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Multimeric RNAs for efficient RNA-based therapeutics and vaccines

Dajeong Kim [1], Sangwoo Han [1], Yoonbin Ji, Sunghyun Moon, Hyangsu Nam, Jong Bum Lee [*]

Department of Chemical Engineering, University of Seoul, 163 Seoulsiripdaero, Dongdaemun-gu, Seoul, South Korea

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ABSTRACT

There has been a growing interest in RNA therapeutics globally, and much progress has been made in this area, which has been further accelerated by the clinical applications of RNA-based vaccines against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Following these successful clinical trials, various technologies have been developed to improve the efficacy of RNA-based drugs. Multimerization of RNA therapeutics is one of the most attractive approaches to ensure high stability, high efficacy, and prolonged action of RNA-based drugs. In this review, we offer an overview of the representative approaches for generating repetitive functional RNAs by chemical conjugation, structural self-assembly, enzymatic elongation, and self-amplification. The therapeutic and vaccine applications of engineered multimeric RNAs in various diseases have also been summarized. By outlining the current status of multimeric RNAs, the potential of multimeric RNA as a promising treatment strategy is highlighted.

1. Introduction

RNA is regarded as a powerful therapeutic agent with its myriad biological functions [1], which has contributed to significant advances in the treatment of various diseases such as cancers, viral infections, and inflammatory disorders in the last few years [2]. Various therapeutic RNAs such as small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotide (ASO), messenger RNA (mRNA), and CRISPR-Cas9 genome editing guide RNA (gRNA) have been used in gene therapy for several diseases [3,4]. While these RNA-based drugs have led to successful therapeutic outcomes, there is still room for improvement in terms of aspects such as blood circulation time, stability, and RNA loading capacity [5,6].

With recent advancements in RNA nanotechnology, clinical translation has been achieved leading to the successful Phase III trial and approval of RNA-based drugs by the US Food and Drug Administration (FDA) such as Patisiran (NCT01960348), Givosiran (NCT03338816), and Lumasiran (NCT03681184). However, only a few RNA therapeutics have been approved, and the delivery of RNA-based drugs remains a challenge due to the inherent instability and low encapsulation efficiency of a single RNA unit in various carriers [7–10]. Thus, a successful clinical translation of RNA molecules requires an efficient delivery platform to overcome various physiological barriers to reach the target cells [11]. With this aim, numerous strategies have been developed to improve the stability and the cargo-loading efficiency of RNA itself, including chemical modification, inorganic conjugation, and RNA multimerization [12–15]. In particular, multimeric RNA, which is a single structure composed of connecting repetitive units, provides superior advantages, as it enhances the stability of unmodified RNAs and the loading density of RNA drugs in drug carriers, resulting in improved efficacy with low cytotoxicity.

Herein, we have reviewed a dynamic range of strategies to prepare a variety of RNA structures by the periodical assembly of repetitive RNA sequences. Recent studies regarding multimeric RNAs for RNA delivery systems have been summarized, including chemically conjugated multimeric RNAs, RNA nanoarchitecture-based sequence-specific assembly, enzymatically amplified multimeric RNAs, and self-amplifying RNAs (saRNAs) generating multiple replicons (Fig. 1). The advantages, beneficial applications, and future perspectives are also discussed, highlighting the novel platform that can overcome the limitation of conventional RNA drugs. This review would shed light on the next-generation RNA drug platform for sustainable and effective RNA delivery in gene therapy, immunotherapy, and vaccine development.

2. Strategies for multimeric RNA preparation

A multimeric RNA molecule is a single, long, and repetitive version of a single unit of RNA (RNA monomer). It can be composed of up to

* Corresponding author.
E-mail address: jblee@uos.ac.kr (J.B. Lee).
[1] These authors contributed to the work equally.

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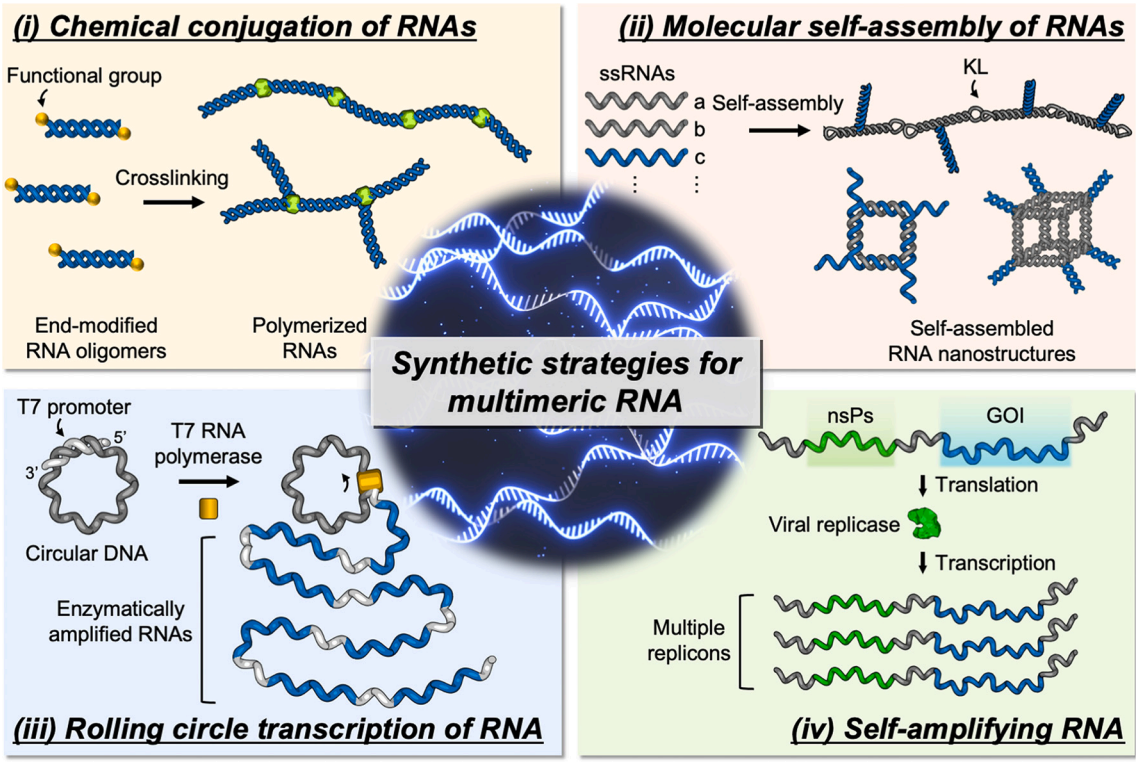

Fig. 1. Schematic describing synthetic strategies for multimeric RNA: (i) the crosslinking of end-modified RNA oligomers to generate multimeric RNA drugs, (ii) the molecular-interaction-mediated self-assembly of multiple RNAs into RNA nanostructures, (iii) the generation of repetitive RNA by RCT from the circular DNA scaffold, and (iv) saRNA including GOI for replicating numerous replicons.

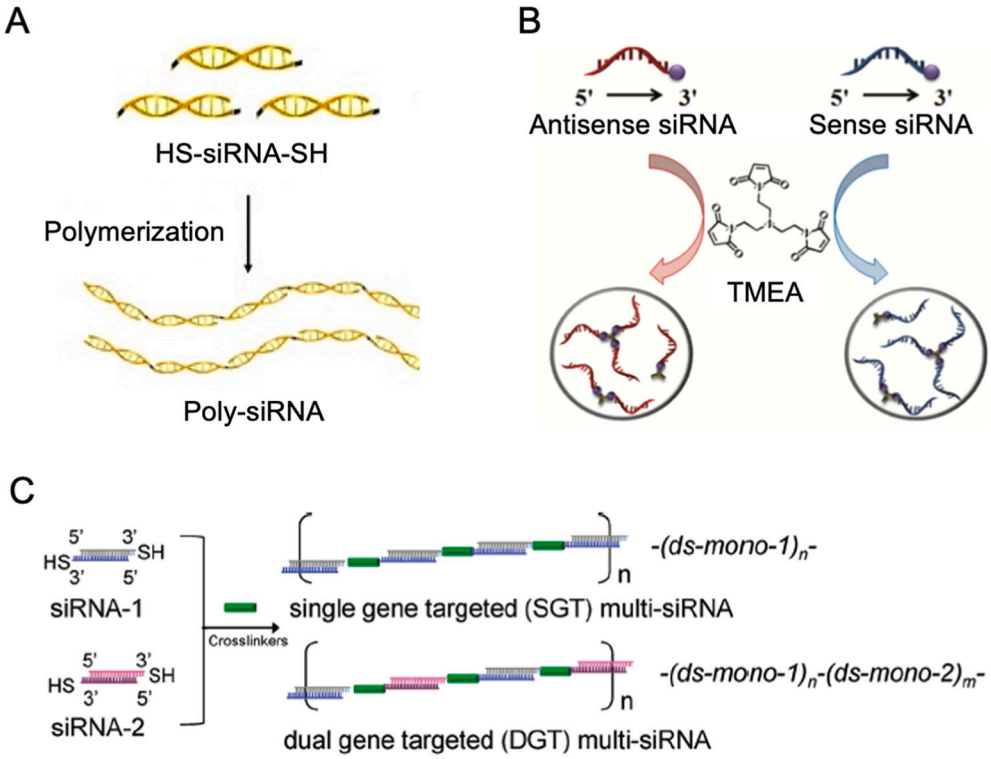

Fig. 2. Various strategies for multiple siRNAs delivery via chemical conjugation. (A) Synthesis of RNA concatemer with thiol group-modified RNA strands. (A) reprinted with permission from [21]. (B) Dendrimeric RNA structure with trigonal crosslinker. (B) reprinted with permission from [22]. (C) Dual gene-targeted siRNA delivery system with bidirectional crosslinker. (C) reprinted with permission from [23].

