In vitro circularization of RNA

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ABSTRACT
Over the past 2 decades, different types of circular RNAs have been discovered in all kingdoms of life, and apparently, those circular species are more abundant than previously thought. Apart from circRNAs in viroids and viruses, circular transcripts have been discovered in rodents more than 20 y ago and recently have been reported to be abundant in many organisms including humans. Their exact function remains still unknown, although one may expect extensive functional studies to follow the currently dominant research into identification and discovery of circRNA by sophisticated sequencing techniques and bioinformatics. Functional studies require models and as such methods for preparation of circRNA in vitro. Here, we will review current protocols for RNA circularization and discuss future prospects in the field.

Abbreviations: AMP, adenosine monophosphate; AppRNA, 5'-adenylated RNA; ATP, adenosine triphosphate; bp, base pair; CEV-A, citrus exocortis viroid strain A; circRNA, circular RNA; ciRNA, circular intronic RNA; CuAAC, copper catalyzed azide alkyn cycloaddition; EDG, ethyl-3-(3'-dimethylaminopropyl)-carbodiimide; HIV, human immunodeficiency virus; IEciRNA, intron-exon circular RNA; nt, nucleotide; PIE, permuted intron exon; T4 Dnl, T4 DNA Ligase; T4 RN, T4 RNA Ligase

Introduction
In recent years, the interest in circular RNAs (circRNA) as a novel type of endogenous non-coding RNA has been strongly grown. CircRNAs are found in all kingdoms of life; thousands have been identified across species from Archaea to humans. CircRNAs have been known for a long time in form of genomes of viroidal plant pathogens or Hepatitis delta virus. Furthermore, circRNAs can be formed from introns (hence termed circular intronic RNA (ciRNA)), or from exons with introns retained between the exons (termed exon-intron circRNA (IEciRNA)). However, the vast majority of circRNAs apparently is transcribed and spliced from exons of protein and non-coding genes. Initially discovered as ubiquitous molecules in mice and human cells, those circRNAs were subsequently found as abundant, conserved and stable species in all eukaryotes studied today.

Whereas a large number of data have allowed suggesting models for their formation, the biological function of circRNAs has remained elusive. For a long time, circRNAs were seen just as side products of aberrant splicing reactions. This view has however dramatically changed due to the fact that with the development of deep sequencing and bioinformatic tools a large number of circRNAs have been discovered in mammalian and other cells. Strikingly, often circRNA is even the more abundant isoform than mRNA. Due to their covalently closed ring structure, circRNAs have a higher stability in the cellular environment as compared with their linear counterparts, implying that possible functions of circRNA may be associated with the longer life time of these molecules. In fact, based on recent research in the field, circRNAs have been suggested to act as miRNA and protein sponges, to be involved in the modulation of gene expression and splicing, and to be associated with diseases initiation and progression. Thus, circRNAs have also been considered as biomarkers for diseases prognostics and diagnostics and as targets or tools for disease treatment. The field of investigations into circRNA and in particular into their putative function within the complex network of regulation of gene expression and splicing is currently very active. In addition to the technical and conceptual challenges, raised for example by sequence overlap with mRNA or linear non-coding RNA transcribed from the same locus, synthetic circRNAs are needed as models for structure and function studies. This has put demand on the side of chemistry and biochemistry to elucidate and optimize protocols for the generation of circRNA. Bascially, procedures for intermolecular RNA-ligation may be adapted to proceed in intramolecular fashion. The key issue is the design of the ligation assays in favor of intramolecular end joining (circularization) versus intermolecular reaction (oligomerization). Circular RNAs can be made from their linear counterparts by enzymatically or chemically supported reactions and in addition by the help of specially designed ribozymes. RNA circularization by chemical means can produce a natural phosphodiester bond. In addition, a number of powerful chemistries may be used, which however, would lead to the formation of non-natural linkages. This is not necessarily a problem, because one modified linkage among the many natural phosphodiester bonds may be tolerated without significantly affecting the function. Here we will review the methods available for formation of nucleic acid
phosphodiester bonds, highlight those protocols that have been used already for production of circRNAs, but also show potential strategies that might be used in the future.

