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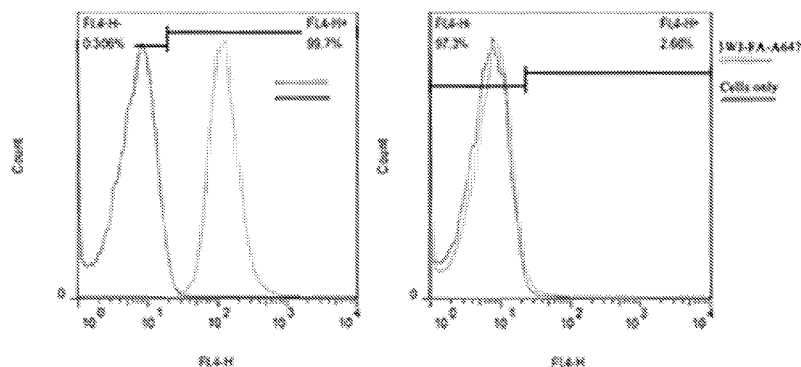


FIG. 2

(57) Abstract: The presently-disclosed subject matter relates to RNA-based composition and method to treat gastric cancer in a sub-
ject. More particularly, the presently disclosed subject matter relates to a RNA nanostructure and composition containing a multiple
branched RNA nanoparticle, a gastric cancer targeting module, and an effective amount of a therapeutic agent. Further, the presently
disclosed subject matter relates to a method of using the RNA nanoparticle composition to treat gastric cancer in a subject having or
at risk of having gastric cancer.

RNA NANOPARTICLE FOR TREATMENT OF GASTRIC CANCER

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RELATED APPLICATIONS

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This application claims the benefit of U.S. Provisional Patent Application No. 62/130,527, filed March 9, 2015, the entire disclosure of which is hereby incorporated by reference in its entirety.

GOVERNMENT INTEREST

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This invention was made with government support under CA151648 and EB012135 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

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The presently-disclosed subject matter relates to RNA nanostructure molecule and method to treat gastric cancer in a subject. More particularly, the presently disclosed subject matter relates to a RNA nanostructure and composition containing a multiple branched RNA nanoparticle, a gastric cancer targeting module, and an effective amount of a therapeutic agent. Further, the presently disclosed subject matter relates to a method of using the RNA nanoparticle composition to treat gastric cancer in a subject having or at risk of having gastric cancer.

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INTRODUCTION

Gastric cancer is the one of the most common cancers, and the second leading cause of cancer-related death in the world (1,2). The effective treatment remains challenging primarily because most diagnosed patients present advanced stages of the disease. It remains

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very difficult to cure effectively, primarily because most patients present advanced stages of the diseases. Up to date, surgery, radiation and chemotherapies are generally very effective for early and *in situ* gastric cancers, but advanced and metastatic cases do not respond to chemo- or radiation therapies (3-5) and resistance to chemotherapy-induced apoptosis is a major cause for the failure of conventional therapies (6-8). The current prognosis of gastric cancer is very poor with 5-year survivals of less than 24% (9). Therefore, developing novel strategies and approaches for recognizing, tracking and killing gastric cancer cells in early disease stage is urgently demand.

Previously, multifunctional nanoprobes has been studies to realize targeted imaging and simultaneous therapy of gastric cancer (10,11). The previous studies show that subcutaneous and *in situ* gastric cancer tissues with 5 mm in diameter could be recognized and treated using multifunctional nanoprobes such as BRCA1(breast cancer associated antigen 1, AF208045) monoclonal antibody-conjugated fluorescent magnetic nanoparticles (12), Her2 monoclonal antibody-conjugated RNase-A-associated CdTe quantum dots (13), folic acid conjugated upper conversion nanoparticles (14), Folate conjugated gold nanorods (15), ce6-conjugated carbon dots (16), ce6-conjugated Au nanoclusters(Au NCs) (17,18). However, clinical translation of these prepared nanoprobes still presents great challenge because all these prepared nanoprobes are not only distributed to the site of gastric cancer, but also partially accumulated in other organs. Therefore, the development of safe and effective nanoprobes for *in vivo* targeted delivery, imaging and simultaneous therapy of early gastric cancer is desirable.

SUMMARY

The presently-disclosed subject matter meets some or all of the above-identified needs, as will become evident to those of ordinary skill in the art after a study of information provided in this document.

This Summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

The presently disclosed subject matter in some embodiments relates to an artificial RNA nanostructure molecule. The RNA nanostructure molecule includes a multiple branched RNA junction motif comprising at least one RNA oligonucleotides, and a gastric cancer targeting module conjugated to the RNA junction motif. In some embodiments, the RNA nanostructure further includes at least one bioactive agent. In some embodiments, the RNA nanostructure binds to gastric cancer cells. A non-limiting example of the bioactive agent is a therapeutic agent. In some embodiments, the bioactive agent includes drugs, fluorescent dyes, or chemicals. In some embodiments, the bioactive agent includes an imaging module. Non-limiting examples of the imaging module is fluorescent dye, including a non-limiting example Alexa647. In some embodiments, the diameter of the molecule is at least about 40 nm or less. In some embodiments, the molecule has zeta potential ranging from about -150 mV to about 150 mV. In some embodiments, the molecule has zeta potential ranging from about -125 mv to about 75 mV. In some embodiments, the molecule is substantially stable in a pH value ranges from about 2 to about 13.

In some embodiments, the nanostructure comprises at least one chemical modification at 2' position. Non-limiting examples of the chemical modification includes 2'Fluoro, 2'Amine, and 2'O-Methyl. In some embodiments, the multiple branched RNA comprises sequence 5' -CCACAUAAGGGCCACUAuuCCCACAUAACUUUGUUGAUCC-3'. In some embodiments, the multiple branched RNA includes sequence 5'-UAGUGGGCCCUUUAUGUGG-3'.

In some embodiments, the multiple branched RNA junction motif is a three-branched RNA junction motif. In some embodiments of the present disclosure, the RNA molecules form dimers, trimers, hexamers, and patterned superstructures. Further, In some embodiments, a branch of the three-branched RNA junction motif includes an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); or a c3WJ RNA module (SEQ ID NO: 3). In one embodiment, the three-branched RNA junction motif comprises an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); and a c3WJ RNA module (SEQ ID NO: 3). The SEQ ID NO: 1 includes nucleotide sequence 5'-UUG CCA UGU GUA UGU GGG-3', the SEQ ID NO: 2 comprises nucleotide sequence 5'-CCC ACA UAC UUU GUU GAUCC-3', and the SEQ ID NO: 3 comprises nucleotide sequence 5'-GGA UCA AUC AUG GCA A-3'.