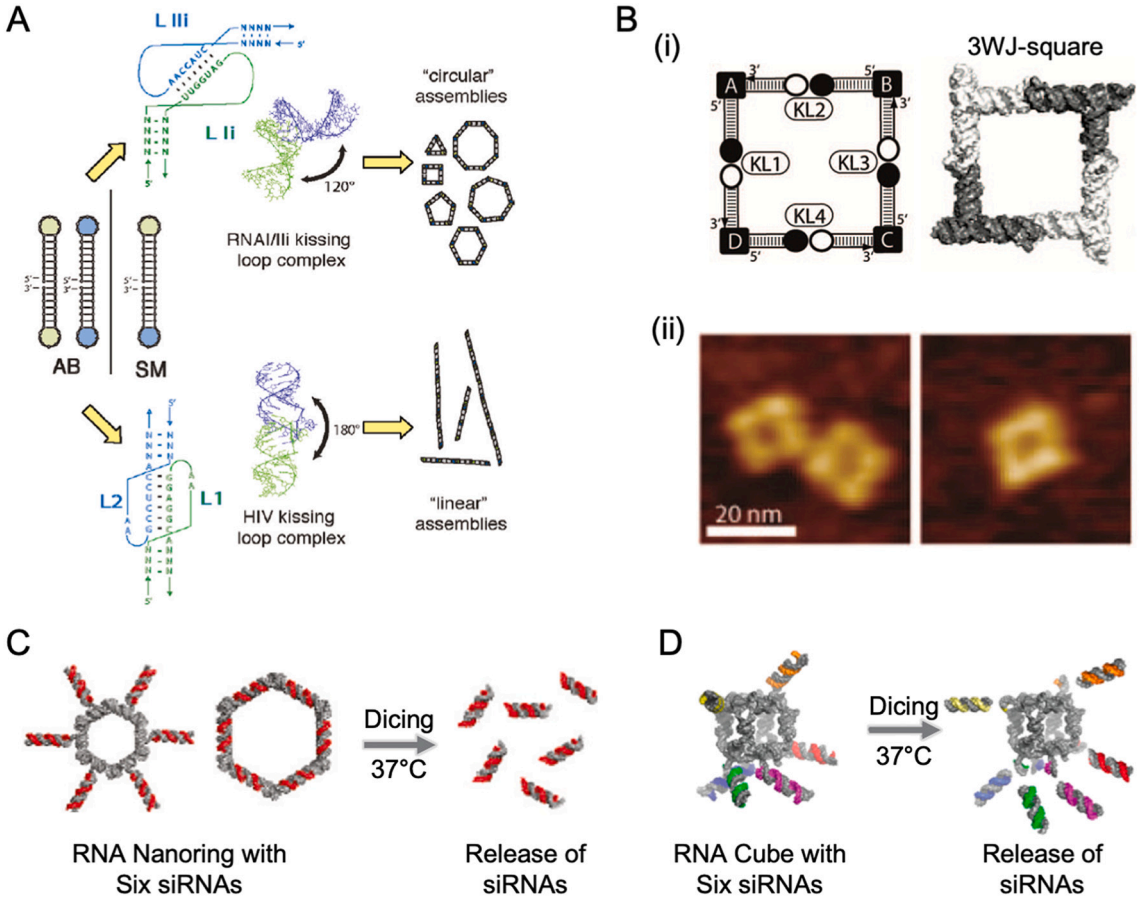

Fig. 3. Multiple functional RNAs delivery with programmable RNA nanoarchitectures. (A) Schematic of circular and linear RNA assemblies with various KL complex strategies. AB and SM indicate two different assembly systems. (A) reprinted with permission from [34]. (B) KL interaction-based RNA tectosquare assembly (i) and AFM images of RNA tectosquare (ii). (B) reprinted with permission from [28]. (C, D) RNA nanoring (C) and RNA nanocube (D) polygons for multiple siRNA release system. (C) reprinted with permission from [34]. (D) reprinted with permission from [29].

several thousand repeats of RNA monomers. These RNA concatemers have various unique properties that are beneficial for RNA delivery, such as extensively hindered 5′ and 3′ ends, highly condensed structure, and an ability to self-assemble. Four major RNA synthetic strategies have been outlined in this review (Fig. 1), including serial elongation via the conjugation of functional moieties on the ends of RNA, molecular interactions of RNA such as base paring or kissing loop (KL) interactions, and enzymatic transcription by RNA polymerase. saRNAs were also reviewed due to their ability to replicate the gene of interest (GOI) from a single saRNA unit in cells, resulting in the delivery of multiple RNAs. With an overview of the emergence and development of each technology, we introduce the application fields and pre-clinical studies where each method is advanced.

2.1. Chemical elongation method

The chemical conjugation strategy multimerizes RNA strands by crosslinking chemical groups (Fig. 1(i)). Single units of RNA oligomer with chemically modified 5′ or 3′ ends are connected sequentially to fabricate dimers, trimers, tetramers, and polymeric RNA structures by direct or crosslinker-mediated conjugation at once. The chemical crosslinkers endow multimeric RNAs with superior properties compared to those of monomeric RNAs, enabling the systemic release and the enhanced delivery of therapeutic RNAs [16]. For example, multimeric RNAs connected with disulfide bonds can be completely cleaved into oligomeric units by glutathione (GSH)-mediated reduction under conditions similar to the intracellular environment [16]. As such, functional chemicals that can utilize disulfide bonds have been widely used for enhanced drug delivery [17–20].

Chemical conjugation-based strategies have also been developed for stable and efficient siRNA delivery. For instance, repetitive siRNA was fabricated by disulfide bonds using the thiol group-modified 5′ ends of the sense and antisense strands (Fig. 2A) [21]. The resulting siRNA concatemer mixture included dimeric, trimeric, and polymeric conjugates. Polymeric siRNA (poly-siRNA), of over 300 bp long, was dominant in the total composition, in which the disulfide bond underwent rapid cleavage to generate monomeric or dimeric siRNA via treatment with 1,4-dithiothreitol (DTT). The multimeric structure was maintained for 2 days in 50% serum condition, significantly longer than monomeric siRNA [18]. The trigonal crosslinker tri-[2-maleimidoethyl]-amine (TMEA) was also used to fabricate multiple siRNA-embedded dendrimer structures to improve the structural stability (Fig. 2B) [22]. Also, homobifunctional crosslinker, dithio-bis-maleimidoethane (DTME), enabled the concatemerization of multiple siRNAs for the synthesis of single and dual gene-targeted siRNA delivery (Fig. 2C) [23]. These studies indicate that chemically crosslinked multimeric RNA platforms could lead to advances in RNA drug delivery, providing significant enhancement of stability and efficient therapeutic RNA release profiles.

2.2. RNA nanoarchitecture-based assembly

Multimeric RNA can also be generated with the assembled RNA nanostructures by the programmable base-pairing of RNA (Fig. 1(ii)). The multiple copies of functional RNA are embedded in supramolecular RNA structures as drug-loading scaffolds [24]. Furthermore, the polymerization of double crossover (DX) RNA tiles also can provide larger

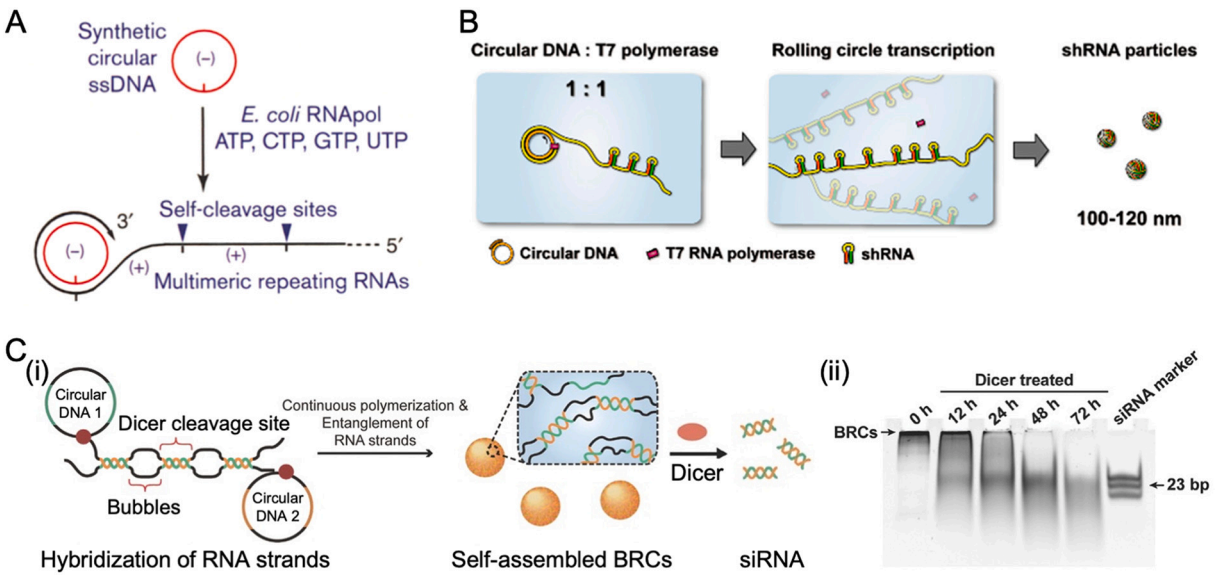

Fig. 4. Enzymatic fabrication of repetitive RNA by RCT. (A) Transcription of multimeric ssRNA via enzymatic elongation from synthetic circular DNA scaffold. (A) reprinted with permission from [37]. (B) RCT-mediated production of multiple copies of short hairpin RNA (shRNA) with both sense and antisense of siRNA, and their self-assembly into nano-sized particles. (B) adapted with permission from [44]. (C) Enzymatic elongation from two complementary circularized DNA scaffolds where RNA transcripts are hybridized as bubbled siRNA structures (i). Dicer-responsive siRNA generation from multimeric siRNA-bearing bubbled RNA-based cargo (BRC) nanoparticle. Controlled release of siRNAs from BRCs via Dicer treatment (ii). (C) adapted with permission from [49].

multimeric RNA structures, enhancing the loading efficiency of therapeutic RNAs [25]. With the notable advances in RNA nanotechnology, delivery vehicles of multimeric RNAs have been developed based on precise controllability and the diversity of RNA assemblies.