**RNA synthesis and functionalization**

**Chain assembly**

At the beginning of all circRNA preparations is the synthesis of the linear RNA with suitable 5' and 3'-terminal functionalities for end-joining. For enzymatic protocols these functionalities are usually 5'-phosphate and 3'-OH. However, for chemical ligation, the required terminal functionalities may be rather diverse (Fig. 1). Therefore, chemical RNA synthesis allowing the site-specific introduction of a desired functionality at the 5'- and/or 3'-end is a valuable option. Chemical RNA synthesis is limited to oligomers of about 50 to 70 nucleotides length. However, protocols for joining of RNA fragments are available, which could be implemented into protocols for RNA circularization. For example, recently we have prepared a modified 129mer RNA for fluorescence studies by enzymatic and chemical ligation of 2 RNA fragments.23 As an alternative to chemical synthesis, RNA can be produced by in vitro transcription, which allows preparation of RNAs of virtually unlimited length, however, requires post-synthetic functionalization strategies.

**5'-terminal functionalization**

In case of chemical RNA synthesis, 5'-terminal modifications such as phosphates, amines, thiols, azides or alkynes to be used

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**Figure 1.** Oligonucleotide circularization strategies. (a) enzymatic ligation of a 5'-phosphate with a 3'-OH terminus; (b) chemical ligation of a phosphate with OH-terminus (the 5'- or the 3'-end can be phosphorylated); (c) chemical ligation of a 3'-thiophosphate with a tosylated 5'-end; (d) chemical ligation of a 3'-thiophosphate with a iodinated 5'-end; (e) chemical ligation of a 3'-aldehyde with a 5'oxoamine (oxime circularization); (f) chemical ligation of a 5'- or 3'-azide with a 3'- or 5'- alkyne (Click circularization); (g) circularization by metal chelation (M = Zn^{2+} or Fe^{3+}, l = terpyridine).
directly for cyclization or, prior to cyclization, for further conjugation chemistry to introduce the required functionality, can be introduced by coupling the respective phosphoramide as last building block in the chain assembly proceeding from the 3' to the 5'-end.23

A characteristic feature of in vitro transcribed RNA is the 5'-terminal triphosphate, because 5'-triposphates of nucleosides are used as building blocks for enzymatic polymerization. As mentioned above, enzymatic end-joining for circularization mostly requires a 5'-monophosphate, and also chemical procedures rely on specific functionalities. Therefore, the 5'-terminal triphosphate needs to be removed prior to further functionalization and ligation. This can be easily achieved by treatment with alkaline phosphatase or any other dephosphorylation enzyme, and subsequent introduction of the required 5'-functionality. A phosphate group can be easily attached onto the 5'-OH by reaction with ATP in the presence of T4 Polynucleotide kinase. This protocol would also allow for further chemical modification, if instead of ATP γ-thio-ATP is used, such that the sulfur of the transferred thiophosphate can be used for further chemical conjugation of a desired functionality. Another option is the reaction of the unique primary alcohol at the 5'-end with triphenylphosphonium iodide to generate a good leaving group and thus making the 5'-terminus suitable for reaction with a nucleophile, as for example an azide.23,24 The azide may be directly used for Click cyclization (see below) or, upon reduction to an amino group, for conjugation with a desired functionality via peptide chemistry.25

Specific 5'-RNA functionalization can also be achieved by transcription priming, adding to the transcription mixture an initiator nucleotide that carries no triphosphate for polymerization and therefore can be used only for priming RNA synthesis at the 5'-end.26,27 This protocol has been mostly used with T7-RNA-Polymerase and therefore requires guanosine conjugated via a 5'-phosphate with the desired functionality, as initiator nucleotide (the 5'-phosphate is necessary for recognition/acceptance of the modified building block by the polymerase). In our laboratory, we have made use of this strategy for enzymatic preparation of RNAs carrying a biotinylated nucleoside28 or a monophosphate29 at the 5'-end.

3'-terminal functionalization

As for 5'-terminal modification, 3'-end modification can be easily achieved by chemical synthesis provided that a suitable modified building block attached to a solid support is available or alternatively, can be made in the laboratory. Preparation of 3'-phosphorylated RNAs, as well as of RNAs with 3'-terminal amines, thiols, azides or alkynes can be easily performed and belongs to the standard repertoire of solid phase phosphoramidite chemistry. Moreover, protocols for post-synthetic modification of the 3'-terminus are available, therein using the characteristic features of the unique 3'-terminal cis-diol. The cis-diol, in the presence of periodate, is easily oxidized to a dialdehde, which subsequently is reacted with N-nucleophiles, such as amines or hydrazines to introduce a desired functionality.30 In our hands, this procedure works very well, and we have prepared a number of RNAs modified with fluorescence dyes or with flavine derivatives.31

