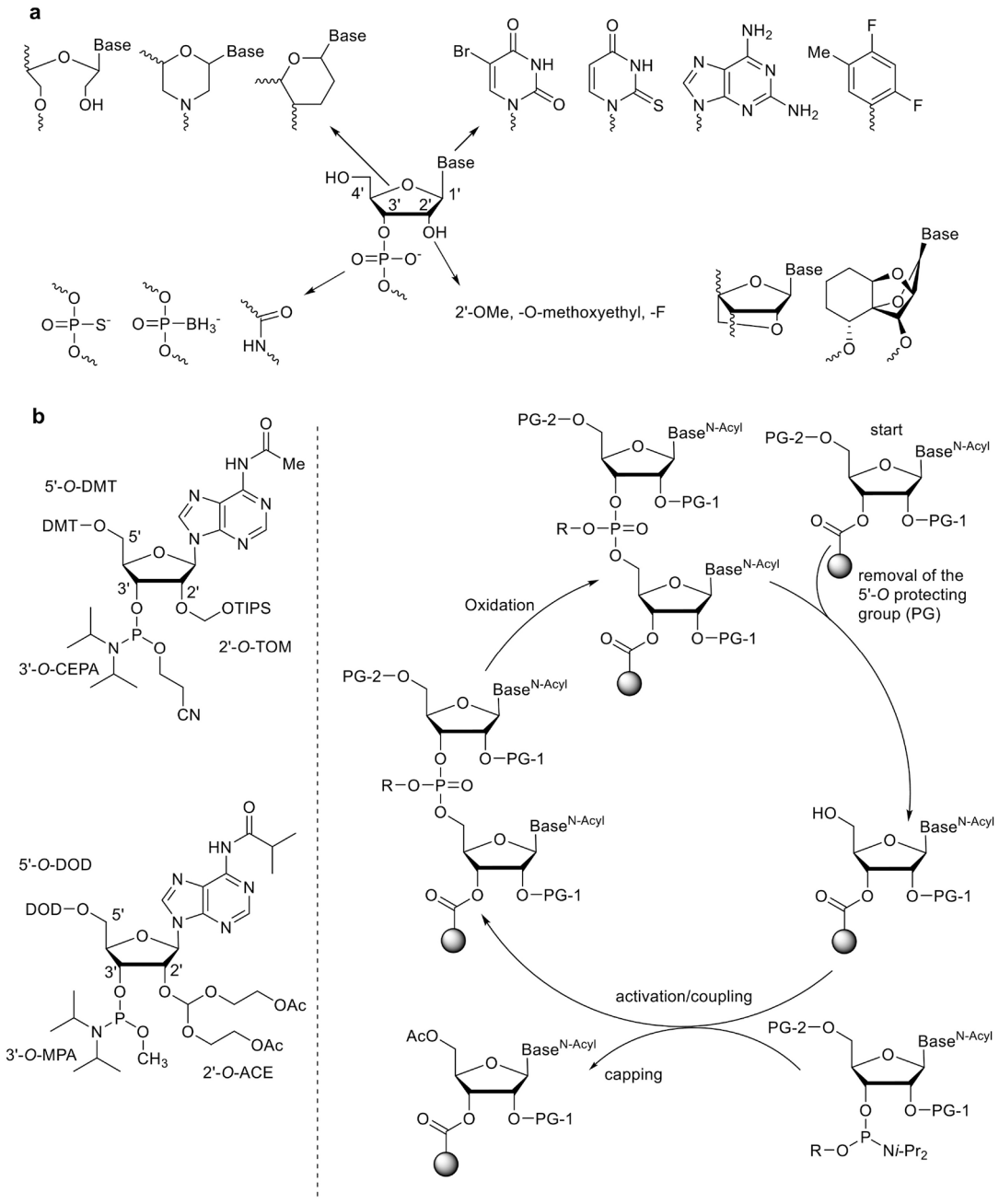
