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(71) Applicants: **BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM** [US/US]; 210 W. 7th Street, Austin, TX 78701 (US). **EMERVAX, INC.**; 2710 Reed Street, Suite 160, Houston, TX 77051 (US).

(72) Inventors: **GARCIA-BLANCO, Mariano, A.**; c/o University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555 (US). **BUKREYEV, Alexander**; c/o University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555 (US). **MALONEY, Adam**; c/o Emervax, Inc., 2710 Reed Street, Suite 160, Houston, TX 77051 (US).

(74) Agent: **FLORES, Edwin S. et al.**; CHALKER FLORES, LLP, 14841 North Dallas Parkway, Suite 575, Dallas, TX 75254 (US).

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(54) Title: APTAMER-BASED PURIFICATION OF CIRCULAR RNAS

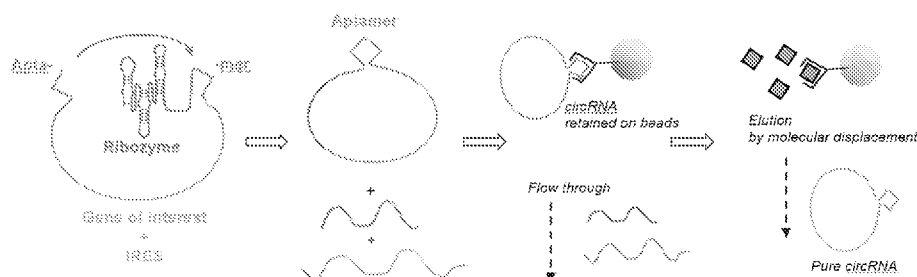


FIG. 1

(57) Abstract: Provided herein are constructs and methods for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA. In certain aspects, the circular RNAs are vaccines.

APTAMER-BASED PURIFICATION OF CIRCULAR RNAS**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional Application Serial No. 63/518,717, filed August 10, 2023, the entire contents of which are incorporated herein by reference .

STATEMENT OF FEDERALLY FUNDED RESEARCH

[0002] Not applicable.

INCORPORATION-BY-REFERENCE OF MATERIALS FILED ON COMPACT DISC

[0003] The Sequence Listing in an XML file, named as _____.xml of _ KB, created on _____, and submitted to the United States Patent and Trademark Office via Patent Center, is incorporated herein by reference.

BACKGROUND

[0004] Without limiting the scope of the disclosure, its background is described in connection with the purification of nucleic acids.

[0005] The development and deployment of mRNA vaccines, such as the COVID vaccine, made evident the importance of this vaccination modality and has stimulated technological innovations to enhance mRNA vaccine efficiency and decrease reactogenicity. An important novel modality uses circular RNAs (circRNA) that, because of their lack of termini, do not stimulate innate immune and inflammatory reactions leading to decrease reactogenicity and improved expression of desired antigens. The lack of termini provides a second advantage to circRNAs: since they cannot be degraded by exonucleases these RNAs are more stable and thus express proteins for longer periods of time.

[0006] A significant problem for the development of circRNAs as vaccine candidates and for other applications where a circRNA is desired is the efficient purification circRNAs from their linear precursors. This challenge is especially burdensome for large RNA molecules (>1000 nt).

[0007] Despite many advances, current purification methods involving chromatography and treatment with exonucleases result in low yields and/or low enrichment. This results in significant carryover of linear RNA which can greatly increase the potential toxicity of circRNA-based vaccines. There is a need for novel methods for the purification of circRNAs that can be used at scale for vaccine production or for any other uses where purity of the circRNA will confer critical advantages by minimizing adverse reactions.

SUMMARY

[0008] As embodied and broadly described herein, the present disclosure relates to a construct for isolating circRNAs comprising: a linear polyribonucleotide comprising, the two halves of an aptamer at the 5'-end and 3'-end of the polyribonucleotide, and between the two half aptamer sequences, a polyribonucleotide containing one or more sequence of interest, wherein intramolecular ligation of the 5'-end and the 3'-end of the construct forms a circRNA comprising a fully contiguous aptamer across the ligation site, and wherein the complete aptamer formed by the ligation is present within the circRNA but absent in the non-ligated form of the RNA. The presence of the complete aptamer in the circRNA provides a binding site for an aptamer ligand that is used for affinity purification of the circRNA. Embodiment of the disclosed invention include ligating the 5'-end and 3'-end of the polyribonucleotide construct using previously described methods for joining of RNA ends, including ligation catalyzed by a ribozyme, or a protein enzyme or chemically induced ligation such as click chemistry.

In one aspect, the complete aptamer binds selectively, and with K_d of $< 10 \mu\text{M}$, and with $K_d < 1, < 2, < 3, < 4, < 5, < 6, < 7, < 8, < 9$ or $< 10 \mu\text{M}$, to specific small molecule ligand, or polymeric ligand, or polypeptide ligand. Examples of aptamer ligands that could be used with the invention described herein, include, but are not limited to: streptavidin, Sephadex, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures.

[0009] In another aspect, the intramolecular ligation of the two ends of the polyribonucleotide, a reaction that generates the complete aptamer and the circRNA, is effected by a natural ribozyme or by an artificial ribozyme. In another aspect, the intramolecular ligation of the two ends of the polyribonucleotide, a reaction that generates the complete aptamer and the circRNA is effected by a natural ribozyme or an artificial ribozyme that is embedded in the polyribonucleotide sequence. In another aspect, the complete aptamer binds to an aptamer ligand that is immobilized on a substrate. In another aspect, the circRNA can direct the expression of one or more proteins when introduced into a eukaryotic cell. In another aspect, the circRNA is a vaccine. In another aspect, an aptamer ligand is used to capture the circRNA where said ligand is tethered to a substrate and the substrate is a gel, beads, agarose, polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof. In another aspect, the substrate is in a vessel, a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof. In another aspect, the circRNA is released after isolation by eluting, with a molecule selected from, but not limited to: biotin,

streptavidin, Sephadex, dextran, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, Dithiothreitol (DTT), poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures, a polypeptide or another molecule or polymer capable of displacing the original target molecule used to bind to the aptamer or RNA denaturing agents including but not limited to TRIzol, urea, phenol, EDTA, or guanidinium isothiocyanate.

[0010] As embodied and broadly described herein, an aspect of the present disclosure relates to a construct for isolating circRNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a 3' splice-site recognition sequence, a 5' splice site recognition sequence, a splicing ribozyme, a first half aptamer, a polyribonucleotide containing one or more sequence of interest, a second half of the aptamer. Incubating the construct under conditions that allow the splicing ribozyme to catalyze the splicing reaction at the splice sites embedded in the construct resulting in the joining of the two half aptamer sequences, generating a circRNA containing the fully contiguous aptamer sequence and the polyribonucleotide sequences of interest, while the splicing ribozyme is released as a byproduct of the circularization reaction and excluded from the circRNA. The complete aptamer within the circRNA, but absent in the linear form of the RNA, provides a binding site for an aptamer ligand which is used for affinity purification of the circRNA.