In some embodiments, the gastric cancer targeting module includes a ligand that binds to at least one gastric cancer cell surface marker. In some embodiments, the ligand binds to a folate receptor, an epidermal growth factor receptor 2 (ErbB-2/HER2), an epidermal growth

factor receptor (EGFR), a HER2 or a combination thereof. In some embodiments, the ligand is an aptamer. Non-limiting examples of the aptamer are aptamers binding to EGFR, PDGFR, folate receptor, or a combination thereof. In one example, the ligand is a EGFR targeting aptamer. In one example, the ligand has sequence 5'- G CCU UAG UAA CGU GCU UUG
 5 AUG UCG AUU CGA CAG GAG GC -3'. In some embodiments, the targeting module is a folate. Non-limiting examples of folate are folic acid, 5 -methyltetrahydro folate, 5-formyltetrahydrofolate, dihydrofolate, tetrahydrofolate, or a combination thereof.

In some embodiments, the bioactive agent is a drug, a fluorescent dye, a chemical, or a combination thereof. In some embodiments, wherein the bioactive agent is a siRNA, a
 10 miRNA, an anti-mRNA, a ribozyme RNAs, or an antisense RNAs. In some embodiments, the bioactive agent is directed a gastric cancer marker. In some embodiments, the bioactive agent is a siRNA sequence. Non-limiting examples of the siRNA are siRNA is directed to Survivin, Bcl-2, XIAP, BCL-XL, or BRCA1. Non-limiting examples of the siRNA include siRNA sequence 5' GGACCACCGCAUCUCUACAdTdT 3', 5'

15 dTdTCCUGGUGGCGUAGAGAUGU 3', 5'-AAGCUGuCACAGAGGGGCUAC-3',
 5'GUAGCCCCUCUGUGACAGCUU-3, 5'-CCAUGUGCUAUACAGUCAUUACUUU-3',
 5'-AAAGUAAUGACUGUAUAGCACAUGG-3', 5'-UUGGACAAUGGACUGGUUGA-3',
 or 5'- UCAACCAGUCCAUUGUCCAA-3. In one embodiment, siRNA is a BRCA1
 siRNA. A non-limiting example is a siRNA sequence 5'-CCACAUAAGGGCCACUA-3'.

20 Another non-limiting example is a siRNA sequence 5'-UAGUGGGCCCUUAUGUGG-3'.
 In some embodiments, the bioactive agent is a microRNA sequence. In some embodiments, the bioactive agent is an anti-miRNA molecule for a miRNA comprising miR-9, miR-10b, miR-21, or miR-26. In some embodiments, the bioactive agent is a miRNA molecule for a miRNA comprising let-7a, miR-10b, miR-25, miR-34a, miR-124, miR-145, or miR-181b.

25 Further provided, in some embodiments of the presently disclosed subject matter, is a composition including a therapeutically effective amount of the RNA nanostructure molecule as disclosed above and herein. In some embodiments, the composition further includes a pharmaceutically acceptable carrier.

30 Still further, the presently disclosed subject matter in some embodiments provides a nanoparticle delivery system including a RNA nanostructure molecule as disclosed above and herein. In some embodiments, the delivery system further includes a pharmaceutically acceptable carrier.

Yet further provided in some embodiments, is a method of treating gastric cancer in a subject having or at risk of having gastric cancer. The method includes the step of

administering to the subject a therapeutically effective amount of a composition comprising the RNA nanostructure molecule as disclosed above and herein. In some embodiments, the method includes a composition further including a pharmaceutically acceptable carrier. In some embodiments, the subject is a mammal or a non-mammal vertebrate. In some

5 embodiments, the subject is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

The features of the presently disclosed subject matter are set forth with particularity in the appended claims. A better understanding of the features and advantages of the presently disclosed subject matter will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the subject matter are used, and the accompanying drawings of which. The drawings were originally published in color, incorporated by reference in their entireties (Daxiang Cui, et al., (2015) Nature, 5:10726, DOI: 10.1038/srep10726). The black and white drawings of the instant application

10 correspond to the color ones published.

FIGs. 1A and 1B include diagrams and images illustrating the global structure of the therapeutic RNA nanoparticles with BRCAA1 siRNA. FIG. 1A is the design of the RNA nanoparticles. Left is the one use in animal trial. Right is the extended one to prepare the AFM images. FIG. 1B is the AFM image of extended 3WJ RNA nanoparticles. The RNA complex in left of a is estimated to be around 10nm. Due to convolution of the tip size (~10 nm in diameter) in AFM images, features smaller than the size of the tip cannot be resolved. To characterize the structure of the RNA constructs, the 3WJ nanoparticles were extended by 39-60 base-pairs (in red color), which is within the persistence length of dsRNA and will not affect the 3WJ folding as described before (31), to generate the AFM image as shown.

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FIG. 2 includes graphs showing flow cytometry analysis for specific binding of 3WJ-FA-A647 nanoparticles to MGC803 cells (left, folate positive), GES-1 cells (right, folate negative control).

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FIG. 3 shows the BRCAA1 silencing effects of FA-pRNA-3WJ-BRCAA1siRNA assayed by (a) qRT-PCR (GADPH is the endogenous control)(there existed statistical difference between FA-pRNA-3WJ-BRCAA1siRNA group and FA-pRNA-3WJ-Scramb-siRNA group, $P < 0.01$) and (b) western blot assay (β -actin bands served as loading control).

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FIG. 4 is a graph showing inhibition of the growth of MGC803 cells by the nanoparticle of FA-pRNA-3WJ-BRCAA1siRNA using CCK8 (Cell Counting Kit-8) assays. The "Control" is non-treated MGC803 cells.

FIG. 5 includes graphs showing determination of cell death by flow cytometry of Annexin V-FITC/PI staining in MGC803 cells transfected with 25 nM FA-pRNA-3WJ-BRCAA1siRNA or FA-pRNA-3WJ-Scram-siRNA for 48 h.

FIG. 6A includes images showing representative *in vivo* fluorescence images of MGC803-tumour-bearing mouse after iv-injected with FA-AlexaFluor647-labeled pRNA nanoparticle. The tumor areas are indicated with arrows. **FIG. 6B** shows representative *ex vivo* images of tumors and organs. Labels: 1, tumor; 2, heart; 3, liver; 4, spleen; 5, lung; 6, Stomach; 7, kidneys; 8, bladder; 9, muscle. **FIG. 6C** is a graphs showing the average fluorescence intensities from the tumor areas of post-injection (3 mice per time point). The error bars represent SEM (n=3).