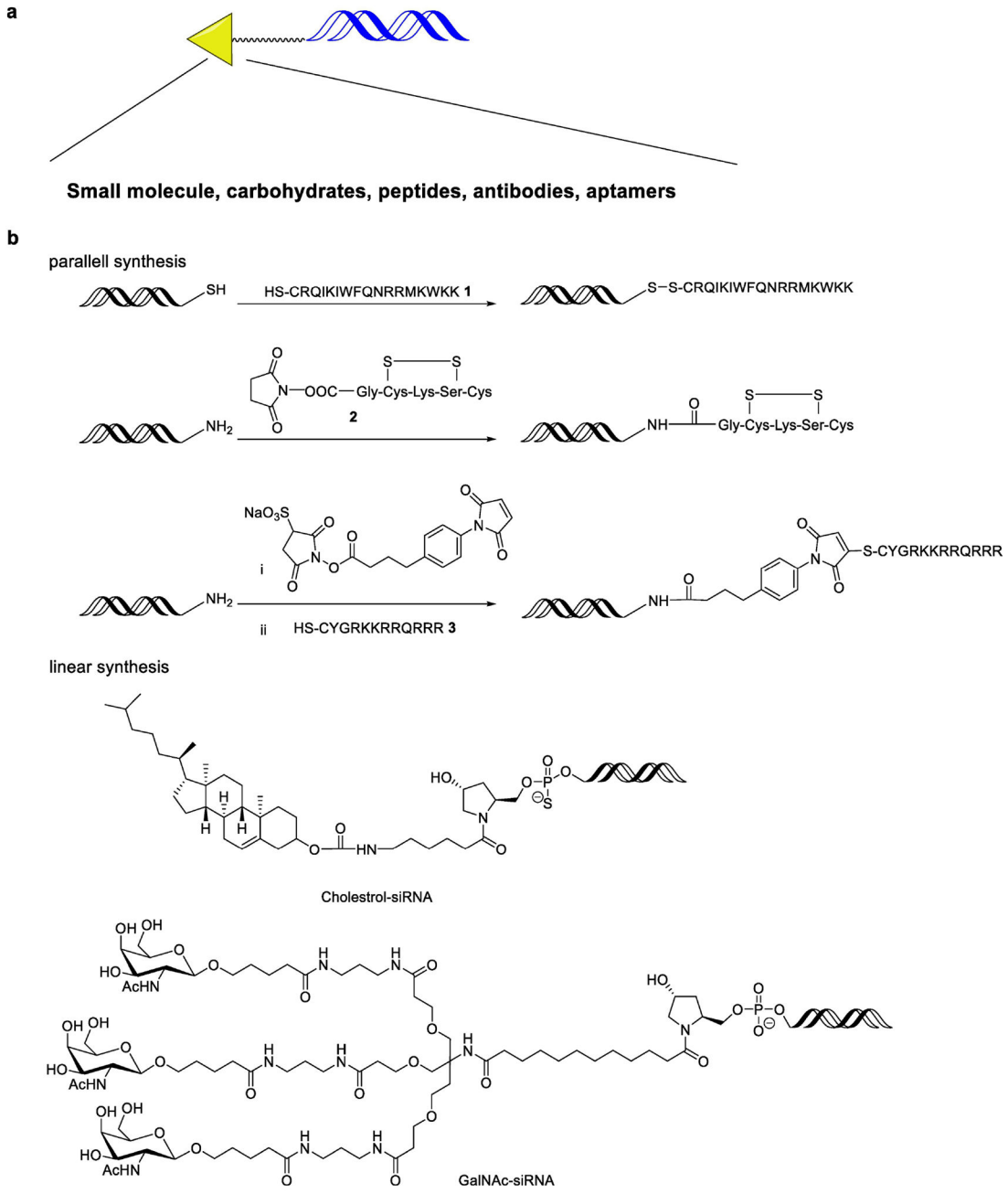