[0011] As embodied and broadly described herein, an aspect of the present disclosure relates to a construct for isolating circRNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, half of a ribozyme sequence, a half of an aptamer, a polyribonucleotide containing one or more sequence of interest, the second half of the aptamer, the second half ribozyme sequence. Incubating the construct under conditions that allow the splicing ribozyme sequences embedded in the construct to catalyze the splicing reaction at the splice sites embedded in the construct resulting in the joining of the two half aptamer sequences, generating a circRNA containing the fully contiguous aptamer sequence and the polyribonucleotide sequences of interest, while the splicing ribozyme is released as a byproduct of the circularization reaction and excluded from the circRNA. The complete aptamer within the circRNA, but absent in the linear form of the RNA, provides a binding site for the aptamer ligand that is used for affinity purification of the circRNA.

[0012] As embodied and broadly described herein, an aspect of the present disclosure relates to a construct for isolating circRNAs comprising: a linear polyribonucleotide comprising, in order, a first half of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second half of the aptamer, wherein a splint oligo sequence is complementary to each one of the half aptamer sequences and can facilitate ligation by a ligation enzyme such as an RNA ligase or a

DNA ligase; wherein intramolecular ligation of the 5'-end and 3'-end of the linear RNA construct generates a circRNA comprising a fully contiguous aptamer at the ligation junction, and wherein the complete aptamer within the circRNA provides a binding site for the aptamer ligand that is used for affinity purification of the circRNA.

[0013] As embodied and broadly described herein, the present disclosure relates to a construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0014] As embodied and broadly described herein, the present disclosure relates to a construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a 3' splice-site recognition sequence, a 5' splice site recognition sequence, a splicing ribozyme, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer; wherein splicing and ligation of the linear polyribonucleotide joins the first and second portions of the aptamer to generate a circular RNA comprising a complete aptamer and the polyribonucleotide containing the one or more sequence of interest; wherein the splicing ribozyme is released as a byproduct of RNA circularization and is excluded from the circular RNA; and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0015] As embodied and broadly described herein, the present disclosure relates to a construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of a ribozyme sequence, a first portion of an aptamer, a polyribonucleotide containing one or more sequences of interest, a second portion of the aptamer, and a second portion of the ribozyme; wherein splicing of the first and second portions of the ribozyme releases the ribozyme and forms a circular RNA comprising a complete aptamer at a splice junction; and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0016] As embodied and broadly described herein, the present disclosure relates to a construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer; wherein a splint oligo sequence is complementary to a portion of the first and the second aptamer to direct ligation by a ligation enzyme; and wherein ligation of

the linear polyribonucleotide forms a circular RNA comprising a complete aptamer that is fully contiguous at a ligation junction, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA. In one aspect, the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione. In another aspect, the complete aptamer binds to a molecule that is immobilized on a substrate. In another aspect, the circular RNA expresses one or more proteins. In another aspect, the circular RNA is a vaccine. In another aspect, the splicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme. In another aspect, the ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1. In another aspect, the circular RNA is released after isolation by eluting with a small molecule selected from: Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione. In another aspect, a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof. In another aspect, the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.

[0017] As embodied and broadly described herein, the present disclosure relates to a method of isolating circular RNAs from the construct described hereinabove: reacting the construct under conditions in which a circular RNA is formed comprising a complete aptamer; and isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer. In one aspect, the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione. In another aspect, the complete aptamer binds to a molecule that is immobilized on a substrate. In another aspect, the circular RNA expresses one or more proteins. In another aspect, the circular RNA is a vaccine. In another aspect, the plicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme. In another aspect, the ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1. In another aspect, a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof. In another aspect, the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof. In another aspect, the circular RNA is released after isolation by eluting with a molecule selected from: biotin, streptavidin, Sephadex, dextran, streptomycin, 4,4'-

methylenedianiline (MDA), imidazole, glutathione, Dithiothreitol (DTT), poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures, a polypeptide or another molecule or polymer capable of displacing the original target molecule used to bind to the aptamer or RNA denaturing agents including but not limited to TRIzol, urea, phenol, EDTA, or guanidinium isothiocyanate.

[0018] As embodied and broadly described herein, the present disclosure relates to a method of making and isolating a circular RNA vaccine comprising providing any of the constructs described hereinabove, wherein the one or more sequence of interest is a viral antigen; reacting the construct under conditions in which the circular RNA is formed; and isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer.

[0019] As embodied and broadly described herein, the present disclosure relates to a method of expressing a protein of interest with any of the constructs described hereinabove comprising isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer; and incubating the circular RNA under conditions in which the protein of interest is expressed from the circular RNA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] For a more complete understanding of the features and advantages of the present disclosure, reference is now made to the detailed description of the disclosure along with the accompanying figures and in which:

[0021] FIG. 1 shows an overview of an aptamer purification scheme. This figure depicts the steps used to purify circRNA using aptamer affinity purification and trans-splicing circularization.

[0022] FIG. 2 shows an EX ThermoFisher agarose gel showing circRNA after aptamer purification. The RNA in lane 1 was purified using the aptamer method in combination with RNase R treatment to produce 90% pure circRNA product shown in lanes 4 and 5. The top band is circRNA while the lower bands are linear precursor, nicked circRNA, or intron RNA.

DETAILED DESCRIPTION

[0023] While the making and using of various aspects of the present disclosure are discussed in detail below, it should be appreciated that the present disclosure provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific aspects discussed herein are merely illustrative of specific ways to make and use the disclosure and do not delimit the scope of the disclosure.

[0024] To facilitate the understanding of this disclosure, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present disclosure. Terms such as “a”, “an” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific aspects of the disclosure, but their usage does not delimit the disclosure, except as outlined in the claims.

[0025] An aptamer is a single-stranded oligonucleotide, typically 20-100 nucleotides in length, that adopts a specific three-dimensional structure capable of binding to a target molecule with high affinity and specificity. The aptamer's binding properties are conferred by its unique sequence and the resulting secondary and tertiary structures, such as hairpins, bulges, loops, and G-quadruplexes. RNA aptamers are identified through an in vitro selection process called SELEX (Systematic Evolution of Ligands by EXponential enrichment), which involves iterative rounds of binding, partitioning, and amplification from a diverse library of randomized sequences. Once selected, RNA aptamers can be chemically modified to enhance their stability against nuclease degradation for use as research tools, diagnostic agents, or therapeutic modalities that bind to proteins, peptides, small molecules, and even whole cells or tissues with antibody-like performance

[0026] An isolated and purified ligand-binding aptamer is identified, and the nucleotide base sequence determined, which, in the context of the present disclosure, is then split at opposite ends of a construct. After the end of the construct are ligated and the circRNA formed, the two halves of the aptamers are joined together, and the full aptamer is formed.