Various RNA nanostructures, ranging from linearly polymerized RNAs with sticky ends to multiple RNA-dangled polygons/polyhedrons with KL interaction, which is an RNA intermolecular interaction formed by two RNA hairpin loops, have been introduced (Fig. 3A) [26,27]. For instance, Jaeger's group developed the KL interaction-based RNA tectosquare (Fig. 3B) [28]. KL-based synthetic strategies have been applied to link separate RNA units to fabricate a variety of RNA nanostructures that embed multiple functional RNAs in the corners or branched sites of

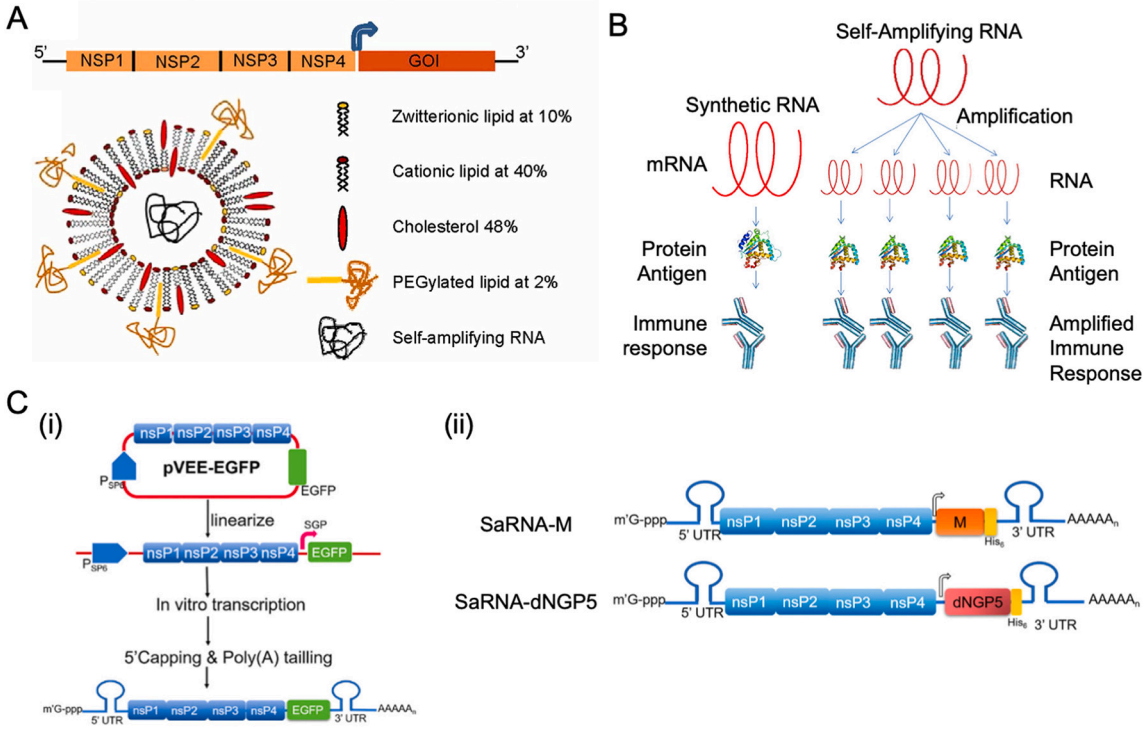

Fig. 5. Constructing saRNA for delivering multiple repeats of RNA-based therapeutic or vaccine. (A) Alphavirus-derived saRNA with GOI encapsulated in zwitterionic lipid nanoparticle. (A) adapted with permission from [54]. (B) Comparison of protein expression and immune response evoked by conventional RNA or saRNA-based vaccine platform. (B) adapted with permission from [55]. (C) Synthesis of Venezuelan equine encephalitis (VEE)-based saRNA for enhanced green fluorescence protein (EGFP) (i) and Porcine reproductive and respiratory syndrome virus (PRRSV) antigen M or dNGP5 (ii). (C) reprinted with permission from [58]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

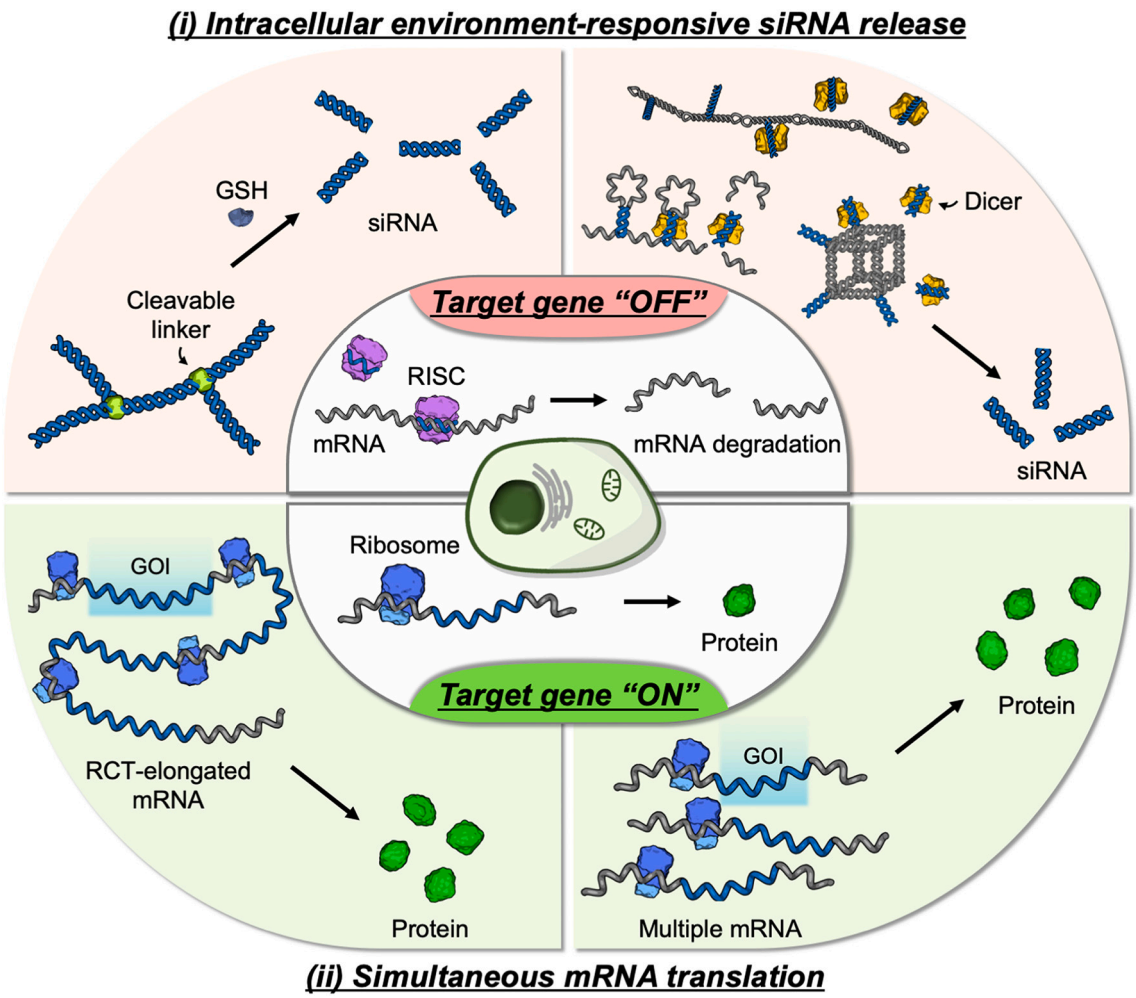

Fig. 6. Working mechanism of multimeric RNAs. (i) Intracellular environment-responsive siRNA release from multimeric RNAs and complexation with RISC for gene knockdown. GSH-responsive siRNA release can be achieved with disulfide bond cleavage on chemically elongated RNA (left). Dicer cleavage-induced siRNA release system from various multimeric RNA structures (right). (ii) Simultaneous mRNA translation from multimeric mRNA for enhanced target gene expression. Proteins can be generated on RCT-based linearly repeated mRNA (left) and multiple mRNAs which are produced from saRNA (right).

each structure. These cargoes-conjugated RNA nanostructures have successfully resisted enzymatic degradation, leading to increased cellular transfection efficiency [24,29]. KL interaction has recently enabled the construction of homooligomeric RNA nanostructures [30]. Numerous nanoassembly designs, including tectosquare [31], RNA fiber structures [32], and highly complex polygons and lattices, have also been developed by adjusting the number of helices (Fig. 3C and D) [29,30,33,34]. By increasing the scale and complexity of nanostructure-based assemblies, multiple biological functions can be endowed upon multimeric RNA-based nanostructures [35].

2.3. Rolling circle transcription (RCT)

Rolling circle transcription (RCT) is a great tool to produce repetitive functional RNAs through an enzymatic elongation of monomer units under isothermal conditions (Fig. 1(iii)) [36–39]. For the enzymatic multimerization, circularized DNA scaffolds are first prepared which encode single or several types of tailor-made RNA-based drugs and the T7 promoter region [40,41]. During the RCT process, T7 RNA polymerase repetitively produces RNA strands with hundreds of amplified functional RNA copies from an endless template DNA (Fig. 4A) [36,39]. Under these conditions, the T7 RNA polymerase, which is the most widely used polymerase due to its excellent strand displacement ability, showed exceptionally high productivity of RNA strands having repeated sequences [42]. The transcription efficiency in the RCT process is affected by the length of the template DNAs and their secondary structures [42].

Through the rational design of circular DNA templates, diverse functional RNAs such as siRNA [9,43–45], miRNA [46], ribozymes [47], and mRNA [48] can be massively produced in the multimerized form via the one-pot isothermal reaction. Lee et al. developed a variety of RCT-based functional RNA structures, ranging from nanoscale to macroscale, with repeated hairpin structures or bubbled structures (Fig. 4B and C) [7,9,49]. In particular, an enzymatically elongated multimeric siRNA exhibited Dicer-responsive controlled release of siRNA for 72 h (Fig. 4C) [49]. In addition to multimerization, automated self-assembly of RNA products from RCT could further enhance their degradation resistance and delivery efficacy, resulting in efficient gene regulation [49,50]. Continuous advances in the fabrication of multimeric RNAs by RCT have shown significant implications for their clinical utilization in biomedical fields [50].