Fig. 2. siRNA synthesis.

a. Chemical modification of nucleic acids including the 2'-position, phosphate linkage, ribose, and nucleobase. **b.** Solid-phase synthesis of RNA strands using automated RNA synthesizer[49].



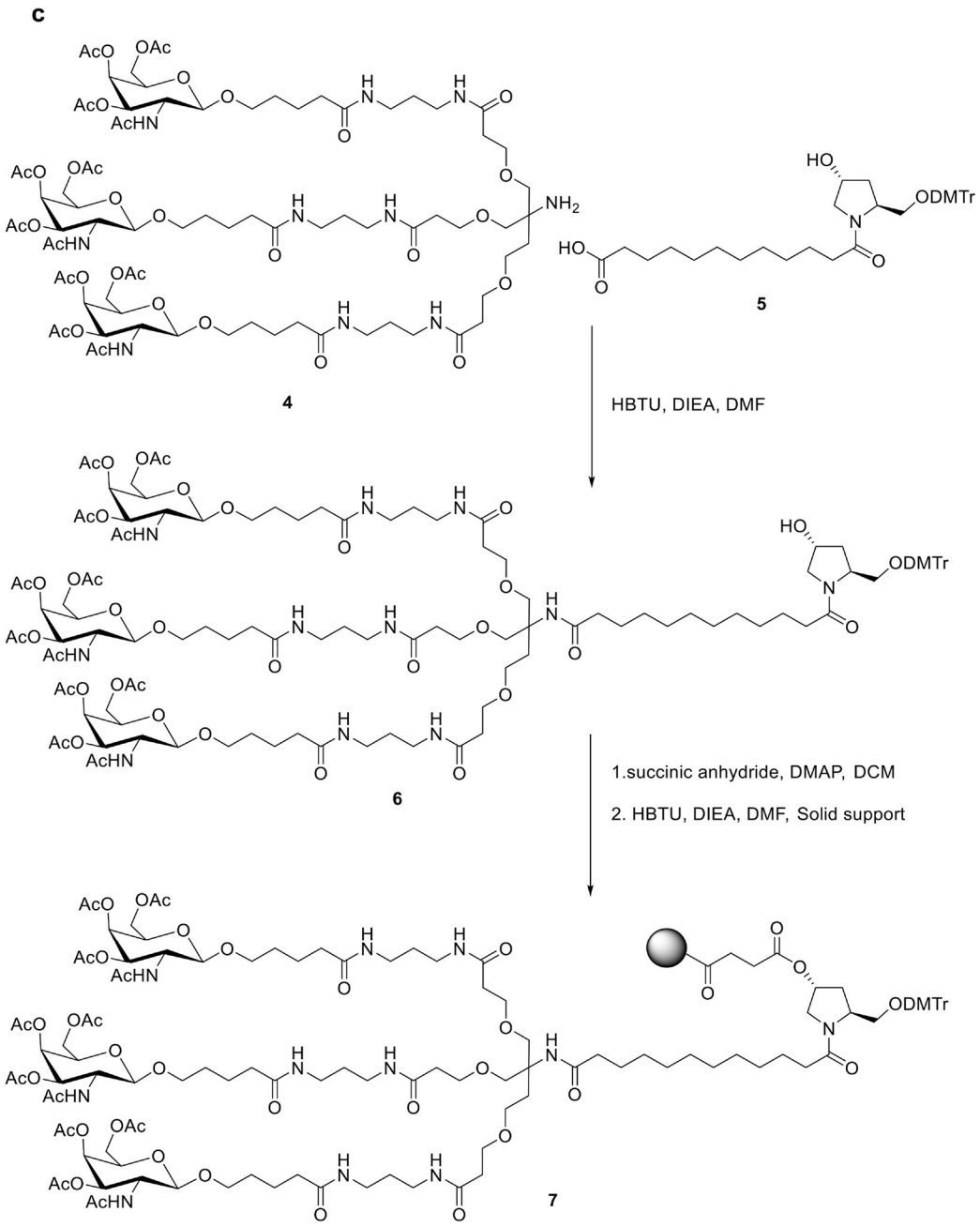


Fig. 3. Chemical strategies for synthesis of siRNA conjugates.

a. siRNA conjugates with ligands including small molecules, carbohydrates peptides, antibodies andaptamers. **b.** Parallel and linear synthesis siRNA-peptide and -cholesterol conjugates. **c.** An example of linear synthesis of GalNAc-siRNA conjugates.