Circularization strategies by 5'-3'-end joining

In general, the preparation of circRNAs from linear precursors is very challenging, due to the negative entropy associated with the circularization step. The most significant side reaction is intramolecular bond forming leading to oligomerization of the linear precursor instead of circularization. This can be counteracted by a clever choice of reaction conditions, in particular by strict control of the concentration regime. Intramolecular reaction would be favored over intermolecular reaction at lower concentration of the linear precursor, which however, has the disadvantage that production of circRNAs at preparative scale becomes rather challenging. Therefore special protocols for pre-orientation of the 2 termini to be linked are of utmost importance. A most favorable scenario is a self-templating effect based on the intrinsic secondary structure of the linear precursor,32,33 (Fig. 2b). Dependent on the specific ligation protocol, the ligation junction can be positioned in a double-stranded area of the folded nucleic acid structure (nick site) or in the single-stranded area with the 2 reacting ends positioned next to each other (e.g. in a hairpin loop). This strategy would allow also for a template-assisted convergent approach of circularization: 2 or even more fragments constituting the full-lengths linear/circular nucleic acid need to be designed to interact in the required way to achieve multi-site ligation and circularization in one.34 Other ways of pre-orienting the 2 reacting ends may include a linear or hairpin helper oligonucleotide (Fig. 2c, d), or a linear oligonucleotide (splint) that interacts with the 5'- and 3'-end of the linear precursor bringing them in a flanking alignment and thus serving as splint for template assisted ligation (Fig. 2e, f).35-37 Thus, the choice of the ligation site and of the explicit strategy depends on the RNA substrate to be circularized and in addition is affected by the decision of enzymatic vs. chemical ligation.

Enzymatic strategies

Several protocols for enzymatic ligation of RNA fragments are available,38 most commonly employing 3 different polynucleotide ligases:

T4 DNA ligase (T4 Dnl). The enzyme requires ATP as cofactor and supports the formation of a phosphodiester bond between a 5'-phosphate and a 3'-hydroxyl group within double stranded regions of DNA or RNA or even hybrid structures. Very importantly, the enzyme catalyzes the covalent joining of the 2 ends only in case of full undistorted complementarity and if no nucleotides are missing at the junction.39 A splint can be used to create the required double stranded region around the ligation site (as depicted in Fig. 2f).40 T4 Dnl-assisted ligation of 2 RNA fragments was pioneered by Moore and Sharp,41,42 and is also suitable for RNA circularization. Using a DNA splint is most favorable, because after reaction the splint can be digested with DNAs. The circularized RNA product can then easily be separated from the linear precursor by polyacrylamide gel electrophoresis. For intermolecular ligation, it has been shown that the length of the splint affects the efficiency of the ligation.40 However, in general it is impossible to predict an optimal splint length of universal validity. Thus, for each individual system, reaction conditions with special focus on
temperature, stoichiometry and splint length need to be defined in order to achieve efficient ligation.

The T4 DNA ligase directed closure of linear DNA is common in the literature, whereas examples of RNA circularization with T4 Dnl are rather rare. A representative example is the T4 Dnl assisted circularization of a 453nt linear RNA precursor that was shown to be translated into protein by the eukaryotic translational apparatus. Furthermore, circular dumbbell RNA/DNA chimeric oligonucleotides were designed for the efficient inhibition of HIV-1 replication. These circular chimeras consisted of a sense RNA and its complementary antisense DNA, connected via 2 ethylene glycol loop structures. Circularization was carried out by enzymatic ligation of the 5′-phosphorylated nicked 46-mer RNA/DNA oligonucleotides with T4 DNA ligase.