FIG. 7 includes images showing tumor sizes in test group and control group under different days **FIG. 7A** is an image showing 0 day at un-treated mouse; **FIG. 7B** is an image showing 0 day in un-treated mouse; **FIG. 7C** shows 14 days at control mouse; **FIG. 7D** is an image showing 14 days in test mouse, and **FIG. 7E** shows tumor tissues from experiment.

FIG. 8 is a graph showing the tumor size curve as the post-treatment time increases. There existed statistical difference between FA-pRNA-3WJ-BRCAA1siRNA treated group and FA-pRNA-3WJ-Scram-siRNA treated group, $P < 0.01$.

FIG. 9 includes images showing the result of HE immunostaining of important organs showing the undetectable damage.

FIG. 10 is an images showing the expression of related apoptosis proteins of MGC803 cells at 48h post-treatment by Western blotting. 1: scrambled control; 2: FA-AlexaFluor647-labeled pRNA nanoparticle; 3: control.

FIG. 11 is a diagram showing the potential mechanism of RNA nanoparticles for gastric cancer therapy.

FIG. 12A is an graph showing the size of 3WJ-BRCAA1 siRNA nanoparticle. **FIG. 12B** is a graph showing the zeta potential of 3WJ-BRCAA1 siRNA nanoparticle. The RNA nanoparticles were dissolved in $1\times$ TMS buffer with concentration at $1.5\ \mu\text{M}$ and then were measured by Zetasizer nano-ZS (Malvern Instrument). The data showed that the size of the nanoparticle is $5.20 \pm 0.83\ \text{nm}$ in diameter, and the zeta potential is $-16.57 \pm 0.75\ \text{mv}$, as shown in Figure S1A and S1B. The data were obtained from three independent measurements.

FIG. 13 includes a graph and an image showing the effects of pH on the fluorescent intensity and stability of RNA nanoparticles. **FIG. 13A** is a graphs illustrating the effects of pH (pH 2 to 13) on the fluorescent intensity of RNA nanoparticles by PerkinElmer LS 55 spectrofluorimeter, and **FIG. 13B** is an image showing the effects of pH (pH 2 to 13) on the

stability of RNA nanoparticles examined by 1.2% agarose gel electrophoresis.

FIG. 14 is a graphs showing the determination of the melting temperature of the 3WJ-BRCAA1 siRNA nanoparticle.

FIG. 15 is an image showing the effects of RNAase A on the stability of RNA nanoparticles. Lane 1: 10U RNAase A; Lane2: 50U RNAase A, Lane 3: 100U RNAase A, Lane 4: 500U RNAase A; Lane 5: 1000U RNAase A; Lane 6: 10000U RNAase A. RNA nanoparticles on different lanes exhibited identical position, similar brightness and no obvious degradation, suggesting that prepared RNA nanoparticles have good stability against RNase A(less than 10000U)-mediated degradation.

FIG. 16 is a graph showing light scattering plots of MGC803 cells at 48h treated with FA-pRNA-3WJ-BRCAA1-siRNA nanoparticles.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom.

In certain instances, nucleotides and polypeptides disclosed herein are included in publicly-available databases, such as GENBANK® and SWISSPROT. Information including sequences and other information related to such nucleotides and polypeptides included in such publicly-available databases are expressly incorporated by reference. Unless otherwise indicated or apparent the references to such publicly-available databases are references to the most recent version of the database as of the filing date of this Application.

While the terms used herein are believed to be well understood by one of ordinary skill in the art, definitions are set forth to facilitate explanation of the presently-disclosed subject matter.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently-disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a cell” includes a plurality of such cells, and so forth.

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

As used herein, ranges can be expressed as from “about” one particular value, and/or to “about” another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

The presently disclosed subject matter in some embodiments relates to an artificial RNA nanostructure molecule. The RNA nanostructure molecule includes a multiple branched RNA junction motif comprising at least one RNA oligonucleotides, and a gastric cancer targeting module conjugated to the RNA junction motif. In some embodiments, the RNA oligonucleotides is at least 6 nucleotides in length. In some embodiments, the RNA nanostructure further includes at least one bioactive agent. In some embodiments, the RNA nanostructure binds to gastric cancer cells. A non-limiting example of the bioactive agent is a therapeutic agent. In some embodiments, the bioactive agent includes drugs, fluorescent dyes, or chemicals. In some embodiments, the bioactive agent includes an imaging module. Non-limiting examples of the imaging module is fluorescent dye, including a non-limiting example Alexa647. In some embodiments, the nanostructure comprises at least one chemical modification at 2' position. Non-limiting examples of the chemical modification includes 2'Fluoro, 2'Amine, and 2'O-Methyl. In some embodiments, the multiple branched RNA comprises sequence 5' -

CCACAUAAGGGCCCACUAuuCCCACAUAUUUGUUGAUCC-3'. In some embodiments, the multiple branched RNA includes sequence 5'-UAGUGGGCCCUUUAUGUGG-3'.

The term "RNA" refers to a molecule comprising at least one ribonucleotide residue.

5 By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β -D-ribofuranose moiety. The terms encompass double stranded RNA, single stranded RNA, RNAs with both double stranded and single stranded regions, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA, or analog RNA, that differs from naturally occurring RNA by the addition, deletion, substitution, and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of an siRNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the presently disclosed subject matter can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or
10 deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of a naturally occurring RNA.
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As disclosed herein, RNA nanotechnology has recently emerged as an important field due to recent finding of its high thermodynamic stability, favorable and distinctive in vivo attributes (US 2014/0179758, hereby incorporate by reference in its entirety). In some
20 embodiments of the present disclosure, as disclosed in US2014/0179758, the RNA molecules form dimers, trimers, hexamers, and patterned superstructures. Further, RNA nanoparticles can be fabricated with precise control of shape, size and stoichiometry, as demonstrated by the packaging RNA (pRNA) of the bacteriophage phi29 DNA packaging motor, which forms dimers, trimers, and hexamers *via* hand-in-hand interactions of the interlocking loops
25 (27,28). The pRNA contains an ultra-stable three-way junction (3WJ) motif (29-31), which can be assembled from three short fragments with extremely high affinity. Recently, the crystal structure of the pRNA-3WJ motif was obtained (32) and a variety of therapeutic RNA nanoparticles using the pRNA-3WJ and pRNA-X motifs as scaffolds have been constructed (33-34). The pRNA-3WJ nanoparticles display thermodynamically stable properties,
30 including high melting temperature with low free energy, resistance to denaturation in 8M urea, and resistance to dissociation at very low concentrations in the blood. Boiling resistant RNA nanoparticles with controllable shapes and defined stoichiometry have recently been reported (36). Various imaging groups, such as fluorophores; targeting ligands, such as

receptor binding aptamers; and therapeutic modules, such as siRNA, miRNA or ribozymes can be integrated into the 3WJ scaffold without affecting the folding and functionality of the core motif and incorporated functional moieties. Upon 2'-Fluoro (2'-F) modifications of Uracil (U) and Cytosine (C) nucleotides, the RNA nanoparticles become resistant to RNase degradation with enhanced *in vivo* half-life while retaining authentic functions of the incorporated modules. Furthermore, the pRNA nanoparticles are non-toxic, non-immunogenic, and display favorable biodistribution and pharmacokinetic profiles in mice.