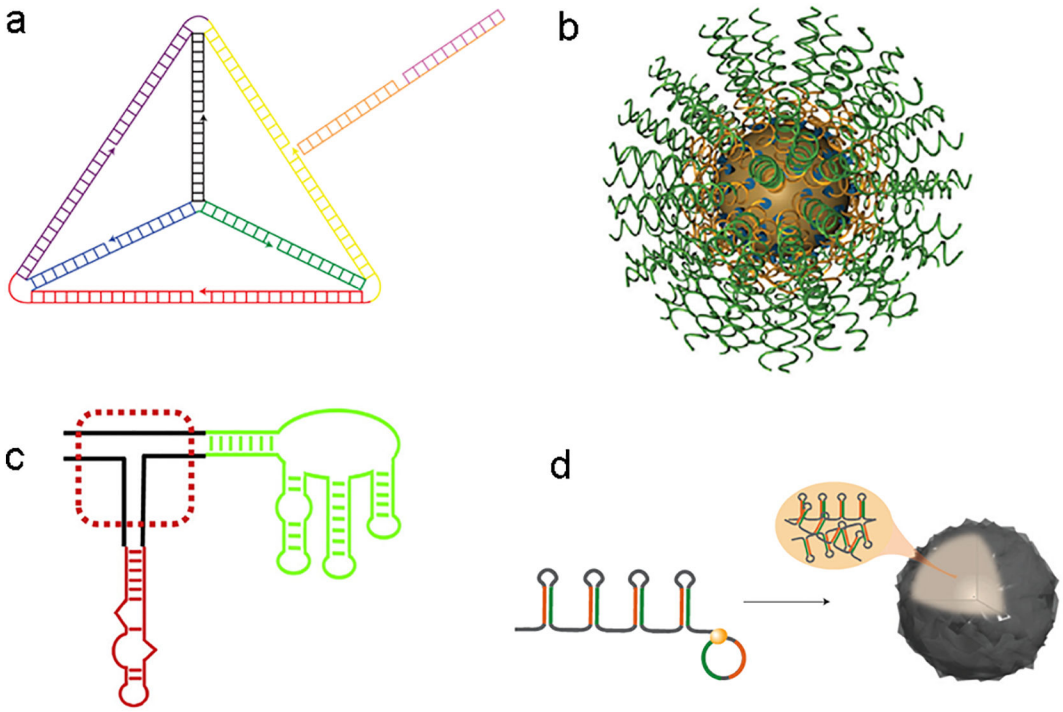


Fig. 4. Nucleotides derived nanoparticles.

a. Tetrahedron DNA origami. **b.** Spherical nucleic acid (SNA) conjugates with a gold core and siRNA shell. **c.** Three-way junction RNA nanoparticles. **d.** RNAi microsponges with multiple copies of shRNA.