[0027] As used herein, the term “synthesizing” refers to chemical methods known in the art of generating a desired sequence of nucleotides including where the desired sequence contains regions with randomized sequence. Typically, such sequences are produced in automated DNA or RNA synthesizers programmed to the desired sequence. Such programming can include combinations of defined sequences and random nucleotides.

[0028] As used herein, a “random combinatorial oligonucleotide library” refers to many oligonucleotides of different sequences where the insertion of a given base at given place in the sequence is random.

[0029] As used herein, a “polymerase chain reaction (PCR) primer nucleotide sequence” refers to a defined sequence of nucleotides forming an oligonucleotide which is used to anneal to a homologous or closely related sequence in order form the double strand required to initiate elongation using a polymerase enzyme.

[0030] As used herein, the term “amplifying” refers to duplicating a sequence one or more times. Relative to a library, amplifying refers to *en masse* duplication of at least a majority of individual members of the library.

[0031] As used herein, the term “modified” is used herein to describe oligonucleotides or libraries in which one or more of the four constituent nucleotide bases of an oligonucleotide are analogues or esters of nucleotides normally comprising DNA or RNA backbones and wherein such modification confers increased nuclease resistance.

[0032] As used herein, amplifying “enzymatically” refers to duplication of the oligonucleotide using a nucleotide polymerase enzyme such as DNA or RNA polymerase. Where amplification employs repetitive cycles of duplication such as using the “polymerase chain reaction”, the polymerase is a heat stable polymerase such as the DNA polymerase of *Thermus aquaticus*.

[0033] As used herein, the term “contacting” in the context of target selection is defined as incubating an oligoribonucleotide library with target molecules.

[0034] As used herein, the term “aptamer ligand” refers to a molecule that selectively and specifically binds to an aptamer with high affinity.

[0035] As used herein, the term “purifying” in the context of circRNA enrichment and isolation refers to separation of circRNA/Aptamer/Ligand complexes, under conditions in which weak binding oligoribonucleotides are removed. In one embodiment circRNA/Aptamer/Ligand complexes are retained on a substrate while oligoribonucleotides non containing the full aptamer and unable to bind the aptamer ligand are washed out.

[0036] As used herein, a “ribozyme” refers to an RNA with catalytic properties that effects transesterification reactions leading to splicing at specific nucleotide positions within an RNA sequence.

[0037] As used herein, a “natural ribozyme” refers to an RNA catalyst found in nature.

[0038] As used herein, an “artificial ribozyme” refers to an RNA catalyst that is generated by *de novo* design and subsequently synthesized, or with a sequence derived from a natural ribozyme but with <30% homology to the original natural ribozyme, and yet still capable of performing the dual steps transesterification reactions leading to RNA splicing and RNA circularization. Such a synthetic derivative can be generated by rational design or in vitro evolution of a natural ribozymes in which the sequence has been randomized in select regions.

[0039] Vaccines/immunogens. The present invention includes vaccines for both active and passive immunization. Immunogenic compositions, suitable for use as a vaccine, include the

circRNAs of the present invention. The circRNAs are prepared in a manner disclosed herein. The circRNA vaccines disclosed herein are not as reactogenic as linear RNAs, that is, circRNAs do not tend to trigger an innate immune response.

[0040] In one example, the circRNAs may be used as part of a vaccine to regulate the development of Th1 or Th2 subsets in a subject or patient. In addition to *in vivo* modulation, the circRNAs may be used *ex vivo* to modify cells *in vitro* that are then administered to the subject. In one example, the circRNAs may include one or more antigens from the pathogen that is targeted. The circRNA vaccine may include more than one circRNA insert in order to target more than one antigen, or to target modifications of the same antigen.

[0041] As used herein, the term “complete aptamer” refers to the ligation of an aptamer that has been split into two portions (which may or may not be equal in length), but that upon excision of an intervening sequence (e.g., an intron) from two junctions, or upon intramolecular ligation, regenerate a contiguous polynucleotide that comprises the full aptamer sequence. Such reconstituted aptamer is then able to bind specific ligands with high affinity. Non-limiting examples of ligand that can bind and aptamer with high affinity and specificity include, streptavidin, Sephadex, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures.

[0042] The complete aptamer binds to a molecule that can be immobilized on a substrate. Non-limiting examples of substrates that can be used to bind the complete aptamer on the circRNA include, a gel, beads, agarose, polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof. In another aspect, the substrate is in a vessel, a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof. The substrate can be placed in, e.g., a vessel that can be, e.g., is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.

[0043] As used herein, the terms “identical” or percent “identity,” in the context of two or more nucleotide sequences, whether RNA or DNA sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotide residues that are the same (i.e., about 60% identity, preferably 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region). In an embodiment, the aptamer sequence about a 60% identity, or 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified nucleotide

(nt) region, which region comprises about 10 nt, 20 nt, 30 nt, 40 nt, 50 nt, 60 nt, 70 nt, 80 nt, 90 nt, 100 nt, 110 nt, 120 nt, 130 nt, 140 nt, 150 nt, 160 nt, 170 nt, 180 nt, 190 nt, 200 nt, 210 nt, 220 nt, 230 nt, 240 nt, 250 nt, 260 nt, 270 nt, 280 nt, 290 nt, 300 nt, 325 nt, 350 nt, 375 nt, 400 nt, 450 nt or about 500 nt of an aptamer sequence.

[0044] The terms “sequence identity” “percent sequence identity” or “percent identical” in the context of nucleotides, whether DNA or RNA, sequences refers to the nucleotides in the two sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the nucleotide sequence or a fragment of the sequence sequence, as desired. Suitably, a fragment is at least about 8 nt in length and may be up to about 700 nt. In an embodiment, an aptamer has a sequence identity of about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% or greater as compared to a known aptamer sequence, such as those disclosed herein. In an embodiment, an aptamer has a sequence identity of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% or greater as compared to a known aptamer sequence, such as those disclosed herein. In an embodiment, an aptamer has a sequence identity of no more than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% or greater as compared to a known aptamer sequence, such as those disclosed herein.

[0045] The term “substantial homology” or “substantial similarity,” when referring to nucleotide sequences or fragments thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleotide sequence (or its complementary strand), there is nucleotide sequence identity in at least about 95, 96, 97, 98, or 99% of the aligned sequences. Preferably, the homology is over full-length sequence, or a nucleotide sequence, for instance an RNA or DNA sequence.