In some embodiments, the multiple branched RNA junction motif is a three-branched RNA junction motif. In some embodiments of the present disclosure, the RNA molecules form dimers, trimers, hexamers, and patterned superstructures. Further, In some embodiments, a branch of the three-branched RNA junction motif includes an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); or a c3WJ RNA module (SEQ ID NO: 3). In one embodiment, the three-branched RNA junction motif comprises an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); and a c3WJ RNA module (SEQ ID NO: 3). The SEQ ID NO: 1 includes nucleotide sequence 5'-UUG CCA UGU GUA UGU GGG-3', the SEQ ID NO: 2 comprises nucleotide sequence 5'-CCC ACA UAC UUU GUU GAUCC-3', and the SEQ ID NO: 3 comprises nucleotide sequence 5'-GGA UCA AUC AUG GCA A-3'.

In some embodiments, the diameter of the molecule is at least about 40 nm or less. The diameter is at least about 35 nm or less, at least about 30 nm or less, at least about 25 nm or less, at least 20 nm or less, at least 15 nm or less, at least 10 nm or less, at least 5 nm or less.

In some embodiments, the molecule has zeta potential ranging from about -150 mV to about 150 mV. The RNA molecule has a zeta potential ranging from about -140 mV to about 140 mV, from about -130 mV to about 130 mV, from about -120 mV to about 120 mV, from about -110 mV to about 110 mV, from about -100 mV to about 100 mV, from about -90 to about 90 mV, form about -80 mV to about 80 mV, from about -70 mV to about 70 mV, from about -60 mV to about 60 mV, from about -50 mV to about 50 mV. The molecule has a zeta potential ranging from about -40 mv to about 40 mV, from about -30 mV to about 30 mV, from about -20 mV to about 20 mV, from about -10 mV to about 10 mV.

In some embodiments, the molecule is substantially stable in a pH value ranges from about 2 to about 13. The RNA molecule is substantially stable in pH about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13. As used herein, the term "substantially stable" can refer to physical and/or chemical stability. As will be recognized by those of ordinary skill in the art, the term

“substantially stable” can refer to stability of the composition under certain conditions, relative to an initial composition (i.e., when a particular batch of the composition is initially prepared). In this regard, as will be recognized by those of ordinary skill in the art, one manner in which stability of a particular embodiment of the composition can be determined is as follows: preparing a batch of the embodiment of the composition, making an initial assessment of a sample of the composition (control sample), subjecting a sample of the composition to conditions of interest (e.g., storage at a particular temperature for a particular time period) (test sample), making an assessment of the test sample, and comparing the assessment of the control sample to the assessment of the test sample. Calculations can be made to determine whether the amounts present in the test sample are $100\% \pm 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, \text{ or } 0.1\%$ of the amount that is in the control sample.

In some embodiments, the RNA nanostructure includes a gastric cancer targeting module. In some embodiments, the gastric cancer targeting module includes a ligand that binds to at least one gastric cancer cell surface marker. In some embodiments, the ligand binds to a folate receptor, an epidermal growth factor receptor 2 (ErbB-2/HER2), an epidermal growth factor receptor (EGFR), a HER2 or a combination thereof. In some embodiments, the ligand is an aptamer. The term "aptamer" as used herein refers to an oligonucleotide that can bind specifically to its target with high affinity. Non-limiting examples of the aptamer are aptamers binding to EGFR, PDGFR, folate receptor, or a combination thereof. In one example, the ligand is a EGFR targeting aptamer. In one example, the ligand has sequence 5'- G CCU UAG UAA CGU GCU UUG AUG UCG AUU CGA CAG GAG GC -3'. In some embodiments, the targeting module is a folate.

The term "folate" as used herein can comprise a genus of well-defined B-vitamin compounds, including but not limited to, 5 -methyltetrahydro folate, 5-formyltetrahydrofolate, dihydrofolate, tetrahydrofolate, folic acid and other folate compounds. As disclosed herein, the targeted delivery systems call for a ligand-receptor pair that is specifically found in cancer cells. Many, but not all, cancer cells, including stomach, ovary, lung, breast, kidney, endometrium, colon and hematopoietic cells, over-expressed folate receptors (FRs) than normal cells for high uptake of folate, since folate is essential component during DNA replication and methylation in highly proliferating cells. Folic acid (FA), a synthetic oxidized form of folate, has been widely used as a ligand conjugate in various cancer targeting materials.

In some embodiments, the bioactive agent is a drug, a fluorescent dye, a chemical, or a combination thereof. In some embodiments, wherein the bioactive agent is a siRNA, a miRNA, an anti-mRNA, a ribozyme RNAs, or an antisense RNAs. In some embodiments, the bioactive agent is directed a gastric cancer marker. In some embodiments, the bioactive agent is a siRNA sequence. Non-limiting examples of the siRNA are siRNA is directed to Survivin, Bcl-2, XIAP, BCL-XL, or BRCA1.

In some embodiments of the present disclosure, a RNA nanotechnology approach is adopted to overcome some of the aforementioned challenges, and report the strategy to target and deliver therapeutic BRCA1 siRNA to *in vivo* stomach cancer tissues using FA-conjugated pRNA-3WJ nanoparticles. The present disclosure relates to the construction of multi-functional, thermodynamically and chemically stable RNA nanoparticles harboring sequences that allow specific binding to stomach cancer specific cell surface antigens or receptors resulting in the internalization of RNA nanoparticles into target cells and delivery of the siRNA, miRNA, and drugs for attaining synergistic effects for the treatment of stomach cancer. Further, the present disclosure relates to effects of prepared RNA nanoparticles on the regression of gastric cancer tissues *in vivo*, and potential molecular mechanism.