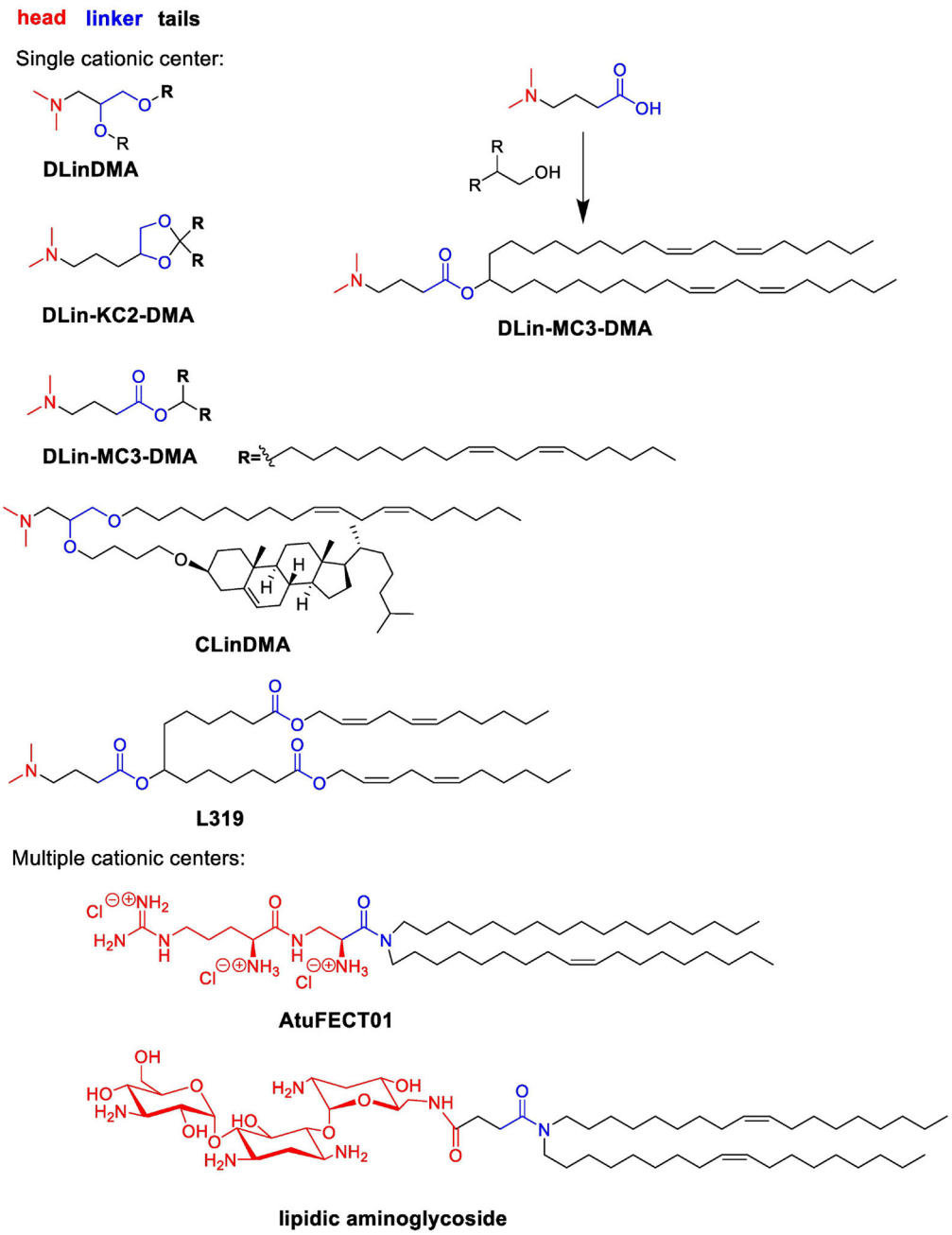


Fig. 5. Lipid analogs with cationic head groups, linker and hydrophobic tails and a representative synthetic route to DLin-MC3-DMA.

Cationic head groups can be single or multiple cationic centers. Linkers span from ester, amide to ketal. Hydrophobic tails can accommodate unsaturated bonds, cholesterol, and ester groups.

