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— *with sequence listing part of description (Rule 5.2(a))*

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(54) Title: IN VITRO AND IN VIVO PROTEIN TRANSLATION VIA IN SITU CIRCULARIZED RNAS

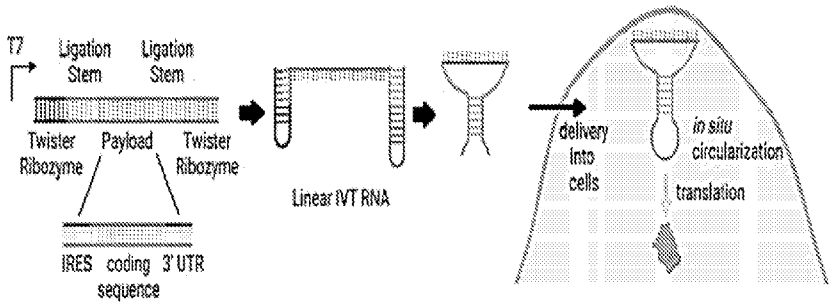

FIG. 1A

(57) Abstract: Provided herein are engineered linear RNA polynucleotide molecules that form circular RNA polynucleotide molecules in cells. Also provided are DNA constructs encoding the engineered linear RNA polynucleotide molecules. The disclosure provides for ribozyme-mediated constructs and systems and methods thereof, for use in a variety of applications, including for protein production systems, inducible gene expression systems, gene therapy, and combinatorial screening.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/62216

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. A61K 31/7105; C12N 15/113; C12N 15/63; C12N 15/67 (2023.01)
ADD.

CPC - INV. A61K 31/7105; C12N 15/113; C12N 15/63; C12N 15/67

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2018/237372 A1 (CORNELL UNIVERSITY) 27 December 2018; [0011], [0012],[0016], [0018], [0026], [0056], [0060], [0068], [0099], [0136]	1-3, 35-37, 54-56, 76-78, 94-105, 116-124
Y	WO 2020/198641 A2 (INTELLIA THERAPEUTICS INC.) 01 October 2020; [0005], [00424], [00427]	1-3, 35-37, 54, 55-56, 76-78, 94-102, 105, 116-124
Y	WO 2021/158964 A1 (UNIVERSITY OF ROCHESTER) 12 August 2021; pg. 1, lines 16-17; pg. 5, lines 4-9; pg. 29, line 31, pg. 94, lines 3-7	94-104, 124
Y	WO 2021/252909 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 16 December 2021; [8], [68]	2, 3/2, 36, 37/36, 55, 56/55
Y	US 2018/0251786 A1 (TOCAGEN INC.) 06 September 2018; [0006], [0007], [0076], [0079]	118-124

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 May 2023 (23.05.2023)	AUG 10 2023
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/62216

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed.

   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),

      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/62216

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 106-115, 125-142
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+, Claims 1-3, 35-37, 54-56, 76-78, 94-105, 116-124, and SEQ ID NO: 1330 (IRES sequence), SEQ ID NO: 1354 (3' UTR sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/62216

-***-Continued From Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-3, 35-37, 54-56, 76-78, 94-105, 116-124, and SEQ ID NO: 1330 (IRES sequence), SEQ ID NO: 1354 (3' UTR sequence) are directed towards linearized ribozyme activated RNA constructs, linearized ribozyme-RNA constructs, DNA constructs, cells comprising constructs, and ribozyme RNA-constructs comprising a sequence of interest positioned between first and second ligation sequences for circularization.

The constructs and cells of Claims 1, 2-3 (each in part), 35, 36-37 (each in-part), 54, 55-56 (each in-part), 76-78, 94-105, 116-124 are believed to encompass the first named invention of Groups I+ and are the claims that will be searched without fee to the extent that they encompass SEQ ID NO: 1330 (first exemplary IRES sequence), SEQ ID NO: 1354 (first exemplary 3' UTR sequence).

This first named invention of Group I+ has been selected to encompass the first species of each of the genera found in claims 2-3 based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines.

Applicant is invited to elect additional IRES sequence(s) and 3' UTR sequence(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), and where available as an option within at least one searchable claim, to be searched. Additional IRES sequence(s) and 3' UTR sequence(s) will be searched upon the payment of additional fees. Applicants must specify the searchable claims that encompass any additionally elected IRES sequence(s) and 3' UTR sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 1331 (IRES sequence) and SEEQ ID NO: 1355 (3' UTR sequence).