It has been proposed for long time that the RNAi can be applied for potential cancer treatment strategy. However, lacking of effect delivery system, the therapeutic small RNAs including siRNAs, miRNAs, anti-sense RNAs, etc., remain undruggable. The RNA nanoparticle-based nano-delivery platform can overcome aforementioned limitations for conventional cancer therapeutic and opens new opportunities for specific delivering RNAi therapeutics to gastric cancer without damaging healthy organs and tissues, reducing the toxicity and side effects, improving the therapeutic effects, and exhibiting great potential in clinical cancer therapy.

The phrase "gastric cancer marker" as used herein refers to genes or gene products (e.g., RNA molecules or proteins) which are characteristic of some or all of the cells in gastric cancer. A gastric cancer marker with diagnostic value can be a gene or gene product expressed in normal, non-cancerous cells, but is characteristic of a type or classification of cancer by, for example, its over-expression or under-expression as compared to its expression in normal, non-cancerous cells. A gastric cancer marker with prognostic value is a gene or gene product for which the over-expression or under-expression confers predictive information about the future aggressiveness of a cancer and/or its response to therapy at the time of diagnosis. In a cancer sample, the patterns of expression of diagnostic and prognostic

cancer markers allow one to accurately identify and determine the future course of the disease, respectively. Non-limiting examples of gastric cancer biomarkers are described in WO2012167112 (herein incorporated by reference in its entirety).

The terms "small interfering RNA", "short interfering RNA", "small hairpin RNA", "siRNA", and shRNA are used interchangeably and refer to any nucleic acid molecule capable of mediating RNA interference (RNAi) or gene silencing. See e.g., Bass, Nature 411:428-429, 2001; Elbashir et al., Nature 411:494-498, 2001a; and PCT International Publication Nos. WO 00/44895, WO 01/36646, WO 99/32619, WO 00/01846, WO 01/29058, WO 99/07409, and WO 00/44914. In one embodiment, the siRNA comprises a double stranded polynucleotide molecule comprising complementary sense and antisense regions, wherein the antisense region comprises a sequence complementary to a region of a target nucleic acid molecule (for example, a nucleic acid molecule encoding Survivin). In another embodiment, the siRNA comprises a single stranded polynucleotide having self-complementary sense and antisense regions, wherein the antisense region comprises a sequence complementary to a region of a target nucleic acid molecule. In another embodiment, the siRNA comprises a single stranded polynucleotide having one or more loop structures and a stem comprising self complementary sense and antisense regions, wherein the antisense region comprises a sequence complementary to a region of a target nucleic acid molecule, and wherein the polynucleotide can be processed either in vivo or in vitro to generate an active siRNA capable of mediating RNAi. As used herein, siRNA molecules need not be limited to those molecules containing only RNA, but further encompass chemically modified nucleotides and non-nucleotides.

In some embodiments, the presently disclosed subject matter takes advantage of the ability of short, double stranded RNA molecules to cause the down regulation of cellular genes, a process referred to as RNA interference. As used herein, "RNA interference" (RNAi) refers to a process of sequence-specific post-transcriptional gene silencing mediated by a small interfering RNA (siRNA). See Fire et al., Nature 391:806-811, 1998 and U.S. Patent No. 6,506,559, each of which is incorporated by reference herein in its entirety. The process of post-transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism that has evolved to prevent the expression of foreign genes (Fire, Trends Genet 15:358-363, 1999).

Disclosed herein are examples of siRNA including Survivin-specific, Bcl-2, XIAP, and BCL-XL siRNA sequences. The Survivin-specific siRNA includes sense sequence: 5'

GGACCACCGCAUCUCUACAdTdT 3' and antisense sequence: 5'
 dTdTCCUGGUGGCGUAGAGAUGU 3' (See, e.g., *Cancer Gene Ther.* 2004 Mar;11(3):186-
 93. Knockdown of survivin expression by small interfering RNA reduces the clonogenic
 survival of human sarcoma cell lines independently of p53.); the Bcl-2 siRNA includes sense
 5 sequence: 5'-AAGCUGuCACAGAGGGGCUAC-3', and antisense sequence: 5'-
 GUAGCCCCUCUGUGACAGCUU-3 (See, e.g., *Nucleic Acids Res.* 2012 Jul;40(13):6319-
 37. doi: 10.1093/nar/gks294. Epub 2012 Mar 30. Delivery of chemo-sensitizing siRNAs to
 HER2+-breast cancer cells using RNA aptamers.); XIAP siRNA includes sense sequence: 5'-
 CCAUGUGCUAUACAGUCAUUAUUU-3, and antisense sequence: 5'-
 10 AAAGUAAUGACUGUAUAGCACAUGG-3 (See, e.g., *Mol Cancer.* 2006 Sep 5;5:34.
 Specific down-regulation of XIAP with RNA interference enhances the sensitivity of canine
 tumor cell-lines to TRAIL and doxorubicin); and BCL-XL siRNA includes sense sequence:
 5'-UUGGACAAUGGACUGGUUGA-3, antisense sequence: 5'-
 UCAACCAGUCCAUUGUCCAA-3 (See, e.g., *Acta Biochim Biophys Sin (Shanghai).* 2005
 15 Aug;37(8):555-60. Silencing of Bcl-XL expression in human MGC-803 gastric cancer cells
 by siRNA.) Further, in one embodiment, siRNA is a BRCA1 siRNA. A non-limiting
 example is a siRNA sequence 5'-CCACAUAAAGGGCCCACUA-3'. Another non-limiting
 example is a siRNA sequence 5'-UAGUGGGCCCUUUAUGUGG-3'.

In some embodiments, the bioactive agent is a microRNA sequence. The term
 20 "MicroRNAs (miRNAs)" as used herein are single-stranded, or double stranded non-coding
 RNAs, at least about 6 nucleotide in length that can regulate gene expression at the post-
 transcriptional level by either degrading their target mRNAs or inhibiting their
 translation(1,2). MiRNAs play important roles in regulating cell cycle, proliferation,
 differentiation, metabolism, and apoptosis(1). A compendium of microRNA and respective
 25 microRNA binding sequences is available at the miRNA registry. (See, e.g., Griffiths-Jones
 et al. (2006) *Nucl. Acids Res.* 34:D140-D144; US20140045709, herein incorporate by
 reference in their entireties.) In particular embodiments, the microRNA and microRNA
 binding sequence employed in the present assay are associated with a disease or condition,
 wherein an antagonist or agonist to the microRNA would be useful in preventing or treating
 30 the disease or condition.