[0046] In an embodiment the period of time wherein the aptamer is used to purify the circRNA is from about 1 minute, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hour, 3 hour, 4 hour, 5 hour, 6 hour, 7 hour, 8 hour, 9 hour, 10 hour, 11 hour, 12 hour, 13 hour 14 hour, 15 hour, 16 hour, 17 hour, 18 hour, 19 hour, 20 hour, 21 hour, 22 hour, 23 hour or 24 hours.

[0047] In an embodiment, use of an aptamer to purify a circRNA increases the amount of circRNA obtained by about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 31%, about

32%, about 33%, about 34%, about 35%, about 36%, about 37%, about 38%, about 39%, about 40%, about 41%, about 42%, about 43%, about 44%, about 45%, about 46%, about 47%, about 48%, about 49%, about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, about 60%, about 61%, about 62%, about 63%, about 64%, about 65%, about 66%, about 67%, about 68%, about 69%, about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% as compared to a circRNA that is not purified using an aptamer of the invention disclosed herein.

[0048] In an embodiment, use of an aptamer to purify a circRNA increases the amount of circRNA obtained by at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 21%, at least 22%, at least 23%, at least 24%, at least 25%, at least 26%, at least 27%, at least 28%, at least 29%, at least 30%, at least 31%, at least 32%, at least 33%, at least 34%, at least 35%, at least 36%, at least 37%, at least 38%, at least 39%, at least 40%, at least 41%, at least 42%, at least 43%, at least 44%, at least 45%, at least 46%, at least 47%, at least 48%, at least 49%, at least 50%, at least 51%, at least 52%, at least 53%, at least 54%, at least 55%, at least 56%, at least 57%, at least 58%, at least 59%, at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% as compared to a circRNA that is not purified using an aptamer of the invention disclosed herein.

[0049] In an embodiment, purification of a circRNA using an aptamer increases the half-life of the circRNA in a formulation by 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, or more.

[0050] In a further embodiment, purification of a circRNA using an aptamer increases the half-life of the circRNA in a nanoparticle by 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8

days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, or more.

[0051] In a further embodiment, purification of a circRNA using an aptamer increases the half-life of the circRNA in a cell following administration of the circRNA to an individual by 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, or more.

[0052] The circRNA that comprises the complete aptamer can then be released from the substrate after isolation by eluting with a molecule selected from, but not limited to: biotin, streptavidin, Sephadex, dextran, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, Dithiothreitol (DTT), poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures, a polypeptide or another molecule or polymer capable of displacing the original target molecule used to bind to the aptamer or RNA denaturing agents including but not limited to TRIzol, urea, phenol, EDTA, or guanidinium isothiocyanate..

[0053] The present invention solves the problem with the isolation of circRNA since it uses affinity chromatography specific for the circRNA over its linear precursor. Furthermore, the method can be generalizable to all circRNAs created by reverse splicing reactions or other ligation methods and should be independent of sequence and length of the circRNA.

[0054] The method described herein uses as an example a permuted group I intron construct that upon splicing (reverse splicing) creates a circRNA that contains an open reading frame (ORF) and necessary elements to mediate translation (e.g., internal ribosome entry site, IRES). However, the method is also compatible with alternative circularization methods (e.g., oligo splint-mediated ligation).

[0055] Development of aptamer purification of circRNA – Use of trans-splicing to incorporate aptamer sequences.

[0056] To develop aptamer purification of circRNA, several technical challenges had to be overcome. One of the primary issues preventing the use of aptamers in circRNA purification, is that any full aptamer sequence transcribed in a precursor construct RNA will be contained in the linear precursor, and the circRNA, which prevents the aptamer from being used for selectively purify the circRNA. To overcome this problem, a strategy was devised to split the aptamer into

two half sequences in the linear precursor RNA so that a full aptamer sequence with ligand-binding capacity would only form in the circRNA after the circularization reaction. This strategy gives the circRNA aptamer high affinity to bind a ligand, but the half sequences in the linear RNAs would not be able to bind the ligand.

[0057] One embodiment of the method disclosed herein uses a modified version of a self-splicing Group I intron, which can splice together two RNAs when specific guide sequences are used. It was found that by making certain sequence changes in the guide sequences, an aptamer could be placed at the splice site of a circRNA (FIG. 1), allowing the original aptamer strategy to be utilized in the context of trans splicing. This method was tested and it was found that the aptamer could be used in combination with other methods to obtain 90% circRNA (FIG. 2), which is higher than can be achieved with conventional purification methods. This innovative method was used to purify circRNAs with high purity and minimal chemical treatment.

[0058] FIG. 1 shows an overview of an aptamer purification scheme. This figure depicts the steps used to purify circRNA using aptamer affinity purification and trans-splicing circularization. The gene of interest and the IRES are formed and flanked with a portion of the aptamer on each end and then ribozyme at one of the ends of the RNA. The ribozyme is then cleaved out and the complete aptamer is formed when the RNA is circularized. Next, the aptamer containing circRNA is captured, in this example, with beads on which an aptamer ligand is bound, and the ribozyme and other RNA contaminants are separated from the beads. Finally, the purified circRNA is eluted from the beads, e.g., by molecular displacement.

[0059] FIG. 2 shows an EX agarose gel (ThermoFisher, USA) showing circRNA after aptamer purification. The RNA in lane 1 was purified using the aptamer method disclosed herein in combination with RNase R treatment to produce the 90% circRNA product shown in lanes 4 and 5. The top band is circRNA where lower bands are linear precursor, nicked circRNA, or intron RNA. Non-limiting examples of aptamers that can be used with the present invention include the ones listed in TABLE 1 in which Aptamer names, sequences and specific ligands are shown.

[0060] TABLE 1. Aptamer names, sequences and specific ligands

Aptamer name	Aptamer Ligand	Aptamer sequence (5'-3')	SEQ ID NO
S1m	Streptavidin	ATGCGGCCGCGACCAGAATCATGCAA GTGCGTAAGAT#AGTCG CGGGTCGGCGGCCGCAT	1
S1	Streptavidin	ACCGACCAGAATCATGCAAGTGCGTAA GATAGTCGCGGGCCGGG	2
D8	Sephadex G-100 and G-200	GTCCGAGTAATTTACGTTTTGATACGG TTGCGGAACTTGC	3

Tobramycin	Tobramycin-modified beads	CCGAGTCGTGCTCACATCGATTTGGAGCGATATGATTCC	4
Streptomycin	Streptomycin-coupled Sepharose	GGATCGCATTTGGACTTCTGCCCAGGGTGGCACCACGGTCGGATCC	5
PP7	PP7 coat protein	TAAGGAGTTTATATGGAAACCCTTA	6
MS2	MS2 coat protein	CGTACACCATCAGGGTACGTTTTTTCGTACACCATCAGGGTACG	7
Csy4 (H29 A)	Csy4 nuclease	GTTCAC TGCCGTATAGGCAG	8
MDA	MDA (4,4'-methylenedianiline)	CUGCGAUCAGGGGUAAAUUUCCGCGCAGGCUCCACGCCGC	9

The # in the S1m aptamer sequence shows the position at which the aptamer was split into half aptamer sequences in the example described in the present disclosure.