Groups I+ share the technical features including: a linearized ribozyme activated RNA construct comprising from 5' to 3' end: a) a first ligation sequence; b) an IRES sequence; c) a polynucleotide sequence of interest encoding a recombinant polypeptide; d) a 3' UTR sequence; e) a poly(A) sequence; and f) a second ligation sequence, wherein the first ligation sequence comprises a 5'-OH end, the second ligation sequence comprises a 2', 3'-cyclic phosphate end, wherein the first and second ligation sequences form a stem substrate for an RNA ligase; a cell comprising a circular RNA construct, wherein the circular RNA construct comprises: a) a first ligation sequence; b) an IRES sequence positioned 3' of the first ligation sequence; c) a polynucleotide sequence of interest encoding a recombinant polypeptide and positioned 3' of the IRES sequence; d) a 3' UTR sequence positioned 3' of the IRES sequence; e) a poly(A) sequence positioned 3' of the 3' UTR; and f) a second ligation sequence positioned 3' of the poly (A) sequence, wherein the first and second ligation sequences are ligated together; a linearized ribozyme-RNA construct comprising from 5' to 3' end: a) a first twister ribozyme; b) a first ligation sequence; c) an IRES sequence; d) a polynucleotide sequence of interest encoding a recombinant polypeptide; e) a 3' UTR sequence; f) a poly(A) sequence; g) a second ligation sequence; and h) a second twister ribozyme; a DNA construct comprising a RNA polymerase II promoter and a nucleic acid sequence encoding a ribozyme-RNA construct, wherein the ribozyme-RNA construct comprises from 5' to 3' end: a) a first twister ribozyme; b) a first ligation sequence; c) an IRES sequence; d) a polynucleotide sequence of interest encoding a recombinant polypeptide; e) a 3' UTR sequence; f) a poly(A) sequence; g) a second ligation sequence; and h) a second twister ribozyme, wherein promoter is operably linked to the nucleic acid sequence encoding the ribozyme-RNA construct; a ribozyme RNA-construct(s) comprising from 5' to 3': an optional primer region, an optional barcode region, a first ribozyme domain, a first ligation stem domain, a payload domain, a second ligation stem domain, and a second ribozyme domain; wherein the payload domain comprises from 5' to 3': an internal ribosome entry site (IRES) or a P2A peptide coding sequence, a coding sequence of at least one polypeptide and/or nucleic acid of interest, and a 3 'UTR sequence; wherein the transcription of the payload domain is activated by or dependent upon the activity of the one or more ribozymes; these shared technical features are previously disclosed by WO 2018/237372 A1 to CORNELL UNIVERSITY (hereinafter 'CORNELL') in view of WO 2021/158964 A1 to UNIVERSITY OF ROCHESTER (hereinafter 'ROCHESTER').

CORNELL discloses a linearized ribozyme activated RNA construct (a linear RNA molecule comprising a ribozyme; paragraph [0012]) comprising from 5' to 3' end: a) a first ligation sequence (a first ligation sequence; paragraph [0012]); b) an IRES sequence (an effector molecule comprising an IRES sequence; paragraphs [0012], [0060]); c) a polynucleotide sequence of interest encoding a recombinant polypeptide (an effector molecule comprising an RNA molecule encoding a peptide sequence; paragraphs [0012], [0060]); and f) a second ligation sequence (a second ligation sequence; paragraph [0012]), wherein the first ligation sequence comprises a 5'-OH end, the second ligation sequence comprises a 2', 3'-cyclic phosphate end (wherein the ligation sequences include a 5'-OH end and a 2', 3'-cyclic phosphate end; paragraphs [0012], [0056]), wherein the first and second ligation sequences form a stem substrate for an RNA ligase (forming a stem substrate for RNA ligase; paragraphs [0012], [0017]); a cell comprising a circular RNA construct (cells comprising a circular RNA molecule; paragraphs [0011], [0012], [0016]), wherein the circular RNA construct (producing a circular RNA molecule; paragraph [0012]) comprises: a) a first ligation sequence (a first ligation sequence; paragraph [0012]); b) an IRES sequence positioned 3' of the first ligation sequence (an effector molecule 3' of the ligation sequence, the effector molecule comprising an IRES sequence; paragraphs [0012], [0060]); c) a polynucleotide sequence of interest encoding a recombinant polypeptide and positioned 3' of the IRES sequence (an effector molecule comprising an RNA molecule encoding a peptide sequence; paragraphs [0012], [0060]); and f) a second ligation sequence positioned 3' of the polynucleotide sequence (a second ligation sequence 3' of the effector; paragraph [0012]), wherein the first and second ligation sequences are ligated together (contacting with a RNA ligase to ligate the ligation sequences; paragraphs [0012], [0017], [0060]); a linearized ribozyme-RNA construct (a linear RNA molecule comprising a ribozyme; paragraph [0012]) comprising from 5' to 3' end: a) a first twister ribozyme (a first ribozyme which is a twister ribozyme; paragraphs [0012], [0037]); b) a first ligation sequence (a first ligation sequence; paragraph [0012]); c) an IRES sequence (an effector molecule comprising an IRES sequence; paragraphs [0012], [0060]); d) a polynucleotide sequence of interest encoding a recombinant polypeptide (an effector molecule comprising an RNA molecule encoding a peptide sequence; paragraphs [0012], [0060]); g) a second ligation sequence (a second ligation sequence; paragraph [0012]); and h) a second twister ribozyme (a second ribozyme which is a twister ribozyme; paragraphs [0012], [0038]); a DNA construct (a nucleic acid sequence which is DNA; paragraphs [0012], [0074]) comprising a RNA polymerase II promoter (a 5' RNA polymerase II promoter; paragraphs [0012], [0066], [0071]) and a nucleic acid sequence encoding a ribozyme-RNA construct (a nucleic acid sequence encoding a linear RNA molecule comprising a ribozyme; paragraph [0012]), wherein the ribozyme-RNA construct