In some embodiments, the present disclosure provides inhibitors of miRNAs (e.g.,
 anti-miR-21). Compositions comprising such inhibitors and methods for inhibiting miR-21
 using such inhibitors are also disclosed herein. Any miRNA inhibitor may be used alone, or
 with other miRNA inhibitor(s) known in the art. In some embodiments, the miRNA inhibitor

comprises an antisense molecule. In some embodiments, the antisense molecule could be a single or a double stranded sequence. Examples of antisense molecule include, but are not limited to, siRNAs, triple-helix-forming agents, ribozymes, RNAi, synthetic peptide nucleic acids (PNAs), antigens (agRNAs), LNA/DNA copolymers, small molecule chemical

5 compounds, and antisense oligonucleotides.

In some embodiments, the microRNA sequence is at least 6 nucleotide in length. In some embodiments, the miRNA molecule or an equivalent, or a mimic thereof is from about 6 to about 30 nucleotides in length. In some embodiments, the miRNA is about 12 to about 30 nucleotides in length. In some embodiments, the miRNA is from about 15 to about 28
10 nucleotides in length. In some embodiments, the miRNA is about 19 to about 25 nucleotides in length. In some embodiments, the miRNA molecule has a length of at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, about 30 nucleotides or more. In some embodiments, an antagomir of a miRNA molecule is from about 6 to about 30 nucleotides in length, from about 10 to about 30 nucleotides in length,
15 from about 12 to about 28 nucleotides in length. In some embodiments, the antagomir of a miRNA molecule has a length of at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, about 30 nucleotides or more.

In some embodiments, the miRNA interferes oncogenic miRNA to regress cancer growth. The RNA nanostructure molecule contains anti-miRNA that silences oncogenic
20 miRNAs, including but not limited to, miR-9, miR-10b, miR-21, miR-17, and miR-26. In some embodiments, the miRNA rescues down-regulated cancer suppressive miRNAs, where the RNA nanostructure introduces cancer suppressive miRNAs, including but not limited to, let-7a, miR-10b, miR-25, miR-34a, miR-124, miR-145, and miR-181b. Further examples is disclosed in US20140045709, which herein incorporate by reference in its entirety.

25 The presently disclosed subject matter further provide a composition including a therapeutically effective amount of the RNA nanostructure molecule as disclosed above and herein. In some embodiments, the composition further includes a pharmaceutically acceptable carrier.

The presently disclosed subject matter in some embodiments provides a nanoparticle
30 delivery system including a RNA nanostructure molecule as disclosed above and herein. In some embodiments, the delivery system further includes a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" as used herein refers to a diluent, adjuvant, excipient, or vehicle with which a heterodimeric probe of the disclosure is administered and which is approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Such pharmaceutical carriers can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. The pharmaceutical carriers can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. When administered to a patient, the heterodimeric probe and pharmaceutically acceptable carriers can be sterile. Water is a useful carrier when the heterodimeric probe is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers also include excipients such as glucose, lactose, sucrose, glycerol monostearate, sodium chloride, glycerol, propylene glycol, water, ethanol, and the like. The present compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The present compositions advantageously may take the form of solutions, emulsion, sustained release formulations, or any other form suitable for use.

The term "therapeutically effective amount," as used herein, refers to the amount of a composition containing administered to a patient already suffering from a disease, condition, or disorder, sufficient to cure or at least partially arrest, or relieve to some extent one or more of the symptoms of the disease, disorder, or condition being treated. The effectiveness of such compositions depend upon conditions including, but not limited to, the severity and course of the disease, disorder, or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician. By way of example only, therapeutically effective amounts may be determined by routine experimentation, including but not limited to a dose escalation clinical trial.

The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of a compound at

levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose can be divided into multiple doses for purposes of administration. Consequently, single dose compositions can contain such amounts or submultiples thereof to make up the daily dose.

5 The dosage can be adjusted by the individual physician in the event of any contraindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. In further various aspects, a preparation can be administered in a “prophylactically effective amount”; that is, an amount effective for
10 prevention of a disease or condition.

Suitable methods for administering to a subject an effective amount of the composition in accordance with the methods of the present disclosure include but are not limited to systemic administration, parenteral administration (including intravascular, intramuscular, intraarterial administration), oral delivery, buccal delivery, subcutaneous
15 administration, inhalation, intratracheal installation, surgical implantation, transdermal delivery, local injection, and hyper-velocity injection/bombardment. Where applicable, continuous infusion can enhance drug accumulation at a target site (see, e.g., U.S. Patent No. 6,180,082).

In some embodiments, the present disclosure provides a method of treating gastric
20 cancer in a subject having or at risk of having gastric cancer. The method includes the step of administering to the subject a therapeutically effective amount of a composition comprising the RNA nanostructure molecule as disclosed above and herein. In some embodiments, the method includes a composition further including a pharmaceutically acceptable carrier. In some embodiments, the subject is a mammal or a non-mammal vertebrate. In some
25 embodiments, the subject is a human. More specifically, in the method, the RNA nanostructure molecule includes a multiple branched RNA junction motif comprising at least one RNA oligonucleotides, and a gastric cancer targeting module conjugated to the RNA junction motif. In some embodiments of the method, the RNA nanostructure further includes at least one bioactive agent. The RNA nanostructure binds to gastric cancer cells. A non-
30 limiting example of the bioactive agent is a therapeutic agent. The bioactive agent includes drugs, fluorescent dyes, or chemicals. In some embodiments, the bioactive agent includes an imaging module. In some embodiment of the method, the diameter of the RNA molecule is at least about 40 nm or less, the molecule has zeta potential ranging from about -150 mV to about 150 mV, and the molecule is substantially stable in a pH value ranges from about 2 to

about 13. In some embodiments of the method, the nanostructure comprises at least one chemical modification at 2' position. Non-limiting examples of the chemical modification includes 2'Fluoro, 2'Amine, and 2'O-Methyl. In some embodiments, the multiple branched RNA comprises sequence 5' -

5 CCACAUAAGGGCCACUAuuCCCACAUAUUUGUUGAUCC-3'. In some embodiments, the multiple branched RNA includes sequence 5'-UAGUGGGCCCUUUAUGUGG-3'. In further embodiments, the multiple branched RNA junction motif is a three-branched RNA junction motif. In some embodiments of the present disclosure, the RNA molecules form dimers, trimers, hexamers, and patterned superstructures. Further, In some embodiments, a branch of the three-branched RNA junction motif includes an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); or a c3WJ RNA module (SEQ ID NO: 3). In one embodiment, the three-branched RNA junction motif comprises an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); and a c3WJ RNA module (SEQ ID NO: 3). The SEQ ID NO: 1 includes nucleotide sequence 5'-UUG CCA UGU GUA UGU GGG-3', the SEQ ID NO: 2 comprises nucleotide sequence 5'-CCC ACA UAC UUU GUU GAUCC-3', and the SEQ ID NO: 3 comprises nucleotide sequence 5'-GGA UCA AUC AUG GCA A-3'.