[0061] Additional small molecule binding aptamers can be those that mimic: anthracyclines, tetracyclines, benzoylmethylecgonine, thalidomide, diclofenac, somatropin, insulin, vasopressin, gonadoliberin, estradiol, aflatoxin, anatox, atrazine, benzylguanine, brevetoxin-2, bromacil, Cadmium, danfloxacin, fumonisin, kanamycin, ketamine, lysergamine, malathion, methamphetamine, n-methyl mesoporphyrin, ochratoxin, ochratoxin, okadic acid, organophosphorus pesticides, oxytetracycline, palladium, polychlorinated buphneyls, progesterone, T-2 toxin, tebuconazole, inabenefide, mefenacet, torabmycin or zealalenone, as taught by, Ruscito and DeRosa, Small-Molecule Binding Aptamers: Selection Strategies, Characterization, and Applications, Front. Chem., 10 May 2016, Sec. Chemical Biology, Volume 4 - 2016 | doi.org/10.3389/fchem.2016.00014, and Strehlitz, et al., "Aptamers for pharmaceuticals and their application in environmental analytics", Bioanal Rev. 2012; 4(1): 1–30, 2011 Dec 17. doi: 10.1007/s12566-011-0026-1, sequence(s) incorporated herein by reference.

[0062] Example 1. A construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0063] Example 2. A construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a 3' splice-site recognition sequence, a 5' splice site recognition sequence, a splicing ribozyme, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer; wherein splicing and ligation of the linear polyribonucleotide joins the first and second portions of the aptamer to generate a circular RNA comprising a complete aptamer and the

polyribonucleotide containing the one or more sequence of interest; wherein the splicing ribozyme is released as a byproduct of RNA circularization and is excluded from the circular RNA; and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0064] Example 3. A construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of a ribozyme sequence, a first portion of an aptamer, a polyribonucleotide containing one or more sequences of interest, a second portion of the aptamer, and a second portion of the ribozyme; wherein splicing of the first and second portions of the ribozyme releases the ribozyme and forms a circular RNA comprising a complete aptamer at a splice junction; and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0065] Example 4. A construct for isolating circular RNAs comprising: a linear polyribonucleotide comprising, in order, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer; wherein a splint oligo sequence is complementary to a portion of the first and the second aptamer to direct ligation by a ligation enzyme; and wherein ligation of the linear polyribonucleotide forms a circular RNA comprising a complete aptamer that is fully contiguous at a ligation junction, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

[0066] Example 5. The construct of any one of examples 1 to 4, wherein the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.

[0067] Example 6. The construct of any one of examples 1 to 5, wherein the complete aptamer binds to a molecule that is immobilized on a substrate.

[0068] Example 7. The construct of any one of examples 1 to 6, wherein the circular RNA expresses one or more proteins.

[0069] Example 8. The construct of any one of examples 1 to 7, wherein the circular RNA is a vaccine.

[0070] Example 9. The construct of any one of examples 1 to 3, wherein splicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme.

[0071] Example 10. The construct of example 4, wherein ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1.

[0072] Example 11. The construct of any one of examples 1 to 10, wherein a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof.

[0073] Example 12. The construct of example 11, wherein the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.

[0074] Example 13. The construct of any one of examples 1 to 11, wherein the circular RNA is released after isolation by eluting with a small molecule selected from: Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.

[0075] Example 14. A method of isolating circular RNAs from the construct of any one of claim 1 to 4 comprising: reacting the construct under conditions in which a circular RNA is formed comprising a complete aptamer; and isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer.

[0076] Example 15. The method of example 14, wherein the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.

[0077] Example 16. The method of example 14 or example claim 15, wherein the complete aptamer binds to a molecule that is immobilized on a substrate.

[0078] Example 17. The method of any one of examples 14 to 16, wherein the circular RNA expresses one or more proteins.

[0079] Example 18. The method of any one of examples 14 to 17, wherein the circular RNA is a vaccine.

[0080] Example 19. The method of any one of examples 14 to 18, wherein splicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme.

[0081] Example 20. The method of any one of examples 14 to 19, wherein ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1.

[0082] Example 21. The method of any one of examples 14 to 20, wherein a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof.

[0083] Example 22. The method of any one of examples 14 to 21, wherein the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.

[0084] Example 23. The method of any one of examples 14 to 22, wherein the circular RNA is released after isolation by eluting with a molecule selected from: biotin, streptavidin, Sephadex, dextran, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, Dithiothreitol (DTT), poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures, a polypeptide or another molecule or polymer capable of displacing the original target molecule used to bind to the aptamer or RNA denaturing agents including but not limited to TRIzol, urea, phenol, EDTA, or guanidinium isothiocyanate..

[0085] Example 24. A method of making and isolating a circular RNA vaccine comprising: providing a construct of any one of examples 1 to 4, wherein the one or more sequence of interest is a viral antigen; reacting the construct under conditions in which the circular RNA is formed; and isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer.

[0086] Example 25. A method of expressing a protein of interest the construct of any one of examples 1 to 4 comprising: isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer; and incubating the circular RNA under conditions in which the protein of interest is expressed from the circular RNA.

[0087] It is contemplated that any aspects of the disclosure discussed in this specification can be implemented with respect to any method, kit, reagent, or composition of the disclosure, and vice versa. Furthermore, compositions of the disclosure can be used to achieve methods of the disclosure.

[0088] It will be understood that particular aspects described herein are shown by way of illustration and not as limitations of the disclosure. The principal features of this disclosure can be employed in various aspects without departing from the scope of the disclosure. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this disclosure and are covered by the claims.

[0089] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this disclosure pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0090] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0091] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. In aspects of any of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. As used herein, the phrase “consisting essentially of” requires the specified integer(s) or steps as well as those that do not materially affect the character or function of the claimed invention. As used herein, the term “consisting” is used to indicate the presence of the recited integer (e.g., a feature, an element, a characteristic, a property, a method/process step or a limitation) or group of integers (e.g., feature(s), element(s), characteristic(s), propertie(s), method/process steps or limitation(s)) only.