**T4 RNA Ligase 1 (T4 Rnl1).** The enzyme, in the presence of ATP as cofactor, supports the ligation of single stranded nucleic acids, and accepts DNA and RNA fragments as substrates. T4 RNA ligase is useful for joining RNA to RNA, provided that the donor contains a 5′ phosphoryl and the acceptor a 3′-OH (Fig. 1a). When designing the experiment and defining the ligation junction for circularization, it should be taken into account that the ligase has different preferences for the terminal nucleotides of the donor- and acceptor strand. This is A > G ≥ C > U for the 3′-terminal nucleotide of the acceptor strand, and pC > pU > pA > pG for the 5′-terminal nucleotide of the donor strand. T4 Rnl1 assisted RNA ligation has been used to produce large amounts of homogeneous and pure circRNA. A key element of these syntheses was the exploitation of the RNA intrinsic secondary structure for orientation of the 5′- and 3′-termini for efficient ligation. Furthermore, dumbbell-shaped circular RNAs for RNAi applications were designed and synthesized by ligation of 2 RNA strands that form a stem structure with overhanging single stranded regions and covalent joining of the thus pre-oriented 5′-and 3′-termini at both sites with T4 Rnl1. Successful ligation in linear arrangements has been also achieved for tRNAs with the ligation junction positioned in the anticodon and the D loop pre-oriented by the intrinsic tRNA structure. Further examples of RNA circularization by T4 Rnl1 include the synthesis of an infectious circRNA of the *citrus exocortis* viroid strain A (CEV-A), circular versions of the hammerhead ribozyme, and others. Apart from intrinsic secondary structure elements that help to orient the 2 termini to be ligated close to each other, proper orientation of the reactive ends can be achieved by using a splint that by hybridization with both the donor and the acceptor brings the substrates close together, however, leaves the terminal 2 to 3 nucleotides of both strands single stranded at the ligation junction (Fig. 2c). In our laboratory, best results were obtained with a DNA splint leaving 2 nucleotides of both donor and acceptor single stranded at the ligation junction. This setup allowed us to achieve, though in an intermolecular set-up, ligation yields of up to 45%.

**T4 RNA ligase 2 (T4 Rnl2).** The enzyme ligates RNA acceptor strands (3′-OH group) with RNA- or DNA-donor strands (5′-phosphate group), preferentially in nicked dsRNA substrates. It is a close relative of T4 Rnl1 and was first described by Ho and Shuman in 2002. We have successfully used T4 Rnl2 to circularize a 104nt linear precursor RNA with donor and acceptor ends positioned in a double stranded region. Recently, circular RNAs ranging in size from 129 to 387 nucleotides were prepared by intramolecular ligation of the respective linear precursor in the presence of a 20nt DNA helper oligonucleotide.

In summary, RNA circularization by enzymatic ligation is a valuable option. All three enzymes mentioned above are ATP-dependent and catalyze the joining of RNA termini with 5′-phosphate and 3′-OH via 3 nucleotidyltransfer steps: (i) the enzyme reacts with ATP to form a covalent ligase-AMP intermediate; (ii) AMP of the intermediate is transferred to the 5′-
phosphorylated RNA terminus forming 5′-RNA-adenylate (AppRNA); (iii) AppRNA is attacked by the 3′-hydroxyl group to form a phosphodiester bond, whereby AMP is liberated. Ligation can proceed in an intramolecular fashion leading to circRNA. Formation of side products by intermolecular ligation cannot be completely ruled out. However, the intrinsic secondary structure of the RNA to be ligated in conjunction with the clever choice of the ligation site and the application of helper oligonucleotides may assist intramolecular ligation, thus favoring RNA circularization.

**Chemical strategies**

Chemical ligation of nucleic acid strands in inter- or intramolecular fashion allows formation of natural phosphodiester bonds. However, in addition the chemist’s toolbox provides a large number of powerful reactions that may be used for joining 2 appropriately functionalized fragments or termini of one and the same molecule. Whereas natural phosphodiester bond formation is favorable in terms of generating an unmodified linkage as it occurs in natural biomolecules, yields are often unsatisfactory. On the contrary, more efficient reactions can be achieved by specific functionalization/activation of reacting ends, which however creates unnatural linkages in the ligated/circularized molecule.

**Natural phosphodiester linkages.** As an alternative to enzymatic ligation, phosphodiester formation can be achieved also by pure chemical strategies, and it is up to the experimental design, if ligation proceeds inter- or intramolecularly. Small circles can be prepared by chemical cyclization of protected oligonucleotides in solution or still at solid support. For longer sequences however, template-based strategies as explained above and illustrated in Fig. 2 are needed. Natural phosphodiester bonds are formed by condensation of 5′-phosphate with 3′-OH or vice versa in the presence of phosphate activating agents, such as cyanogen bromide (BrCN) or water-soluble ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Fig. 1b). Chemical ligation was originally developed for linking DNA strands, and since then has been used quite extensively for the synthesis of RNA circles. The most significant problem associated with chemical ligation of RNA is the formation of 2′, 5′-phosphodiester bonds, due to phosphate migration. To circumvent this problem, 2′-deoxynucleotides may be used at the 3′-end of oligonucleotides to be ligated/circularized. As a representative example, Wang et al. synthesized a 34mer circular RNA by chemical ligation of the 5′-phosphorylated linear precursor with BrCN/imidazole/Ni²⁺. Favorable orientation of the 2 reacting ends was achieved by triplex formation with a short DNA oligonucleotide (as shown in Fig. 2).