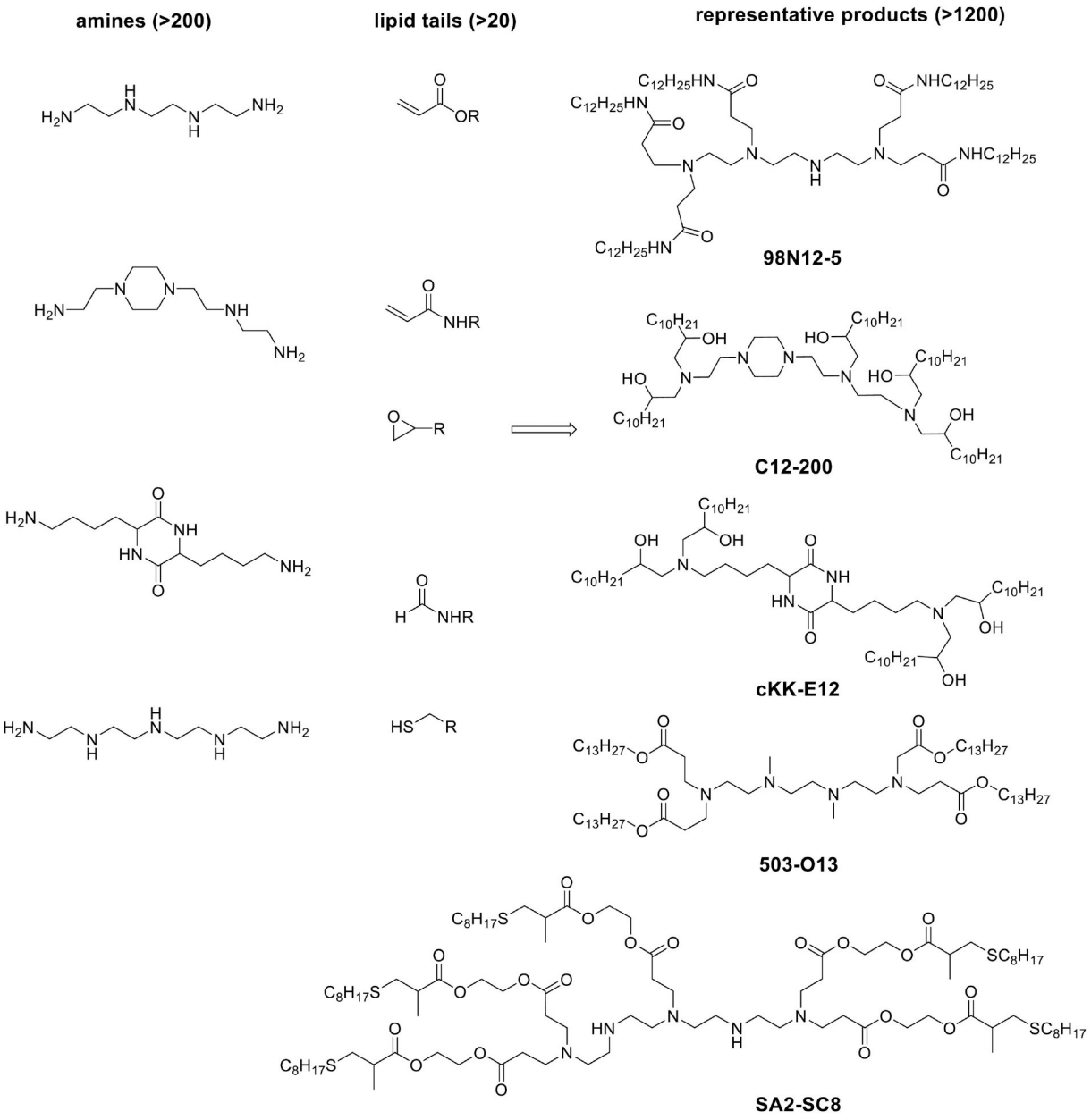


Fig. 6. Combinatorial and high throughput strategies of lipid-like materials.

Thousands of lipid-like compounds were synthesized through Michael addition reactions, epoxide ring-opening reactions, reductive amination reactions and thiol-ene reactions. Materials were screened with high throughput bioassays both *in vitro* and *in vivo*.