[0092] The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0093] As used herein, words of approximation such as, without limitation, “about”, “substantial” or “substantially” refers to a condition that when so modified is understood to not necessarily be absolute or perfect but would be considered close enough to those of ordinary skill in the art to warrant designating the condition as being present. The extent to which the description may vary will depend on how great a change can be instituted and still have one of ordinary skilled in the art recognize the modified feature as still having the required characteristics and capabilities of the unmodified feature. In general, but subject to the preceding discussion, a numerical value herein that is modified by a word of approximation such as “about” may vary from the stated value by at least ± 1 , 2, 3, 4, 5, 6, 7, 10, 12 or 15%.

[0094] Additionally, the section headings herein are provided for consistency with the suggestions under 37 CFR 1.77 or otherwise to provide organizational cues. These headings shall not limit or characterize the disclosure(s) set out in any claims that may issue from this disclosure. Specifically, and by way of example, although the headings refer to a “Field of Invention,” such claims should not be limited by the language under this heading to describe the so-called technical field. Further, a description of technology in the “Background of the Invention” section is not to be construed as an admission that technology is prior art to any disclosure(s) in this disclosure. Neither is the “Summary” to be considered a characterization of the disclosure(s) set forth in issued claims. Furthermore, any reference in this disclosure to “invention” in the singular should not be used to argue that there is only a single point of novelty in this disclosure. Multiple inventions may be set forth according to the limitations of the multiple claims issuing from this disclosure, and such claims accordingly define the invention(s), and their equivalents, that are protected thereby. In all instances, the scope of such claims shall be considered on their own merits in light of this disclosure but should not be constrained by the headings set forth herein.

[0095] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of preferred aspects, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the disclosure. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the disclosure as defined by the appended claims.

[0096] To aid the Patent Office, and any readers of any patent issued on this application in interpreting the claims appended hereto, applicants wish to note that they do not intend any of the appended claims to invoke paragraph 6 of 35 U.S.C. § 112, U.S.C. § 112 paragraph (f), or

equivalent, as it exists on the date of filing hereof unless the words “means for” or “step for” are explicitly used in the particular claim.

[0097] For each of the claims, each dependent claim can depend both from the independent claim and from each of the prior dependent claims for each and every claim so long as the prior claim provides a proper antecedent basis for a claim term or element.

WHAT IS CLAIMED IS:

1. A construct for isolating circular RNAs comprising:
a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and
wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.
2. A construct for isolating circular RNAs comprising:
a linear polyribonucleotide comprising, in order from 5' to 3' direction, a 3' splice-site recognition sequence, a 5' splice site recognition sequence, a splicing ribozyme, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer;
wherein splicing and ligation of the linear polyribonucleotide joins the first and second portions of the aptamer to generate a circular RNA comprising a complete aptamer and the polyribonucleotide containing the one or more sequence of interest;
wherein the splicing ribozyme is released as a byproduct of RNA circularization and is excluded from the circular RNA; and
wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.
3. A construct for isolating circular RNAs comprising:
a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of a ribozyme sequence, a first portion of an aptamer, a polyribonucleotide containing one or more sequences of interest, a second portion of the aptamer, and a second portion of the ribozyme;
wherein splicing of the first and second portions of the ribozyme releases the ribozyme and forms a circular RNA comprising a complete aptamer at a splice junction; and
wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.
4. A construct for isolating circular RNAs comprising:
a linear polyribonucleotide comprising, in order, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, and a second portion of the aptamer;
wherein a splint oligo sequence is complementary to a portion of the first and the second aptamer to direct ligation by a ligation enzyme; and

wherein ligation of the linear polyribonucleotide forms a circular RNA comprising a complete aptamer that is fully contiguous at a ligation junction, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

5. The construct of any one of claims 1 to 4, wherein the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.
6. The construct of any one of claims 1 to 5, wherein the complete aptamer binds to a molecule that is immobilized on a substrate.
7. The construct of any one of claims 1 to 6, wherein the circular RNA expresses one or more proteins.
8. The construct of any one of claims 1 to 7, wherein the circular RNA is a vaccine.
9. The construct of any one of claims 1 to 3, wherein splicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme.
10. The construct of claim 4, wherein ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1.
11. The construct of any one of claims 1 to 10, wherein the circular RNA is released after isolation by eluting with a small molecule selected from: Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.
12. The construct of any one of claims 1 to 11, wherein a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof.
13. The construct of claim 12, wherein the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.
14. A method of isolating circular RNAs from the construct of any one of claim 1 to 4 comprising:

reacting the construct under conditions in which a circular RNA is formed comprising a complete aptamer; and

isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer.

15. The method of claim 14, wherein the complete aptamer binds Streptavidin, Sephadex, Streptomycin, MDA (4,4'-methylenedianiline), Imidazole, His tag, MS2, PP7, Csy4, or Glutathione.

16. The method of claim 14 or claim 15, wherein the complete aptamer binds to a molecule that is immobilized on a substrate.
17. The method of any one of claims 14 to 16, wherein the circular RNA expresses one or more proteins.
18. The method of any one of claims 14 to 17, wherein the circular RNA is a vaccine.
19. The method of any one of claims 14 to 18, wherein splicing is trans-splicing with a trans-splicing ribozyme or group I intron ribozyme.
20. The method of any one of claims 14 to 19, wherein ligating is with a ligase selected from T4 RNA Ligase 1, T4 RNA Ligase 2, T4 RNA Ligase 2 truncated, RtcB Ligase, TS2126 RNA Ligase 1.
21. The method of any one of claims 14 to 20, wherein a substrate is used to capture the circular RNA that is a gel, column, bead, agarose, polymer, a polyacrylamide, a UV-curable polymer, a PEG based hydrogel, emulsion bead-nucleic acid conjugate, a polymer bead-nucleic acid conjugate, or a combination thereof.
22. The method of any one of claims 14 to 21, wherein the substrate is in a vessel is a tube, a syringe, a micro-container, a spin column, a flow cell, or a combination thereof.
23. The method of any one of claims 14 to 22, wherein the circular RNA is released after isolation by eluting with a molecule selected from: biotin, streptavidin, Sephadex, dextran, streptomycin, 4,4'-methylenedianiline (MDA), imidazole, glutathione, Dithiothreitol (DTT), poly-histidine, bacteriophage coat protein MS2, bacteriophage coat protein PP7, Csy4 protein, or other protein or bacteriophage coat protein that bind to RNA structures, a polypeptide or another molecule or polymer capable of displacing the original target molecule used to bind to the aptamer or RNA denaturing agents including but not limited to TRIzol, urea, phenol, EDTA, or guanidinium isothiocyanate.
24. A method of making and isolating a circular RNA vaccine comprising:
 - providing a construct of any one of claims 1 to 4, wherein the one or more sequence of interest is a viral antigen;
 - reacting the construct under conditions in which the circular RNA is formed; and
 - isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer.
25. A method of expressing a protein of interest with the construct of any one of claim 1 to 4 comprising:
 - isolating the circular RNA by capturing the aptamer with a ligand that specifically binds the aptamer; and

incubating the circular RNA under conditions in which the protein of interest is expressed from the circular RNA.