**Non-natural linkages.** There are a number of alternative chemistries for oligonucleotide ligation, which however, rely on specific functionalization of the reacting ends, and as mentioned above, in consequence yield unnatural linkages at the ligation junction (Fig. 1c-g). Nevertheless, such alternative chemistries have been used, because of high efficiency and the possibility to be carried out at larger scale. Furthermore, one non-natural linkage within a circular DNA or RNA may well be tolerated without considerable effects on the function. Basically, the required functionalities for chemical ligation/circularization are introduced during oligonucleotide synthesis or alternatively, are conjugated post-synthetically. Circularization then is initiated by either self-templating effects or helper oligonucleotides as described above, bringing the 3′- and 5′-end in close proximity. Whereas a number of reports describe the synthesis of circular DNAs with a non-natural linkage at the ligation site, application of such specific protocols in the field of circRNA synthesis is still rather rare. For example, DNA has been circularized using S₂O₂- reactions between 5′-termini with tosylate or iodo leaving group and 3′-phosphorothioates (Fig. 1c, d). Reaction of bifunctionalized oligonucleotides with terminal S-oxymine and 3′-aldehyde delivered circDNA with oxime linkage (Fig. 1e). Oxime circularization was also used for circularization of RNA, although only small circles (tetrarmers) were prepared. Other circularization procedures involve copper catalyzed azide alkyne cycloaddition (CuAAC) (Fig. 1f), strategies for photoligation, or conjugation of metal binding ligands such as terpyridine to the 5′- and 3′-end of the oligonucleotide to be circularized (Fig. 1g). The latter method was used to produce a circular 2′-OMeRNA. Terpyridine was coupled to the 5′- and 3′-end of a short linear RNA by phosphoramidite chemistry and circularization was achieved by addition of Zn²⁺ or Fe²⁺.

Many chemical ligation protocols still suffer from rather low yields and as described above often require specific functionalization of the linear precursor. It remains questionable if the available strategies can be used for circularization of large circular RNA molecules, and if they can compete with enzymatic ligation. Click circularization by CuAAC is certainly one of the most powerful methods for linking the 2 ends of a linear RNA bearing a terminal alkynyl and azide function (Fig. 1f). However, these functionalities first need to be introduced, and the resulting triazole linkage has to be tolerated. Thus, the choice of the circularization strategy should always consider the intended application of the circular product.

**Ribozymes for RNA circularization**

A recent study of human circRNAs revealed that these molecules are usually composed of 1–5 exons. Considering that about 80 % of exons are in the size range of 20 to 200 bp, biologically relevant circular RNAs may greatly vary in size, being composed of up to 1000 bases. Thus, in addition to enzymatic and chemical strategies described above that are more favorable to the synthesis of smaller RNA circles, methods for production of large circular RNAs are needed.