2.4. Constructing the saRNA

A single saRNA encoding target RNA sequences has been applied to deliver multiple tandem copies of RNA to the cell (Fig. 1(iv)). As the structure of saRNA generally originates from RNA viruses such as alphaviruses, which can amplify their own RNAs, they contain both the

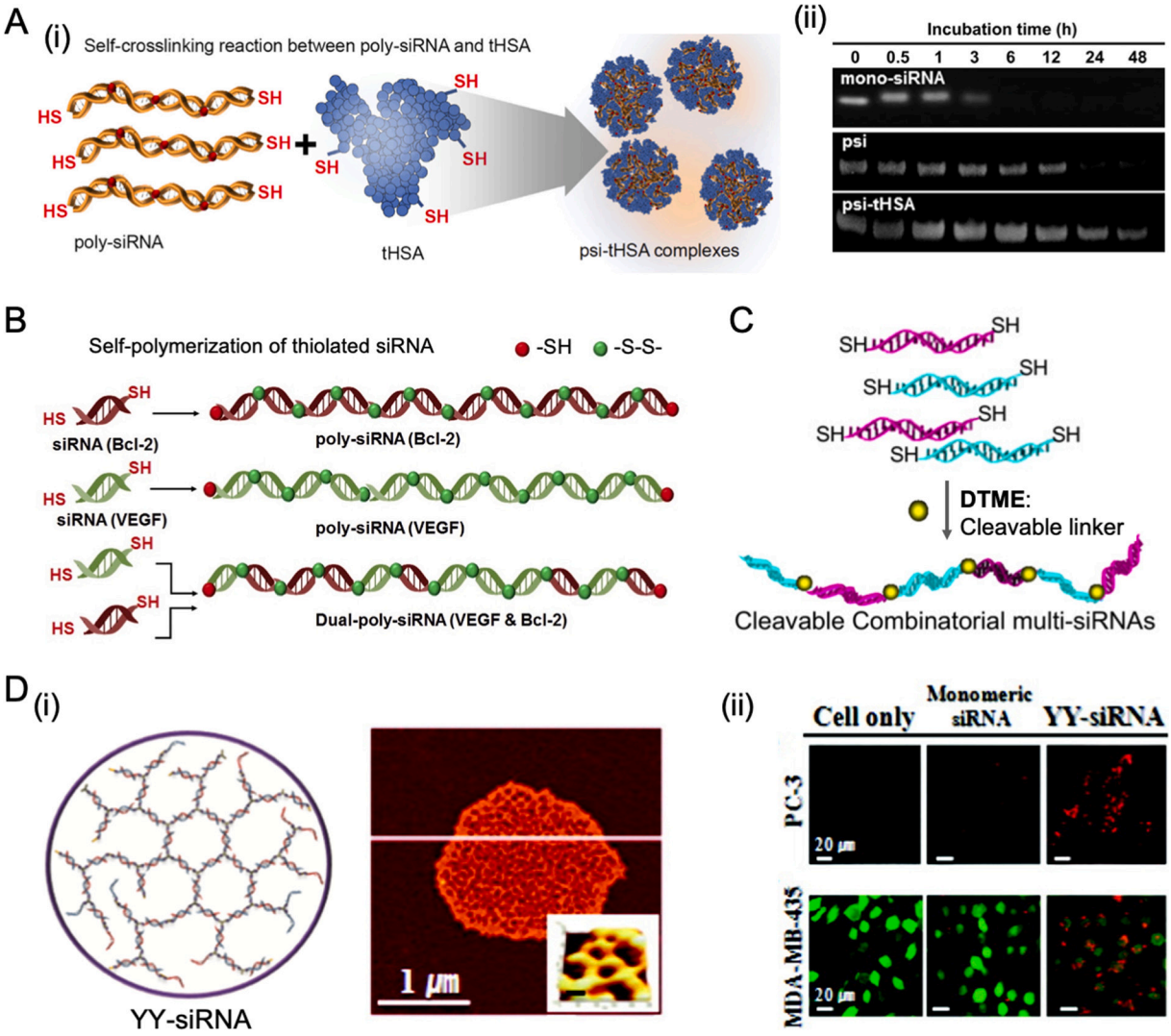

Fig. 7. Chemically conjugated multimeric RNAs for gene regulation. (A) Crosslinking of thiol-modified siRNAs and tHSA fabricating polymeric siRNA-tHSA nanocomplexes (psi-tHSA) (i), the enhanced stability of psi-tHSA (ii). (A) reprinted with permission from [63]. (B) Generation of dual-poly-siRNA by disulfide bonds. (B) reprinted from with permission from [66]. (C) DTME-mediated multimerization to generate Multi-siRNA. (C) reprinted with permission from [18]. (D) The branched polymerization of siRNAs by trigonal TMEA crosslinker to generate multimeric YY-siRNA, the assembled microhydrogel structure of YY-siRNA (i), and the efficient cellular uptake of YY-siRNA (ii). (D) reprinted with permission from [22].

GOI region and viral replicase [51]. With cytosolic internalization, the initially translated viral replicase further accelerates the generation of RNA replicons to maintain the long-term expression of amplicons.

As the saRNA was first used as a vaccination agent, very small amounts (<1 ng) of infectious but attenuated flavivirus RNA were used as a self-amplifying vaccine to substitute live vaccines by deleting 470 nt in the noncoding region from the whole genome [52]. These infectious saRNAs have since been developed into non-infectious saRNA platforms with improved safety which contain viral replicase-encoding non-structural protein 1–4 (nsP1–4), and GOI region under the subgenomic promoter (Fig. 5A) [53,54]. The saRNA also exhibits a higher target antigen expression efficiency and durability than that of same amount of non-replicating mRNA platforms due to its high amplification (Fig. 5B) [55]. This non-viable saRNA platform can elicit protective immunity against influenza that is comparable with that of mRNA at a 64-fold higher content [55]. In other words, utilizing saRNA allows the drug dosage to be reduced remarkably, further guaranteeing its safety. Since the GOI region can be manipulated to encode a variety of functional genes such as therapeutics and vaccines, this rapidly scalable saRNA-based strategy provides a novel template to generate multiple RNA strands from single RNA in host cells without any gene-integrative products (Fig. 5C) [56–58].

2.5. *Working mechanism of multimeric RNAs*

The multimeric RNAs often underwent the release process for the desired function [16,29,49]. By the release of therapeutic RNA under various cellular environments, it can achieve the controlled and prolonged therapeutic effects of multiple RNAs. In particular, chemically conjugated multimeric siRNAs are rationally cleaved in the reductive intracellular environment such as the presence of GSH [16,17]. The Dicer, intracellular endoribonuclease, is also responsible for the release and function of siRNA drugs from the chemically multimerized RNA [18]. Then, the monomerized siRNAs can be associated with the RNA-induced silencing complex (RISC) for mRNA degradation similar to the traditional siRNA delivery approach (Fig. 6(i)) [59]. In the case of branched RNA nanoarchitectures, the precursor siRNAs are often embedded in the extended arms of the nanoarchitectures (Fig. 6(i)) [29,34]. These strategies could rationally design the release mechanism of siRNA by Dicer. The RCT-based multimerized siRNAs can be cleaved

by Dicer as well [9]. Since the human Dicer is known that it preferentially processes the dsRNA from the termini [60], the elongated RNA transcripts bearing dsRNA regions were gradually degraded, allowing the sustained release of therapeutic RNAs (Fig. 6(i)) [9,49]. Therefore, the gene-silencing strategy by multimeric RNA potentially allows long-term downregulation of the target protein for efficient therapeutics. In contrast, the release process for the desired function was not necessary for the multimeric mRNAs such as RCT-based mRNA or saRNA (Fig. 6 (ii)) [48,56]. In addition, the multimerized mRNA showed significantly enhanced stability with the repetitive ribosome binding sites (RBS) and protein-encoding region, potentially allowing efficient and long-term upregulation of target proteins without additional modification [48]. Since the traditional mRNA-vaccinations have been normally required the additional modifications to enhance the stability and translation [61], multimeric mRNA could provide new opportunity for efficient vaccination.

3. Applications of multimeric RNAs as therapeutics and vaccines

3.1. Chemically conjugated multimeric RNA for stimuli-responsive siRNA delivery

Multimerization via chemical elongation of RNA drugs can significantly increase the delivery efficiency and therapeutic efficacy due to the enhanced stability, loading efficiency, and feasibility of stimuli-triggered release. For example, disulfide bond, one of the widely used chemical bonds, is used to synthesize from dimeric to poly-siRNAs of over 300 bp via the oxidation-mediated direct conjugation of dithiol-modified siRNAs at the 5′ or 3′ ends of the sense and antisense strands [21,62]. Due to the highly concentrated RNAs, the charge density of poly-siRNA was 12 times that of monomeric siRNA after complexation with polyethyleneimine (PEI), resulting in its enhanced silencing efficiency and nuclease resistance [21].

During the thiol group-mediated conjugation process, thiolated human serum albumin (tHSA), a natural albumin-based carrier with high biocompatibility, is easily involved in the multimerization of siRNA (Fig. 7A) [63]. The stable complexation of tHSA to nanosized structures further enhances the stability of polymerized siRNAs in serum conditions (Fig. 7A) and their tumor-targeting ability, resulting in significant inhibition of tumor growth [63]. Similarly, the successful crosslinking of biocompatible thiolated gelatin with polymerized siRNAs and their self-assembly into nanoparticles also increases tumor accumulation via the enhanced permeation and retention (EPR) effect [64]. When used against Notch1, which is related to the progression of rheumatoid arthritis, polymerized siRNA was successfully self-assembled with thiolated glycol chitosan (tGC) via electrostatic interactions and disulfide bonds, resulting in its high accumulation at arthritic sites [65]. The successful reduction of inflammatory reactions and damage of joint tissues via specific knockdown of Notch1 has demonstrated the potential of chemically elongated and complexed siRNAs for the treatment of intractable diseases [65].

In addition to homogeneous siRNA, heterogeneous siRNA polymers targeting vascular endothelial growth factor (VEGF) and B-cell lymphoma 2 (Bcl-2) can also be achieved via copolymerization resulting in dual-gene-targeted siRNA polymers (dual-poly-siRNAs) for synergistic anticancer therapy via angiogenesis inhibition and apoptosis simultaneously (Fig. 7B) [66]. By incorporating tumor-homing tGC into the polymerization, a single nanoparticle containing dual-poly-siRNA can be co-delivered to cancer cells, evoking synchronized VEGF and Bcl-2 silencing.

Additional moieties can also be adopted for crosslinker-mediated multimerization of siRNAs to improve the gene silencing effect [16]. In particular, each sense and antisense strand modified with a thiol group at the 3′ end can be dimerized with a cleavable homobifunctional crosslinker dithio-bis-maleimidoethane (DTME), which has a disulfide bond and can be serially hybridized to fabricate multimeric siRNAs (multi-siRNAs) [16]. Dimeric siRNAs are initially synthesized in a switched sequence direction (5'-RNA-3′-crosslinker-3'-RNA-5′) to arrange the sequence in a certain direction with complementarity with opposite strands [16,21]. Due to the linearly multimerized structure, the degradation resistance of the multi-siRNA complexed with PEI would be 2-fold increased than that of naked siRNA. The cleavable multi-siRNA guides the gene silencing with high target specificity due to the more precise cleavage of the intended single siRNA compared to that of the non-cleavable form, which might randomly generate non-target-specific siRNAs [16]. Similarly, the short dimeric form can also lead to significantly enhanced gene silencing of green fluorescent protein (GFP) expression (~66%) compared to that of the monomeric siRNA (~14%) [17].