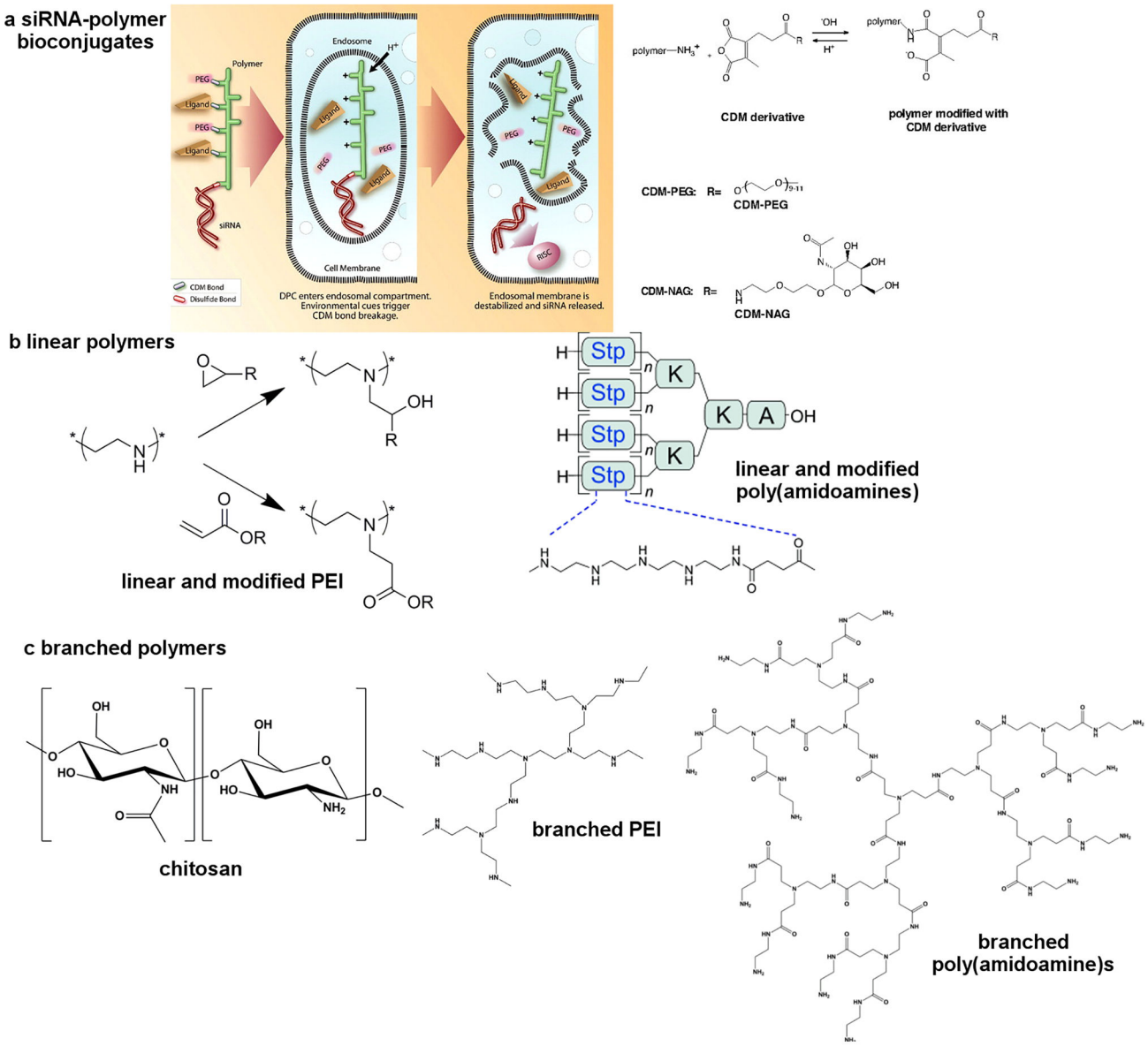


Figure 7.
Selected examples of conjugate, linear, and branched type architectures for siRNA delivery.

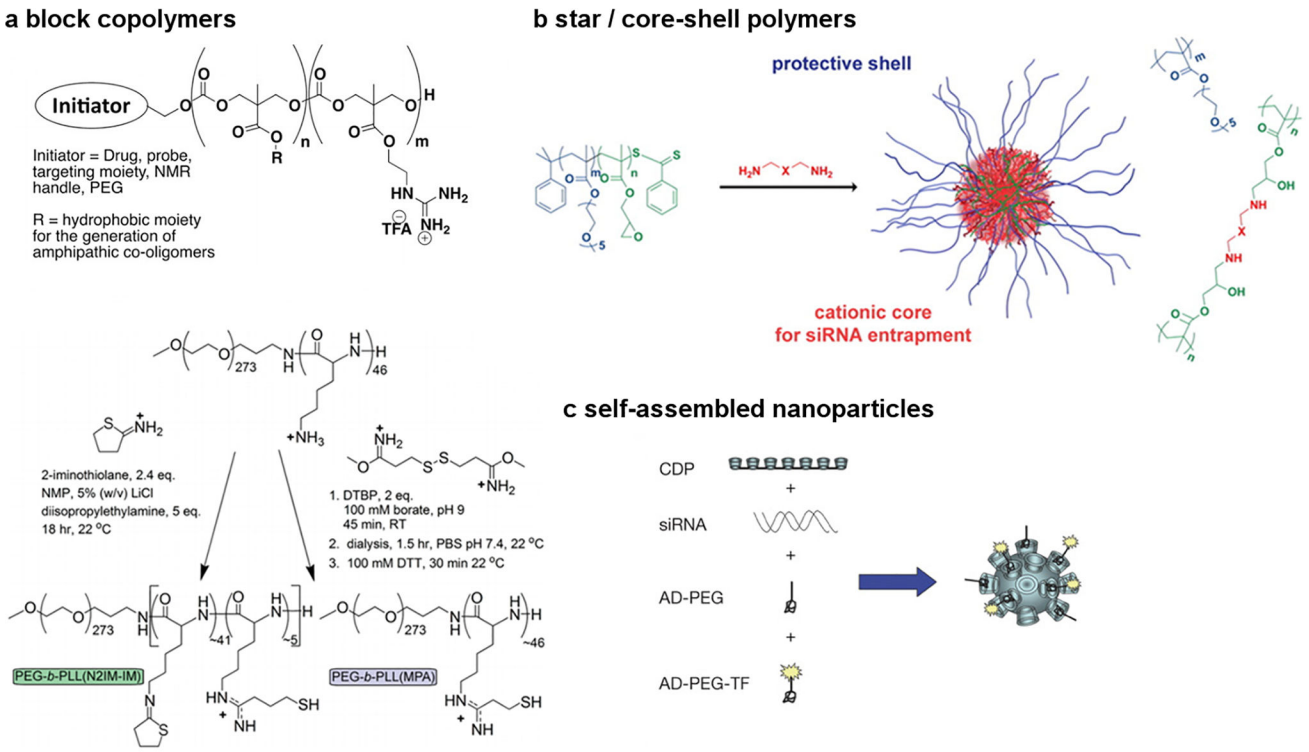


Figure 8.

Selected examples of block copolymer, star / core-shell, and self-assembled designs for siRNA delivery.