Many ribozymes occurring in nature are capable of catalyzing phosphodiester bond formation within specific sequence context. However, this natural occurring activity has rarely been used for engineering into tools that would support RNA ligation/circularization at preparative scale. Previously, we have engineered hairpin ribozyme variants that fold into 2 alternative cleavage active conformations, thus cutting off their 5′- and 3′-terminus, and thereby producing a 5′-OH group and a 3′-terminal 2′,3′-cyclic phosphate. These characteristic ends are required for ligation, which then can take place in an intramolecular manner as depicted in Fig. 3. The driving force of this reaction is the characteristic cleavage-ligation equilibrium of the hairpin ribozyme: Ligation proceeds via ring opening of the cyclic phosphate, thus being enthalpically favored...
over cleavage. Entropically, ligation is disfavored, owing to the decrease in degrees of conformational freedom upon connecting the 2 fragments. However, the entropic cost of ligation is rather small and can be compensated by the favorable enthalpic contribution. In addition, ligation is about 2 times faster than cleavage. Thus, the internal equilibrium of the hairpin ribozyme is shifted toward ligation. Translated into practical use this means that the 2 activities can be controlled by structural modulation. Hairpin ribozymes that form a stable structure, such that fragments remain bound, favor ligation, whereas hairpin ribozymes that are less stable, such that cleavage fragments can easily dissociate, favor cleavage. We have demonstrated hairpin ribozyme supported RNA circularization for the generation of 83mers. However, as shown in Fig. 3, the hairpin ribozyme structure may be embedded in RNA sequences of variable length (gray lines), thus paving the way toward preparation of large RNA circles. A disadvantage of the procedure is that the generated circRNAs are not stable due to the dynamic equilibrium of hairpin ribozyme catalyzed cleavage and ligation. Previously, we have shown that hairpin ribozyme activity can be allosterically controlled by external cofactors. Therefore, a possible scenario of hairpin ribozyme supported RNA circularization could exist of enzymatic or chemical synthesis of linear precursors containing the hairpin ribozyme structure at the ligation junction, but requiring an additional cofactor, such as an oligonucleotide effector or an allosteric activator, for induction of ligation. After reaction, the cofactor can be removed, thus switching off hairpin ribozyme activity and leaving behind the ligated RNA as stable circle.

Another method for ribozyme-supported production of circular RNAs relies on a spontaneous group I intron self-splicing system (Fig. 4). A circularly permuted group I intron precursor RNA consisting of end-to-end fused exons that interrupt half intron sequences, self-splices to generate a circular RNA. As it was demonstrated later, foreign sequences can be integrated into the exon of such a permuted self-splicing system, thus allowing the synthesis of circRNAs with desired sequence elements. Based on the permuted intron exon (PIE) method, a variety of circular RNAs up to the length of 1130 nts have been generated in vitro as well as in vivo, mostly in E.coli cells. Because RNA ligation for circularization is catalyzed by the intronic RNA, the system is also known as RNA cyclase ribozyme. There is an early report on PIE mediated production of circular RNA in yeast. The circularly permuted group I intron precursor RNA was expressed as a eucaryotic mRNA from a regulated promotor, and the accumulation of circRNAs was demonstrated. However, it remains questionable if the PIE method is reliably applicable in eucaryotic cells, because so far further evidence is missing. There are also reports on modified group II introns for inverse splicing that generates circRNA. In a way similar to the PIE method, exons need to be arranged in a consecutive way with the branch point upstream and intronic sequences up- and downstream of the exons. Group II intron self-splicing systems designed in this manner have shown good circularization efficiency and are favorable in terms of complete exon sequence variability. However, due to the mechanism of group II intron self-splicing, the produced circRNA have a 2‘, 5’-phosphodiester linkage at the ligation junction.

**Summary**

The appearance of circRNAs in all kingdoms of life implies that these abundant stable RNA species have important biological functions, which however are not yet fully understood. The growing interest in circRNA function has put demand on
chemists to develop strategies for synthesis of circular RNA. Enzymatic and chemical strategies for RNA ligation have been adapted to proceed at intramolecular level, assisted by self-templating effects or helper oligonucleotides. In addition, naturally occurring ribozymes have been engineered to support circularization of linear precursors in self-processing reaction schemes. The choice of the specific protocol should be made on the basis of where the circRNA should be produced (in vitro or in vivo), the nature of the RNA components (modified versus natural nucleotides), and the size of the circRNA. Ribozyme supported self-circularization (hairpin ribozyme or cyclase ribozyme) allows in vivo production of circRNA, whereas chemical and enzymatic procedures can be applied exclusively in vitro. Natural linear RNAs can easily be produced by in vitro transcription; the integration of modified nucleotides or other functionalities however, requires chemical synthesis of linear precursors followed by chemical or enzymatic ring closure. Circularization of linear precursors is entropically disfavored, in particular when it comes to circles of larger size. Therefore, self-splicing scenarios that as a result of intrinsic mechanistic features deliver circular RNAs are advantageous over enzymatic/chemical ring closure reactions for the synthesis of large circRNAs. On the contrary, smaller RNA circles can be easily generated by intramolecular ligation.

The field of investigation into biogenesis and function of circRNA will continue and it is on the chemists to refine RNA circularization strategies or even to develop new protocols to deliver circRNA models on demand.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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