Chemical molecule-mediated RNA multimerization can be easily engineered to include siRNAs with combinatorial targets or external moieties such as a targeting aptamer for efficient cancer therapy [18,19]. A dual-gene-targeted multimeric siRNA (DGT multi-siRNA) against anti-apoptotic gene expressions, survivin and Bcl-2, was produced via heterogeneous multimerization of siRNA strands that were modified at both the ends and crosslinked by DTME (Fig. 7C) [18]. Since heterogeneous DGT multi-siRNA could be complexed with PEI with high mechanical stability, DGT multi-siRNA evoked downregulation of genes and sequential cell apoptosis more efficiently compared to single siRNA multimers or a physical mixture of each multimer [18]. A multivalent siRNA structure was also developed by crosslinking single-stranded antisense strands followed by the serial hybridization of the DNA aptamer-tethering sense strand, resulting in an increased siRNA delivery in cancer cells [19].

Chemical crosslinking endows RNA multimers with structural diversity by incorporating branched RNAs. Using the trigonal crosslinker TMEA, Y-shaped trimeric RNA can be fabricated through the spontaneous crosslinking of thiol group-modified RNA strands [22]. The trimeric RNA with sense sequence of siRNA can be further subjected to hybridization with complementary antisense RNA-based trimer to generate Y-shaped siRNA structures (YY-siRNA) (Fig. 7D) [22]. Moreover, multi-arm junctions can extend the nanosized multimers to construct dense and compact microhydrogels, allowing the improved cellular delivery and gene silencing effect to be achieved to a greater extent compared to that of linearly elongated siRNA (Fig. 7D). In short, rationally designed chemical multimerization of siRNA could grant stable RNA delivery platforms, which improve the gene silencing efficiency.

3.2. RNA nanoarchitecture-based multimeric RNA for multifunctional RNA delivery

A variety of modular design strategies, such as simple interaction between complementary sticky ends, double crossover assemblies, and KL interaction, have been developed to construct self-assembled multimeric RNAs. For instance, the hybridization was utilized for the construction of a simple multimeric siRNA-based architecture [26]. Supramolecular siRNA nanostructures were also developed via linear concatemerization through the serial hybridization of the sticky ends of poly A and poly T overhangs at the monomer siRNA units. The concatemer formed by the longer $(dA)_n/(dT)_n$ bridge exhibited the greater delivery potency with efficient siRNA protection from nucleases, offering an excellent gene silencing efficiency [26].

With the double crossover method, more complex and well-defined nanostructures that enable to deliver multiple therapeutic RNAs have been extensively investigated. For instance, a de novo RNA tile monomer embedding siRNA, characterized by two helices held together by two DXs, was assembled into a periodic lattice structure via programmed sticky end interactions [25]. As a multimeric RNA delivery scaffold, the self-assembly of RNA lattices offered several benefits, including easy incorporation of therapeutic RNAs in the lattices, improving the stability of the RNA molecules, precise stoichiometric

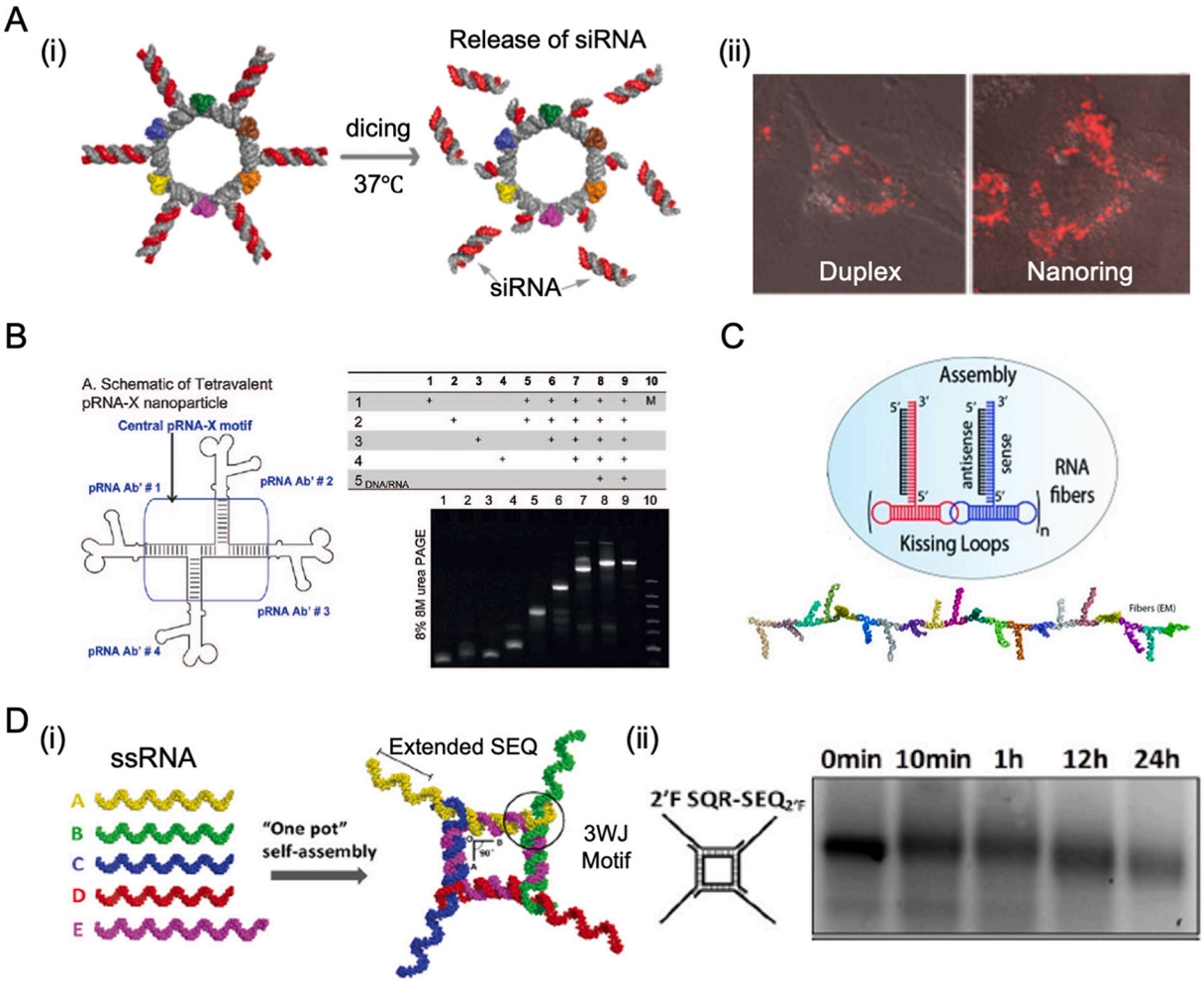

Fig. 8. Various RNA nanoarchitecture-based multimeric RNA delivery and their therapeutic effects. (A) RNA nanoring assembly through the KL interaction with functionalized multiple siRNAs (i), improved cellular uptake efficiency of functionalized-RNA nanoring compared to that of monomeric siRNA (ii). (A) reprinted with permission from [27]. (B) The design and fabrication of tetravalent pRNA-based X-motif for multimeric siRNA delivery via the stepwise assembly. (B) adapted with permission from [67]. (C) The assembly of RNA fiber functionalized with Dicer-cleavable siRNAs. (C) adapted with permission from [32]. (D) Assembly of immunogenic 2′F-modified RNA square (2′F RNA-SQR) (i), 10% serum stability of 2′F RNA-SQR until 1 day (ii). (D) adapted with permission from [76].

control of therapeutic RNAs, and multifunctionality with functional RNAs (siRNA, miRNA, antisense RNA, or mRNA). The resulting RNA tiles and lattices exhibited comparable uptake efficiency and target gene regulation with enhanced serum stability [25].

With KL interaction, Shapiro's group reported various RNA nanostructures such as RNA nanorings, nanocubes, and tetrahedra via the interaction between RNA tertiary motifs [24,27]. These RNA nanostructures have shown potential as delivery vehicles for multimeric siRNAs with multiple functional moieties (Fig. 8A) [24,27]. The double-stranded RNA (dsRNA)-packaged nanoring exhibited superior uptake efficiency due to its compact and stable structure (Fig. 8A). Furthermore, the knockdown efficiency of RNA nanoring was comparable to that of free siRNA molecules with six times lower than free siRNA doses [27]. In another study, tetravalent siRNA nanoparticles were assembled using phi29 DNA packaging RNA (pRNA)-based X-motif as the core component (Fig. 8B) [67]. The tetravalent siRNA nanostructure exhibited greater gene silencing effects than the single siRNA-conjugated X-motifs, implying the superiority of the multimeric formulation in RNA delivery. In addition, a tertiary-branched motif-based multimeric RNA nanostructure was studied as a potential siRNA delivery carrier. Guo's group reported controllable self-assembled RNA dendrimers for multimeric, functional RNA delivery based on the three-way junction motif (3WJ) in the pRNA motif as a core building block [68]. The RNA dendrimer exhibited an enhanced delivery into the cancer cells through a folate ligand conjugation, implying a great therapeutic potential of the nanostructures.

In addition, the KL-mediated polymerization of siRNA units has enabled the synthesis of multimeric siRNA nanofibers, which serve as a scaffold for siRNA coordination [32]. This scaffold provides branched sites for the loading of multiple siRNAs, allowing the simultaneous delivery of multimeric siRNAs. Every siRNA-dangled, dumbbell-shaped RNA unit was connected through KL interaction, achieving a brush-like structure (Fig. 8C) [32]. This RNA nanofiber was efficiently delivered into the human breast cancer and melanoma cells with a significant target gene knockout efficiency without any cellular damage.