Further, the gastric cancer targeting module includes a ligand that binds to at least one gastric cancer cell surface marker. In some embodiments, the ligand binds to a folate receptor, an epidermal growth factor receptor 2 (ErbB-2/HER2), an epidermal growth factor receptor (EGFR), a HER2 or a combination thereof. In some embodiments, the ligand is an aptamer. Non-limiting examples of the aptamer are aptamers binding to EGFR, PDGFR, folate receptor, or a combination thereof. In one example, the ligand is a EGFR targeting aptamer. In one example, the ligand has sequence 5'- G CCU UAG UAA CGU GCU UUG AUG UCG AUU CGA CAG GAG GC -3'. In some embodiments, the targeting module is a folate. Non-limiting examples of folate are folic acid, 5 -methyltetrahydro folate, 5-formyltetrahydrofolate, dihydrofolate, tetrahydrofolate, or a combination thereof.

The RNA nanostructure of the method further includes at least one bioactive agent. The bioactive agent is a drug, a fluorescent dye, a chemical, or a combination thereof. Further, the bioactive agent includes a siRNA, a miRNA, an anti-mRNA, a ribozyme RNAs, or an antisense RNAs. In some embodiments, the bioactive agent is directed a gastric cancer marker. In some embodiments, the bioactive agent is a siRNA sequence. Non-limiting examples of the siRNA are siRNA is directed to Survivin, Bcl-2, XIAP, BCL-XL, or BRCAA1. Non-limiting examples of the siRNA include siRNA sequence 5'

GGACCACCGCAUCUCUACAdTdT 3', 5' dTdTCCUGGUGGCGUAGAGAUGU 3', 5'-
AAGCUGuCACAGAGGGGCUAC-3', 5'GUAGCCCCUCUGUGACAGCUU-3, 5'-
CCAUGUGCUAUACAGUCAUUACUUU-3', 5'-

AAAGUAAUGACUGUAUAGCACAUGG-3', 5'-UUGGACAAUGGACUGGUUGA-3', or

5' UCAACCAGUCCAUUGUCCAA-3. In one embodiment, siRNA is a BRCA1 siRNA.

A non-limiting example is a siRNA sequence 5'-CCACAUAAGGGCCACUA-3'. Another non-limiting example is a siRNA sequence 5'-UAGUGGGCCCUUAUGUGG-3'.

In some embodiments, the bioactive agent is a microRNA sequence. In some embodiments, the

bioactive agent is an anti-miRNA molecule for a miRNA comprising miR-9, miR-10b, miR-

21, or miR-26. In some embodiments, the bioactive agent is a miRNA molecule for a miRNA comprising let-7a, miR-10b, miR-25, miR-34a, miR-124, miR-145, or miR-181b.

As used herein, the term "subject" refers to a target of administration of the pharmaceutical composition. The subject of the herein disclosed methods can be a vertebrate, such as a mammal, a fish, a bird, a reptile, or an amphibian. Thus, the subject of the herein

disclosed methods can be a human or non-human. Thus, veterinary therapeutic uses are provided in accordance with the presently disclosed subject matter. As such, the presently

disclosed subject matter provides for administration to mammals such as humans and non-human primates, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by

humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and

dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; rabbits, guinea pigs, and rodents. Also

provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e.,

poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including,

but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like. The term does not denote a particular age or sex.

Further, in some embodiments, the RNA nanoparticle based nano-delivery platform is expected to outperform treatment strategies for gastric cancer therapy in several aspects: (1) RNA nanoparticles have defined size, structure and stoichiometry. Unpredictable side effects arising from heterogeneous particles can thus be avoided. (2) Nanoscaled size of the RNA nanoparticles facilitates tissue penetration and target to tumor. (3) RNA nanoparticles are

easy to construct by self-assembly, highly soluble and not prone to aggregation. (4) The RNA nanoparticles are thermodynamically stable and, therefore, the entire construct will remain intact at ultra-low concentrations in the body. (5) The polyvalent nature of the RNA nanoparticle allows for easy integration of targeting modules, imaging modules and therapeutic modules into a single form. (6) RNA nanoparticles display low or no immunogenicity and/or toxicity even at high doses. The 3WJ-pRNA scaffold displays favorable pharmacokinetic and pharmacodynamic profiles in vivo (half-life of 5-10 hours compared to 0.25-0.75 hours of the most stable 2'F modified siRNA counterparts); non-toxic; and no induction of interferon or cytokines.

The presently disclosed subject matter is further illustrated by the following specific but non-limiting examples. The following examples may include compilations of data that are representative of data gathered at various times during the course of development and experimentation related to the present disclosure.

EXAMPLES

This study reports the use of the thermostable three-way junction (3WJ) of bacteriophage phi29 motor pRNA to escort folic acid, a NIR image marker and BRCA1 siRNA for targeting, imaging, delivery, gene silencing and regression of gastric cancer in animal models. In vitro assay revealed that the RNA nanoparticles specifically bind to gastric cancer cells, and knock-down the BRCA1 gene. Apoptosis of gastric cancer cells was observed. Animal trials confirmed that these RNA nanoparticles could be used to image gastric cancer in vivo, while showing little accumulation in crucial organs and tissues. The volume of gastric tumors noticeably decreased during the course of treatment. No damage to important organs by RNA nanoparticles was detectable. All the results indicated that this novel RNA nanotechnology can overcome conventional cancer therapeutic limitations and opens new opportunities for specific delivery of therapeutics to stomach cancer without damaging normal cells and tissues, reduce the toxicity and side effect, improve the therapeutic effect, and exhibit great potential in clinical tumor therapy.

MATERIALS AND METHODS

Construction and characterization of FA conjugated BRCA1-siRNA pRNA-3WJ nanoparticles

The pRNA-3WJ nanoparticle consisted of three fragments, a_{3WJ} , b_{3WJ} and c_{3WJ} , was functionalized with folate, as targeting ligand; Alexa₆₄₇, as imaging module; and BRCA1 siRNA (or scrambled control), as therapeutic module. The RNA fragments were then synthesized chemically (TriLink), self-assembled into RNA nanoparticles, and characterized

by 1.2% agarose gel shift assays and AFM imaging as well as Zeta potential/Particle Sizer, as described previously (52). The construct contains sequences as following:

Strand 1: 5' (folate)UUGCCAUGUGUAUGUGGG 3';

Strand 2: 5' CCACAUAAGGGCCCCACUAuuCCCACAUAACUUUGUUGAUCC 3'

Strand 3: 5' (Alexa 647)GGAUCAAUCAUGGCAA 3'

Strand 4: 5' UAGUGGGCCCUUUAUGUGGuu 3'

In order to evaluate the effects of a wide pH range on the stability of RNA nanoparticles, the prepared RNA nanoparticles were dispersed in varied pH buffers for 12h, RNA nanoparticles/ buffer = 1:1(v/v), and pH ranged from 2 to 13 (Table 1: details of preparation of a series of buffer solutions), then 1.2% agarose gel electrophoresis was used to characterize the stability of prepared RNA nanoparticles. Effects of pH on the fluorescent intensity of RNA nanoparticles were investigated by measuring the fluorescent intensity of RNA samples with different pH via the photoluminescence (PL) spectra (Perkin Elmer LS55 spectrofluorimeter).