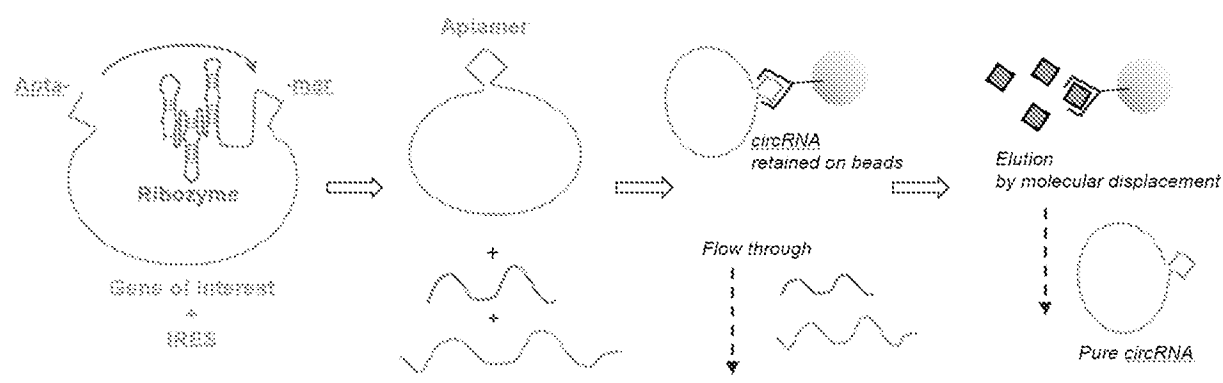


FIG. 1

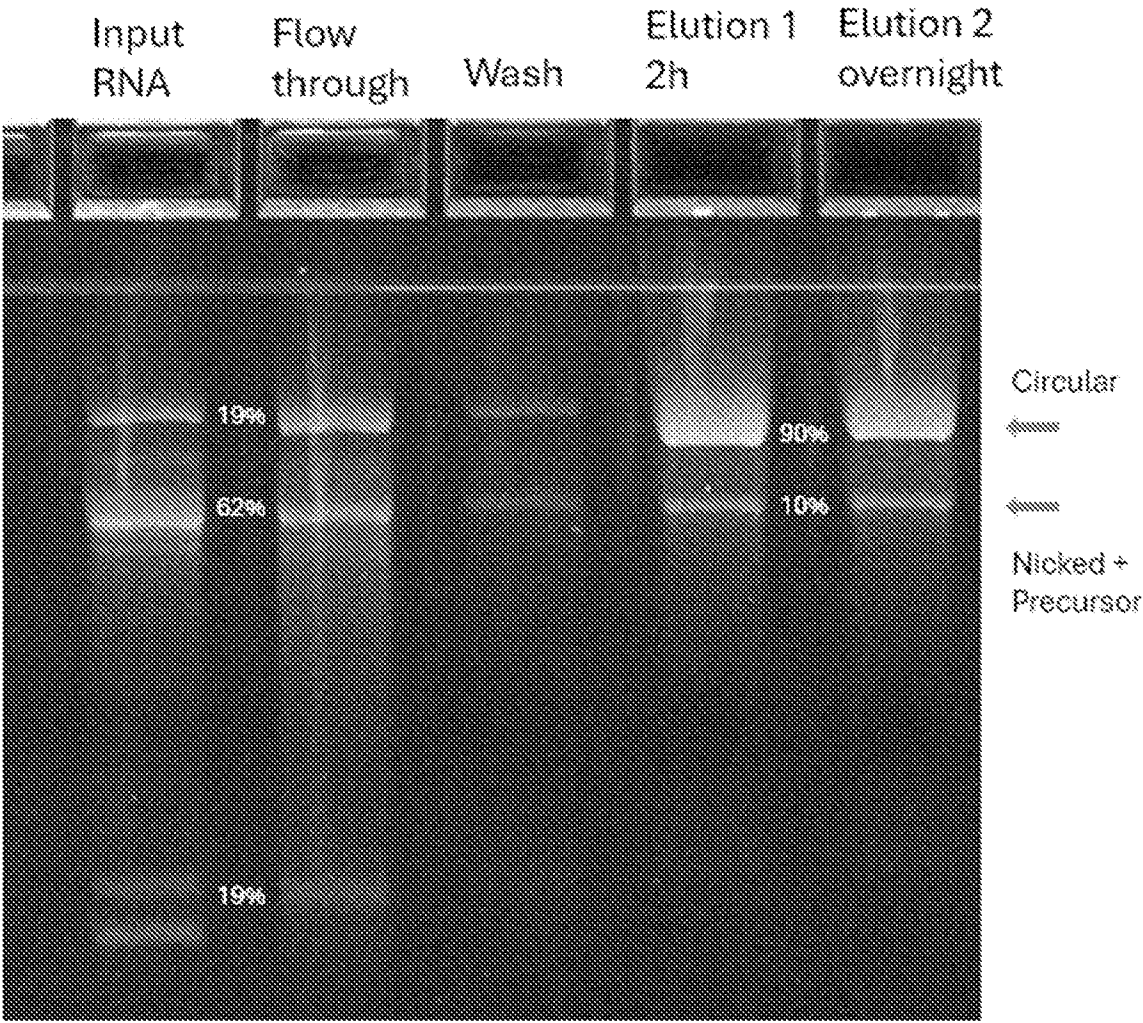


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/041775

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61K 31/713** (2024.01); *C12N 15/11* (2024.01); *A61K 39/12* (2024.01); *A61P 31/14* (2024.01)CPC: **A61K 31/713**; *A61K 2039/53*; *A61K 2039/575*; *C12N 2760/12134*

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 data base consulted during the international search (name of data base 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 (27.12.2018) para [0020], para [0026], para [0041], para [0051], para [0054], para [0099], para [0143], para [0154], para [0164], para [0168], Figure 4A, Figure 10A, Claim 1, Claim 10.	1-5, 9-10
Y	WO 2023/031856 A1 (SANOFI) 09 March 2023 (09.03.2023) para [0006], para [0007], para [0098] - para [0100], Title.	1-5, 9-10
Y	WO 2021/158964 A1 (UNIVERSITY OF ROCHESTER) 12 August 2021 (12.08.2021) Abstract, Claim 27, Claim 29, Claim 31	1-5, 9-10
Y	US 10,041,107 B2 (SOMAGENICS, INC.) 07 August 2018 (07.08.2018) col 11, ln 52 - col 11, ln 64.	4, 5/4, 10
Y	SASSOLAS et al. Homogenous assays using aptamers. Analyst, 2011, Vol 136, No 2, pg 257-274. pg 257-274. p. 258, col 2, para 3.	1-5, 9-10



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

11 October 2024 (11.10.2024)

Date of mailing of the international search report

17 December 2024 (17.12.2024)

Name and mailing address of the ISA/US

COMMISSIONER FOR PATENTS
MAIL STOP PCT, ATTN: ISA/US
P.O. Box 1450
Alexandria, VA 22313-1450
UNITED STATES OF AMERICA

Authorized officer

KARI RODRIQUEZ

Facsimile No. 571-273-8300

Telephone No. PCT Help Desk: 571-272-4300

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.: **6-8, 11-13, 16-23**
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:

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.