In the meantime, immunogenic RNAs such as viral single-stranded RNA (ssRNA) or dsRNA that can be recognized by cell surface (TLR3) [69], endosomal (TLR3/7/8) [70–72], and cytosolic (RIG-1, MDA5) receptors [73–75], have also been reported, demonstrating that they can activate host immune responses. There have been ongoing efforts to develop immunomodulatory nanostructures with multiple RNAs for cancer immunotherapy or as vaccine adjuvants by leveraging the immunogenic properties of RNAs. Guo et al. designed RNA polygon structures with the extension of immunogenic RNA at the vertices (Fig. 8D) [76]. Triangle, square, pentagon, and tetrahedron structures were assembled using 2′F-modified RNA, and their shape-specific

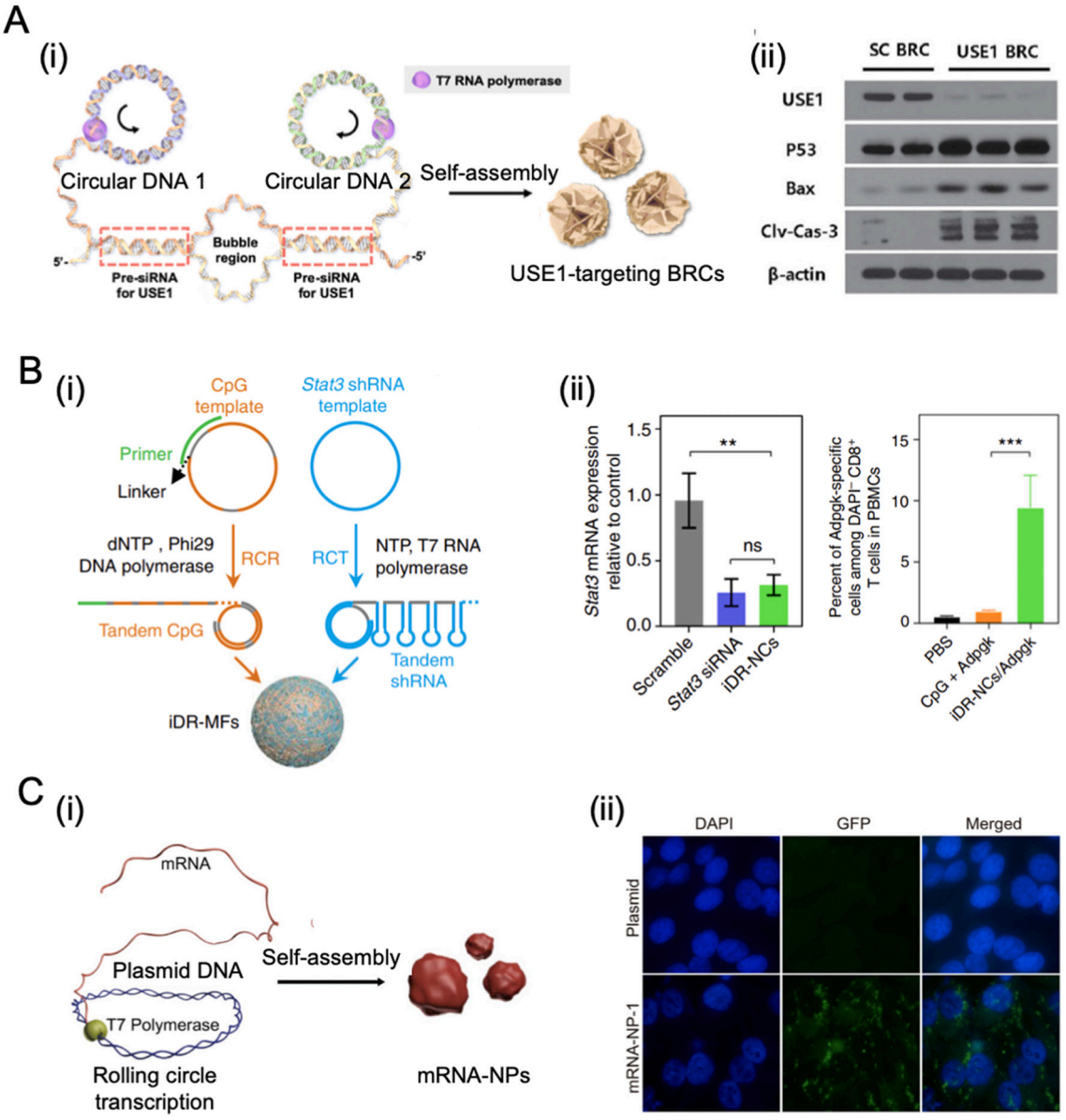

Fig. 9. Various therapeutic applications of RCT-based multimeric RNAs. (A) cRCT process for producing multimeric siRNA targeting USE1 and self-assembly of BRCs (i), and BRC-mediated USE1 gene silencing effect (ii). (A) reprinted with permission from [50]. (B) Synthesis of multi-functional repetitive RNA-DNA hybrid-based drug through dual enzymatic polymerization (i), and STAT3 gene knockdown efficacy and iDR-NC-induced increase of neoantigen-specific $CD8^+$ T cells (ii). (B) adapted with permission from [86]. (C) RCT-based multimeric mRNA synthesis from pDNA template (i) and target protein expression after multimeric mRNA nanoparticle treatment (ii). (C) adapted with permission from [48].

immunogenicity was evaluated. The 2′F RNA polygon structure showed high stability in serum conditions (Fig. 8D), allowing an improved cellular uptake efficiency to be achieved compared to that of ssRNA. When the immunogenic RNAs were attached to the RNA polygon, macrophages showed enhanced cytokine secretion, whereas the RNA polygon structure itself did not show any immunogenicity. The immunogenicity also increased from a two-dimensional (2D) triangular structure to a three-dimensional (3D) tetrahedron with immunogenic RNAs. Various artificial RNA stoichiometries have been applied to construct numerous multimeric RNA delivery systems for the improvement of therapeutic efficiency. Research on the RNA structural designs for multiple RNA delivery suggests that precisely assembled RNA scaffolds are highly suitable as efficient platforms for various therapeutic purposes such as RNA interference (RNAi) and immunotherapy.

3.3. Enzymatically elongated multimeric RNA for long-term RNA delivery and its potential for vaccination

As one of the enzymatic elongation approaches, the RCT technique has drawn attention to producing therapeutic RNAs as it enables the scalable synthesis of RNA with tandem repeats from a single circular DNA [9]. The most well-known application would be the RCT-mediated production of multimeric short hairpin RNA (shRNA) utilized for improved RNAi therapy [9]. During the RCT process, repetitive shRNA strands were simultaneously self-assembled into densely packed sheet structures and then gradually developed into a highly porous sponge-like superstructure called an RNAi microsponge [9]. A large amount of shRNA could be embedded in a single spherical particle, providing significantly improved cargo protection compared to that of naked RNA. Via the sustained generation of siRNA by Dicer-induced digestion, the repetitive RNAi microsponge successfully reduced the firefly luciferase

expression. As the RCT process can easily be controlled by adjusting the reaction components, multimeric RNA structures with tailor-made diameters can be synthesized [44,77,78].

In addition to ssRNAs, dsRNAs can also be multimerized by the complementary RCT (cRCT) method, which utilizes two complementary circular DNAs for the fabrication of multimeric siRNAs [79,80]. Two kinds of complementary RNAs were multimerized from each circular DNA to form the consecutive library siRNA which was cleavable at every dsRNA site by Dicer, showing successful down-regulation of target proteins [80]. To improve the siRNA generation efficiency from the repetitive RNAs, partially complementary RNAs including ssRNA spacers were introduced between siRNAs, enabling a precise recognition and specific cleavage to be achieved by Dicer [49]. This bubbled RNA-based cargo (BRC) design would rapidly generate siRNAs with a better gene silencing effect compared to that of commercially available siRNAs [49]. Further pre-clinical studies have been conducted to investigate the therapeutic potential of BRC as a gene therapy platform to treat lung cancer (Fig. 9A) [50]. When using the BRC bearing siRNA against UBA6-specific E2 conjugating enzyme 1 (USE1) protein (USE1 BRC) which is overexpressed in lung cancer patients, the target gene was successfully downregulated, resulting in the suppression of tumor growth [50]. In addition, further conjugation with microbubbles increased the delivery efficiency of the multimeric siRNA drug to the tumor site via focused ultrasound as well as therapeutic efficacy [81]. These studies have shown the significance of RCT-based multimeric RNAs as a potential platform for clinical applications.

The scalable productivity of the RCT technique enables the fabrication of multimeric RNA-based 3D biomaterials comprising large amounts of functional RNAs. In 2014, a macroscopic RNA membrane structure fabricated by cRCT with subsequent evaporation-induced self-assembly (EISA) process was first reported [82]. As the RNA membrane was made up of numerous dsRNAs, it could be used as a platform for loading large amounts of multimeric siRNAs [82,83]. With further ultrasonication, the siRNA-containing RNA membrane could be easily torn into a number of nanosheets made up of repetitive siRNAs. The separated siRNA nanosheets showed efficient target gene knockdown [83]. In addition, the rigidity and free-standing property of the RNA membrane could allow the facile coating of large amounts of multimeric RNA on microneedles, enabling the transdermal delivery of multimeric RNA drugs for potential vaccination applications [84].

The multimerization of RNA also allowed to develop synergistic RNA therapeutics with repeatedly functionalized molecules on multimeric RNAs [85]. To achieve the synergistic therapeutics, dual-enzymatic polymerization was introduced to fabricate multimeric RNA-DNA hybrid materials in combination with repetitive target-specific DNA or immunostimulatory DNA moieties with multimeric RNAs [86,87]. The intertwining DNA-RNA nanocapsules (iDR-NCs) bearing multimeric STAT3 shRNA and CpG DNA were developed via the simultaneous polymerization of DNA and RNA by phi29 DNA polymerase and T7 RNA polymerase, respectively (Fig. 9B) [86]. The multimeric CpG DNA and STAT3 shRNA, which were complementary to one another, synergistically activated antigen-presenting cells and generated 8-fold neoantigen-specific peripheral $CD8^+$ T cells than did monomeric CpG DNA (Fig. 9B). In another study, an RNA nanovector consisting of polymerized siRNA with aptamer DNA showed great potential as a carrier-free therapeutic for target gene knockdown [87]. Furthermore, the dual-enzyme polymerization method was utilized to synthesize the multimeric RNA-DNA hybrid hydrogel, embedding numerous siRNA-aptamer complexes into its own system. This tumor-targeted gene silencing composite demonstrated efficient tumor-targeting ability and protein downregulation effects [88].

In addition, synthetic monomeric DNA strands have also been employed to embed additional functions [14]. Two types of complementary DNA strands, namely the folate-conjugated DNA and cholesteryl DNA, were hybridized to repetitive siRNAs to introduce targeting ability and amphiphilicity for self-assembly, respectively [14]. The combinatory siRNA structure could be highly accumulated in tumor sites for efficient downregulation of the target gene [14]. Other approaches have also been identified to impart extra functionality, such as the complexation of multimeric RNAs with polymeric carriers. Repetitive siRNA complexed with tGC exhibited higher tumor cell uptake and in vitro gene silencing efficacy without considerable cytotoxicity [89,90]. In 2017, a polymeric single-stranded guide RNA (sgRNA) was first reported that *co*-polymerized with siRNA by RCT [91]. During the RCT and subsequent self-assembly, additional Cas9 proteins concurrently bind to the multimerized sgRNA, leading to the generation of Cas9/sgRNA/siRNA-complexed nanoparticles (poly-RNP) [91]. The poly-RNP bearing chimeric sgRNA and siRNA targeting GFP synergistically evoked the permanent knockout of the target gene with only a single dose transfection [91].