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from previous Supplemental Box-***-

comprises from 5' to 3' end: a) a first twister ribozyme (a first ribozyme which is a twister ribozyme; paragraphs [0012], [0037]); b) a first ligation sequence (a first ligation sequence; paragraph [0012]); c) an IRES sequence (an effector molecule comprising an IRES sequence; paragraphs [0012], [0060]); d) a polynucleotide sequence of interest encoding a recombinant polypeptide (an effector molecule comprising an RNA molecule encoding a peptide sequence; paragraphs [0012], [0060]); g) a second ligation sequence (a second ligation sequence; paragraph [0012]); and h) a second twister ribozyme (a second ribozyme which is a twister ribozyme; paragraphs [0012], [0038]), wherein promoter is operably linked to the nucleic acid sequence encoding the ribozyme-RNA construct (the promoter initiates transcription of a downstream nucleic acid sequence such as the RNA construct; paragraphs [0012], [0066]); a ribozyme RNA-construct(s) (a linear RNA molecule comprising a ribozyme; paragraph [0012]) comprising from 5' to 3': an optional primer region (a 5' RNA polymerase II promoter; paragraphs [0012], [0066], [0071]), an optional barcode region (optional), a first ribozyme domain (a first ribozyme which is a twister ribozyme; paragraphs [0012], [0037]), a first ligation stem domain (a first ligation sequence which forms a stem substrate; paragraph [0012], [0017]), a payload domain (an effector molecule; paragraph [0012]), a second ligation stem domain (a second ligation sequence which forms a stem substrate; paragraphs [0012], [0017]), and a second ribozyme domain (a second ribozyme which is a twister ribozyme; paragraphs [0012], [0038]); wherein the payload domain comprises from 5' to 3': an internal ribosome entry site (IRES) or a P2A peptide coding sequence (an effector molecule comprising an IRES sequence; paragraphs [0012], [0060]), a coding sequence of at least one polypeptide and/or nucleic acid of interest (an effector molecule comprising an RNA molecule encoding a peptide sequence; paragraphs [0012], [0060]); wherein the transcription of the payload domain is activated by or dependent upon the activity of the one or more ribozymes (the ribozymes express the RNA in cells; paragraphs [0016], [0044]) .

CORNELL does not disclose a 3' UTR sequence, a poly(A) sequence; d) a 3' UTR sequence positioned 3' of the IRES sequence; e) a poly(A) sequence positioned 3' of the 3' UTR.

ROCHESTER discloses a 3' UTR sequence (a 3' UTR; page 76, lines 24-26), a poly(A) sequence (a polyA tail; page 76, lines 24-26); d) a 3' UTR sequence positioned 3' of the IRES sequence (a 3' UTR positioned downstream of an open reading frame, where IRES sequences are positioned before open reading frames; page 76, lines 24-26); e) a poly(A) sequence positioned 3' of the 3' UTR (a polyA tail downstream of the 3' UTR; page 76, lines 24-26).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the constructs, as previously disclosed by CORNELL, to include a 3' UTR and poly(A) sequence, as previously disclosed by ROCHESTER, to provide the benefit of moderating the stability of mRNA encoded by the construct (ROCHESTER; page 77, lines 7-12).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the CORNELL and ROCHESTER references, unity of invention is lacking.