Group I, claims 1-5, 9-10, directed to a construct for isolating circular RNAs.

Group II, claims 14-15, 24-25, directed to a method of isolating circular RNAs, making and isolating a circular RNA vaccine and expressing a protein of interest.

The inventions listed as Groups I-II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical features:

Group I has the special technical feature of a construct for isolating circular RNAs, that is not required by Group II.

Group II has the special technical feature of a method of isolating circular RNAs, making and isolating a circular RNA vaccine and expressing a protein of interest, that is not required by Group I.

Common Technical Features:

Groups I-II share the common technical feature of:

a construct for isolating circular RNAs comprising:

a linear polyribonucleotide comprising, in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is obvious over WO 2018/237372 A1 to Cornell University (hereinafter 'Cornell'), in view of WO 2023/031856 A1 to Sanofi, in further view of the article entitled "Homogenous assays using aptamers" to Sassolas et al. (Analyst, 2011, Vol. 136, pg 257-274) (hereinafter 'Sassolas').

Cornell teaches a linear polyribonucleotide comprising a polyribonucleotide containing one or more sequence of interest, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer (Claim 1 - "A RNA molecule comprising a first ribozyme; a first ligation

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

sequence; an effector molecule; a second ligation sequence; and a second ribozyme."; Claim 10 - "The RNA molecule of claim 1, wherein the effector molecule is selected from the group consisting of... an aptamer..."; para [0020] - "FIG. 4A is an illustration showing a construct design for autocatalytically processed circRNA expression. Self-cleaving ribozymes flank the ends of the circularized insert that contains an RNA aptamer sequence. The ligation sequences, which remain on either side after cleavage... FIG. 4C shows fully-cleaved products of transcription in vitro contain appropriate ends for circularization by the endogenous ligase, RtcB..."; Figure 4A; Note, Figure 4A, depicts a linear that becomes circularized after the 5' and 3' ends are ligated together.), and

purification of the circular RNA (para [0099] - "... the RNA molecule of the present invention may be isolated..."), but does not explicitly teach a construct for isolating circular RNAs comprising: wherein the linear polyribonucleotide comprises in order from 5' to 3' direction, a first portion of an aptamer, a polyribonucleotide containing one or more sequence of interest, a second portion of the aptamer, wherein intramolecular ligation of a 5'-end and a 3'-end of the construct forms a circular RNA comprising a complete aptamer that is fully contiguous across a ligation site, and wherein the complete aptamer within the circular RNA provides a binding site for affinity purification of the circular RNA.

Sanofi teaches the use of split RNA aptamers comprising streptavidin aptamers for use with streptavidin-based affinity isolation (Title - "Compositions and methods for RNA affinity purification"; para [0006] - "... mRNA comprises at least one RNA aptamer..."; para [0007] - "RNA aptamer is embedded in an RNA scaffold..."; para [0098] - "... the RNA aptamer is a split aptamer. Split aptamers are analogs to split-protein systems... and rely on two or more short nucleic acid strands that assemble into a higher order structure..."; para [0100] - "... RNA aptamer binds to a ligand. In some embodiments the ligand is utilized in an affinity purification system. In some embodiments, the affinity ligand comprises... streptavidin...").

Sassolas teaches aptazymes comprise an aptamer and a ribozyme (p. 258, col 2, para 3 - "Aptazymes are constituted by an aptamer sequence connected to a ribozyme..."). Since Cornell teaches that circularized RNA is more resistant to degradation (para [0009] - "... various groups have described improved eukaryotic expression by making circular RNA lacking 5' or 3' ends. Such RNA molecules are resistant to eukaryotic exoribonucleases and are therefore degraded more slowly by cellular machinery."), it would have been obvious to one of ordinary skill in the art that the split streptavidin-aptamer taught by Sanofi could be circularized by the method of circularizing RNA comprising an aptamer taught by Cornell, so that the streptavidin-aptamer containing RNA persists better and/or longer in environments comprising nucleases. Since Cornell teaches that RNA aptamers are used as affinity reagents that can bind a target such as streptavidin (para [0051] - "... 'aptamer' refers to a nucleic acid molecule that binds with high affinity and specificity to a target."; para [0168] - "... RNA aptamers are mainly used as affinity reagents..."; para [0054] - "... aptamer that binds a target molecule... Suitable target molecules of interest include... streptavidin..."), it would have been obvious to one of ordinary skill in the art that the circularized RNA comprising a streptavidin-binding aptamer taught by Cornell could be isolated by the streptavidin-based affinity purification system taught by Sanofi, so that the circularized RNA can be effectively purified. Since Cornell teaches a linear RNA that comprises a 5' ribozyme, a first ligation sequence, an effector molecule, a second ligation sequence, and 3' ribozyme (Claim 1; Claim 10), it would have been obvious to one of ordinary skill in the art that the ribozyme segments next to a ligation sequence flanking the linear RNA taught by Cornell are aptazyme segments comprising a ribozyme and an aptamer taught by Sassolas, wherein the flanking aptazymes segments comprise a portion of the split streptavidin-aptamer made obvious by Cornell and Sanofia and are arranged such that the aptamer portions are split between the two flanking aptamer portions of the aptazymes with the ligation sequences in-between the ribozyme and the partial aptamer segment, so that the linear RNA construct comprises in order from 5' to 3' direction, a first portion of a streptavidin-aptazyme, a polyribonucleotide containing one or more sequence of interest, and a second portion of the streptavidin-aptazyme that when is circularized it generates a functional streptavidin-aptamer contiguous across the ligation site that provides a site for the circularized RNA to be bound streptavidin such as in a streptavidin-based affinity purification system.

As the technical features were known in the art, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/041775

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

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.: **1-5, 9-10**

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.