Meanwhile, the potential of elongated RNAs via RCT has also been investigated for the delivery of miRNAs and immunostimulatory RNAs. For instance, a tumor suppressor miRNA-34a was multimerized into miRNA nanoparticles via the RCT of rationally designed scaffold DNA for regulating the expression of the silent information regulator 1 (SIRT1) [46]. These miRNA-34a nanoparticles showed 80% anti-proliferation effect when delivered to human prostate cancer cells [46].

The delivery of mRNAs for target gene expression has been challenging due to the relatively long RNAs and their innate instability. RCT method could also multimerize long RNA strands as well as short RNAs depending on the template DNA. In 2015, a multimeric mRNA was developed using plasmid DNA (pDNA)-based RCT to achieve efficient gene expression (Fig. 9C) [48]. While the naked mRNA fragments rapidly degraded within 5 min in 2% serum condition, the multimeric mRNA maintained its structure even after 1 h of exposure to 10% serum conditions, showing the significantly enhanced stability of multimeric mRNAs [48]. The simultaneous self-assembly into mRNA nanoparticles (mRNA-NPs) during the RCT induced a 6-fold increase in protein expression compared with that of pDNA (Fig. 9C) [48]. In addition, the multimerized mRNA-NPs have potential to allow efficient and long-term upregulation of target proteins without additional modification. For the traditional mRNA-vaccinations, additional modification and storage at cold chain are major issues. Therefore, highly stable multimeric mRNA could provide a new opportunity for efficient vaccination by addressing the aforementioned issues. In another study, an anti-tumoral apoptin-encoding repetitive mRNA was developed via RCT exhibited a synergistic gene-chemotherapy effect by the co-delivery of intercalated doxorubicin to 4T1 breast cancer cells [92].

The immunogenicity of repetitive RNAs comprising ssRNAs or dsRNAs has also been reported [79]. While ssRNA-based multimeric structures showed negligible immunostimulation, dsRNAs often exhibited an immunomodulatory effect with respect to the extent of base pairing, suggesting the controllability of multimeric RNA for purpose-oriented utilization [79]. In summary, engineering and pre-clinical studies have emphasized the great potential of RCT-derived multimeric RNA platforms to improve the cargo loading efficiency, structural stability, and therapeutic efficacy of RNA-based drugs which have been the main obstacles for clinical application of RNA.

3.4. saRNA for enhanced therapeutic and vaccination effects with lower dose

Since saRNAs were derived from alphavirus templates and can elevate the levels of cytosolic RNA replicons and proteins, many researchers have attempted to exploit saRNA platforms in the field of vaccine development against viruses and cancer [93–96]. In the early stages of its development, saRNA was utilized for prophylaxis against Sindbis virus and influenza A as naked RNA through intramuscular injection [97,98]. Besides the virus vaccines, Ying et al. reported saRNA-based therapeutic cancer vaccine by RNA replicase encoding the model antigen, β-galactosidase (β-gal) sequence [53]. Unprecedented to traditional DNA or RNA vaccines, a single intramuscular injection of 0.1

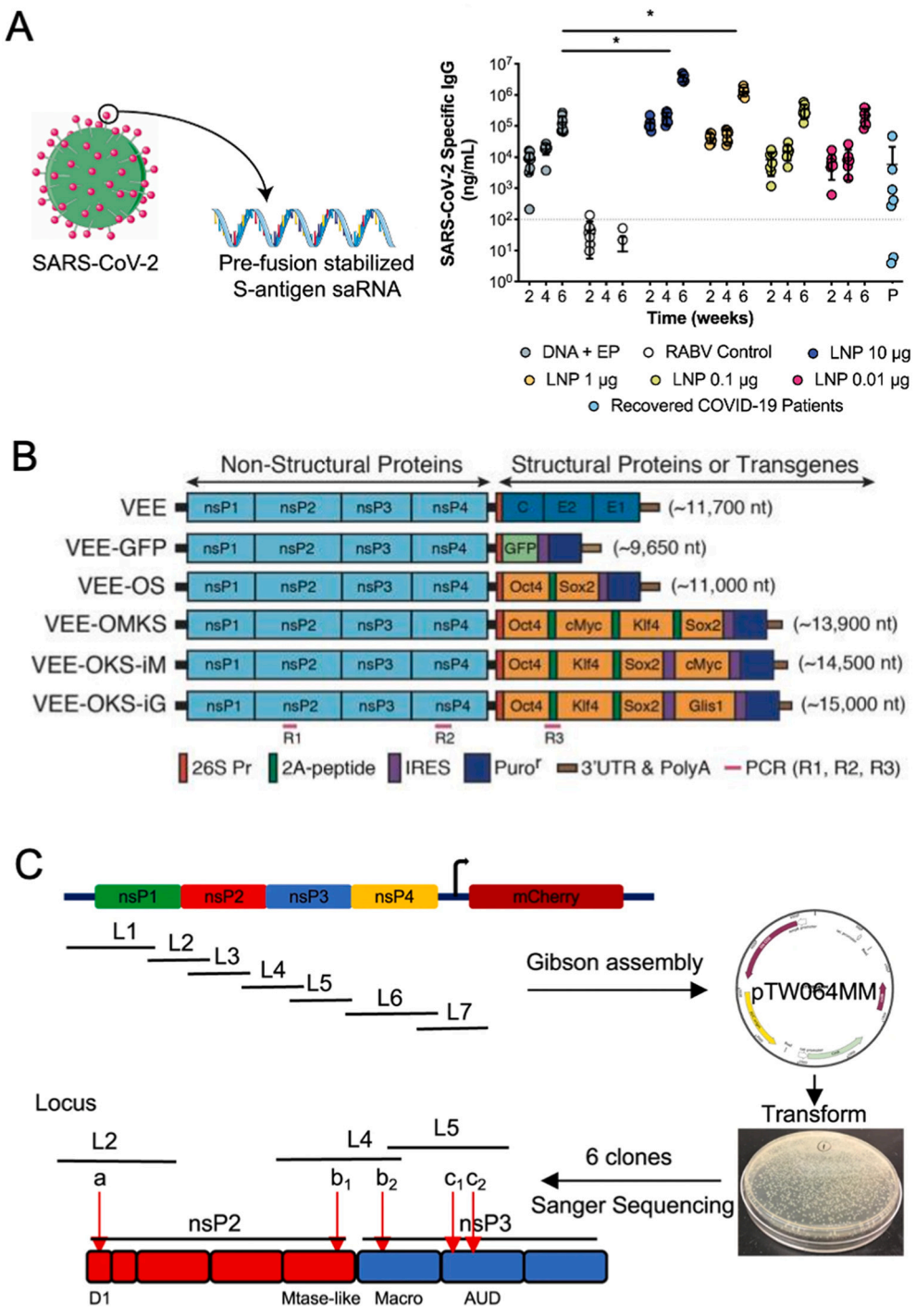

Fig. 10. Application of saRNA in therapeutics or vaccines. (A) The vaccinal saRNA encoding pre-fusion stabilized spike protein of SARS-CoV-2 encapsulated in LNP (i) and quantification of produced SARS-CoV-2-specific IgG in mice (ii). (A) adapted with permission from [105]. (B) Construction of VEE-derived saRNA for expression of reprogramming factors from primary human foreskin fibroblasts (HFFs) for the generation of iPSC colonies. (B) reprinted with permission from [107]. (C) Identified mutations in nsP2 and nsP3 of VEE construct. (C) adapted with permission from [111].

μg saRNA would induce the β-gal-specific $CD8^+$ T cells, achieving a 10–20 day-longer protection from tumor than that of non-amplifying RNA.

In addition, researchers have developed several approaches by using non-viral delivery carriers to further improve the efficacy, safety, and replication of saRNAs [95, 99,100]. In particular, a novel zwitterionic lipid nanoparticle formulation was established to enhance the vaccine efficacy against various viruses, such as the respiratory syncytial virus and influenza [54,101]. When the saRNA-based vaccine candidate encoding the respiratory syncytial virus fusion glycoprotein (RSV-F) was intramuscularly injected, approximately 11.4-fold increase of the F-specific antibody was observed [54]. As a non-viral carrier, a chitosan-coated nanoparticulate was also employed for the cytosolic delivery of saRNA by the protonation effect for translation in dendritic cells [102]. Further studies have shown the potential protection efficacy against human immunodeficiency virus (HIV) in mice and non-human primates [103,104]. In a non-human primate model, a saRNA vaccine encoding HIV-1 gp140 was formulated in a cationic nanoemulsion for the potent antigen-specific immunity and neutralizing antibody production without notable adverse effects [103].

Recently, saRNA has also been utilized against the newly emerged severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) by encoding the pre-fusion stabilized spike protein (Fig. 10A) [105,106]. The saRNA encapsulated by lipid nanoparticles elicited significant

Table 1
Summarized properties of each multimeric RNA therapeutic application.

	Chemical conjugation[23]	RNA nanoarchitecture assembly[76, 114]	RCT[9, 46, 48, 49]	saRNA[57]
Applicable therapeutic RNA	- siRNA	- mRNA - siRNA - miRNA - Immunogenic RNA	- mRNA - siRNA - miRNA - Immunogenic RNA	- mRNA
Therapeutic function	- Gene knockdown	- Gene knockdown - Gene upregulation - Immunostimulation	- Gene knockdown - Gene upregulation - Immunostimulation - mRNA vaccine	- Gene upregulation - mRNA vaccine
Pros	- Useful for stimuli-triggered release of therapeutic RNA - High amount of therapeutic RNAs loading capacity	- Applicable to multi-functional RNAs delivery - Accurate control of the loading amount of therapeutic RNA - Rationally programmable property	-Applicable to multi-functional RNAs delivery - High amount of therapeutic RNA loading capacity - Fabrication into various structures	- High level of RNA amplification with low dose - Flexibility to incorporate multi-pathogen sequences
Cons	- Not applicable to various therapeutic RNAs - Requirement of RNA modification	- Relatively low loading amount of therapeutic RNAs - Not usable of non-functional scaffold region	- Uncontrollable extent of polymerization - Hard for exact quantification of therapeutic RNAs	- Little information is known for immunogenicity of RdRP complex - Uncontrollable productivity of target protein

SARS-CoV-2 specific IgG production, which was superior to control groups electroporated with pDNA or naturally infected groups in order of magnitude [105]. Compared to the conventional mRNA vaccine, the self-transcribing and replicating RNA-based SARS-CoV-2 vaccine induced exceptionally high T cell immune responses and S-specific IgG production, promoting the protection against the SARS-CoV-2 infection with a single administration in a mouse model [106].

In addition to the vaccine platform, several attempts have been made to develop a modular saRNA to induce efficient cellular reprogramming enabled by a single transfection. By reconstituting four major transcriptional factors (Oct4, Klf4, Sox2, and cMyc) in a single saRNA replicon with Venezuelan equine encephalitis (VEE) nsPs, induced pluripotent stem cells (iPSCs)-producing system was achieved (Fig. 10B) [107,108]. Transfection of the VEE-Oct4, Klf4, Sox2, and cMyc (VEE-OKS-iM)-embedded saRNA allowed human fibroblasts to produce all four factors at considerably higher levels [107]. Using VEE-OKS-iM saRNA, iPS colonies with pluripotent gene markers comparable to human embryonic stem cells have been successfully generated [107]. Further research showed that a single transfection system comprising 1 μg of self-replicating RNA produced iPSCs more efficiently than the control group with a daily transfection protocol of 1.2 μg of non-replicating RNA-based platform [108]. Non-integrative, simple, and powerful saRNA-based cellular modulation was applied to generate disease-specific cell lines and in vivo mouse models. The results show the potential of cellular modulation platform addressing the safety issue of DNA vector- or virus-based systems [109,110].

More recently, saRNA has also been employed to encode immunomodulatory cytokines for enhanced cancer immunotherapy [111,112]. RNA replicons for interleukin-2 (IL-2), a potent antitumor cytokine, induced the retardation of tumor growth, which was further enhanced by introducing RNA alteration in nsP2 and nsP3 sequences (Fig. 10C) [111]. The mutation sequence was first identified by selecting and sequencing the mCherry-saRNA-transfected cells. The authors elucidated that the employment of sequence mutations would alter the interaction of amplicons with the host cell immunity, enabling an increase in the subgenomic amplicon level and the subsequent protein expression. As a result, mutant saRNA encoding IL-2 level at the tumor site and antitumor T cell infiltration induced a 5.5-fold and 2.1-fold increase, respectively, leading to greater retardation in tumor progression compared with that of wildtype saRNA [111]. Similarly, ionizable lipid-encapsulated saRNA encoding IL-12 for antitumor T cell immunity achieved persistent expression of target gene for a week by a single injection of encapsulated saRNA [112]. As well as the high expression level of IL-12, immunogenic cancer cell death was synergistically induced by the RNA replicon itself and the ionizable LNP, consequently leading to large tumor eradication [112].

In addition to the mass production of proteins, attempts have also been made to externally turn off protein expression from saRNAs using RNA binding protein L7Ae and FDA-approved small molecule trimethoprim (TMP) [113]. For the small molecule-mediated expression modulation, the authors developed L7Ae-fused destabilizing domain which can be stabilized by TMP. By oral administration of TMP, saRNAs bearing L7Ae-binding structural motifs between subgenomic promoter and GOI interact with stabilized L7Ae, leading to the 15.7-fold reduction of GOI expression [113]. The studies showed that the saRNA system could be successfully applied for enhanced therapeutics and vaccines.

These profound pre-clinical studies have further accelerated the clinical translation of saRNA as a universally applicable platform to overcome the limitations of conventional DNA or mRNA therapeutics. Moreover, saRNA-based vaccine candidate can be manufactured within 8 days due to the feasibility for design and synthesis of saRNAs after the target sequence becomes available [101]. However, there is still a need for meticulous clarification of unexpected immunity or side effects from viral components for the clinical translation from animal models to humans. Aided by the recently approved mRNA vaccines, saRNA formulations are expected to provide a facile strategy for non-viral, integration-free, and efficient RNA amplification.

3.5. Summary and issues to be considered for applications

In Section 3, various clinical applications of each multimeric RNA strategy are reviewed. Each multimeric RNA has their own advantages and disadvantages and these different properties have led them to be utilized to each different application. While RNA nanoarchitecture assembly and RCT-based multimeric RNA have been applied for diverse functional RNA delivery, chemical conjugation strategy has been mainly used for siRNA delivery and saRNA has been typically focused on mRNA delivery. Applicable therapeutic RNAs and pros and cons of each multimeric RNA are summarized in Table 1.

Since multimeric RNAs are unnatural RNAs which are composed of extremely long and three-dimensional structures, additional research on systemic toxicity such as cytotoxicity and immunotoxicity is highly required for the clinical translation in the future. The complete intact form of RNA structures could also be recognized by nucleic acid-sensing receptors such as TLRs [33]. The each of assembled nanoarchitectures exhibited different immunological response and recognition by TLRs regarding on the programmed shapes and sizes of structures of the whole structure [33]. Moreover, the immune responses of the multimeric RNAs were tactically controllable depending on the repetitive dsRNA length while showing the negligible toxicity [33,79]. These studies indicate

Table 2
Comparison of delivery system of multimeric RNAs with mRNA and pDNA.

	Applicable tools for RNA delivery					Additional modification for enhancing delivery
	Naked	Lipid-based carrier	Cationic polymer	Biopolymer	ETC	
Monomeric mRNA	[115–117]	[118–120]	[121,122]	[123,124]	[125,126]	5′ capping [61], Poly A tail [61], Pseudouridine [127]
pDNA	[128]	[129–131]	[132]	[133,134]	[135]	n.a.
Chemically conjugated multimeric RNA	n.a.	n.a.	[16–19,21,22,62]	[63–66]	n.a.	Thiol group [16–19,21,22,62–66] DNA overhang [16–19,21,22,62–66], Amine group [17], Aptamer [19]
RNA nanoarchitectures	[33,68,76]	[24,25,27,29,31,32,67]	n.a.	n.a.	n.a.	DNA overhang [26], Aptamer [27,29], Folate [67,68], 2′-F [67,68,76]
RCT-elongated multimeric RNA	[14,43,79,85,87,88]	[41,44–46,49,50,80,91]	[9,48,92]	[86,89,90]	[81,84]	Folate DNA [14], 2′-OMe DNA [41], Cholesteryl DNA [14,43], DNA aptamer [43,87,88], Antibody [85], Sense RNA [90]
saRNA	[53,97]	[54,83,95,99,100,101,103–108,111,112]	[55,58, 99]	[102]	[52, 96, 109, 113]	5′ capping [52–55,58,95,98,99,101,104–108,111–113], Poly A tail [58,98,104,106–108,113], m5C [108]

that the multimeric RNA could be empowered the immunostimulatory effects for therapeutic purposes, but there is still a concern on unusual recognition and cytokine stimulation possibilities completely different from monomeric RNAs. Thus, the long-term toxicity and safety of the whole multimeric RNA and its derivatives should be thoroughly evaluated to prevent the concerns on any undesirable systemic toxicity and immune responses.

Since effective delivery of RNA is one of the most important issues, various cellular delivery methods have been developed. A variety of traditional delivery methods such as lipid-based nanocarriers, cationic polymers, and electroporation can be easily applied to multimeric RNA (Table 2). In particular, the RNA nanoassemblies can be treated as naked with 2′-fluoro-modified RNA as well as the lipid-based polymers. The nanoassemblies can exploit the ligands for giving targeted delivery [69,77]. Since the RCT-based multimeric RNAs are also particulated, the naked or various carrier-complexed types are applicable. Due to their repetitive sequences, functionalized DNA strands can be readily hybridized to the multimeric RNAs [14,43]. Not only multimeric RNAs can be delivered with conventional delivery tools and enhance the stability and delivery efficiency, but also multimeric RNA carriers should be further developed for improved encapsulation and cellular delivery.

4. Conclusion and perspectives

Over the last decade, the field of multimeric RNA-based drug delivery has been rapidly developed as it grants enormous benefits over conventional RNA therapeutics. Purpose-oriented RNA multimerization strategies have improved the stability of therapeutic RNA itself without additional modifications and encapsulation efficiency of therapeutic RNAs. Moreover, the progress in multimeric RNA engineering has enabled efficient and prolonged therapeutic deliveries customized to treat or prevent various cancer and infectious diseases and to induce the desired cellular reprogramming. As summarized in this review, emerging evidence has highlighted the considerable potential of multimerized RNAs for efficient drug delivery and therapeutic efficacy.

Four major RNA multimerization strategies for efficient RNA-based therapeutics and vaccines have been reviewed. Firstly, the chemical conjugation of monomeric RNAs produced stimuli-responsive polymeric RNAs, which can be rapidly cleaved to the RNA fragments of desired length. Compact condensation of polymeric RNAs with positively charged carriers enabled the achievement of a better delivery of siRNAs than that of monomeric siRNAs. Secondly, rationally designed RNA nanostructures via molecular interactions between RNA itself were utilized to bear several homogenous and heterogeneous small RNA drugs. By hybridizing the bifunctional RNA nanoarchitecture, a linearly polymerized RNA drug with enhanced structural stability was produced. Thirdly, enzymatic replication from circular scaffold DNAs produced several thousands of repetitive nucleobases. Enzymatic reactions with purpose-oriented design of scaffold DNA have been widely applied to produce multimeric RNAs encoding from small interference RNA to mRNA. These linearly extended multimers were self-assembled from nanoscale to microscale structures. These self-assembled structures further improved the stability and cargo transfer efficiency, subsequently reducing the required dosage compared with monomeric RNA. Lastly, the saRNA replicon-based drug delivery strategy carried the genetic information needed to generate a number of RNA therapeutics from a single RNA strand. Compared to other linearized RNA multimers, this strategy could be useful for generating relatively long sequence RNAs, such as mRNA.

Numerous pre-clinical studies have further emphasized the importance of multimerized RNA drugs over conventional monomeric RNA-based medications, accelerating their clinical use. As the properties of multimeric RNAs significantly differ from conventional monomeric RNAs, further investigation of multimeric RNAs with regard to their systemic toxicity, immune responses, residence times, and working durations is required for successful clinical translation. Moreover, non-

canonical structures would impose unknown relationships with biomolecules in the body due to their repetitive sequences [136]. In addition, numerous carriers for monomeric RNAs delivery such as polycationic reagents, peptides, exosomes, or lipid nanoparticles [137] are rarely optimized to multimeric RNA due to the different physical properties including RNA charge density, cargo size and dimension. Therefore, further development of multimeric RNA carriers should be achieved for enhanced cellular delivery. Also, chemical modification can be applied to increase the RNA stability, leading to improved therapeutic efficiency [138,139]. Given the unprecedented therapeutic efficacy of multimeric RNAs over monomeric RNAs, the multimeric RNA structures could offer a promising platform for the development of the state-of-the-art formulations of RNA therapeutics.

CRediT authorship contribution statement

Dajeong Kim: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Sangwoo Han:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Yoonbin Ji:** Writing – original draft, Visualization. **Sunghyun Moon:** Writing – original draft. **Hyangsu Nam:** Writing – original draft. **Jong Bum Lee:** Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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