Table 1. Preparation for a series of buffer solutions (pH 2 to 13)

pH	0.2M glycine (ml)	0.2M HCl (ml)	Deionized water (ml)
2*	5	4.4	10.6
3*	5	1.14	13.86
*Glycine HCl buffer			
	0.2M Na ₂ HPO ₄ (ml)	0.1M Citrate (ml)	
4 [△]	7.71	12.29	/
5 [△]	10.3	9.7	/
6 [△]	12.63	7.37	/
7 [△]	16.47	3.53	/
8 [△]	19.15	0.85	/
[△] Phosphate Citrate buffer			
	0.2M glycine	0.2M NaOH	
9 [▲]	5	0.88	14.12
10 [▲]	5	3.2	11.8
[▲] Glycine NaOH buffer			
	0.1M Na ₂ CO ₃ (ml)	0.1M NaHCO ₃ (ml)	
11 [★]	19	1	/
[★] Crabonate Bicarbonate buffer			
	0.05M Na ₂ HPO ₄ (ml)	0.1M NaOH (ml)	
12 [☆]	10	5.38	4.62
[☆] Phosphate NaOH buffer			
	0.2 M KCl	0.2M NaOH	
13 [•]	5	13.2	1.8
[•] KCl NaOH buffer			

The RNA nanoparticles contained 2'-F modified U and C nucleotides to make them resistant to RNase degradation. However, Effects of RNAase A on the stability of RNA nanoparticles was still investigated. RNase A-free purified water was used to dilute RNase A (Sigma Company), the resulting solutions were respectively exhibited different concentration of RNase A (10U, 50 U, 100U, 500U, 1000U,10000U) , then, each tube was respectively added into 1 μ g RNA nanoparticles, incubated at 37°C for 12h, then 10% SDS-PAGE(sodium dodecylsulfate-polyacrylamide gel electrophoresis) gel electrophoresis is used to observe effects of RNase A on the stability of RNA nanoparticles. The pRNA-3WJ was prepared by diluting 100 μ M of the complexes in diethylpyrocarbonate (DEPC) treated water with PBS at 1:1 (v/v) right before the experiments.

Effects of prepared RNA nanoparticles on cell binding efficiency and specificity

The human gastric cancer MGC803 cells and human gastric epithelial GES-1 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were maintained at 37 °C (5% CO₂) in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 1 mg/mL streptomycin. Cell culture products and reagents were purchased from GIBCO. 200 nM AlexaFluor647 labeled 3WJ-FA-A647 was incubated with 1 $\times$ 10⁵ MGC803 and GES-1 cells at 37 °C for 1 h, after washing with PBS for three times, the cells were collected and resuspended in PBS buffer, followed by analyzed with a FACS Calibur (BD Biosciences).

In order to investigate the specificity of RNA nanoparticles binding to MGC803 cells, MGC 803 cells were cultured in a humidified 5% CO₂ balanced air incubator at 37°C for 2 days. All the cells were collected and implanted onto 18 mm glass coverslips in a 12-well tissue culture plate, and culturing was continued for 3 days. After the cells were rinsed 3 times, 500 μ L of medium containing prepared RNA nanoparticles was added into each dish and incubated for 30 min. Three dishes of all dishes were first incubated with free folic acids for 30 min, then incubated with RNA nanoparticles, then washed with PBS buffer, and then examined under the dark field microscopy. Dark-field images were obtained on an upright Olympus IX71 optical microscope integrated with a CRi Nuance multispectral imaging system(Cambridge Research & Instrumentation, Inc., Woburn, MA, USA).

Effects of RNA nanoparticles on the silencing of BRCA1 gene in MGC803 cells

MGC803 cells were transfected with a positive BRCA1 siRNA control using Lipofectamine 2000 (Invitrogen) as the carrier. Two 3WJ-RNA constructs were assayed for the subsequent BRCA1 gene silencing effects: one harboring folate and BRCA1

siRNA; and, the other harboring folate and BRCA1 siRNA scramble control. After 48 h of treatment, total RNAs from MGC803 cells were isolated by using Trizol (Invitrogen) and Direct-zol™ RNA MiniPrep (Zymo Research) according to manufacturer's instructions. First-strand cDNA was obtained by using 1µg of total RNA and random primers and M-
 5 MLV reverse transcriptase (Promega). All reactions were carried out in a final volume of 25 µl and assayed in triplicate. qRT-PCR was performed using a BioRad iQ5 iCycler Detection System with a three-step PCR protocol (95 °C for 10 min, followed by 40 cycles of 95 °C for 5s, 60 °C for 30 s and 72 °C for 30 s) with Hieff™ qPCR SYBR® Green Master Mix (Yeasen). The data was analyzed by the $\Delta\Delta CT$ method. The primers for BRCA1 and
 10 GAPDH are as follows:

BRCA1: forward: 5'-ACCAAATCTCCCGCAAGG-3';

reverse: 5'-CATATTTTCCAGGTCCGACA-3'.

GAPDH: forward: 5'-GAAGGTGAAGGTCGGAGTC-3';

reverse: 5'-GAAGATGGTGATGGGATTTC--3'.

15 The qRT-PCR data were treated by using comparative Ct method, the calculation formation is as follows: $2^{-\Delta\Delta Ct}$; $\Delta\Delta Ct = (\text{treated group Ct} - \text{treated group GAPDH Ct}) - (\text{control group Ct} - \text{control group GAPDH Ct})$. The results obtained indicate the relative ratio is calculated that target gene mRNA expression levels in the treated group are divided by mRNA expression level in the control group.

20 For western blot assays, the total cell lysates were prepared in high KCl lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 300 mM KCl, 1 mM EDTA, 0.5% Triton X-100 and 0.5% sodium deoxycholate) with complete protease inhibitor cocktail (Roche). Thirty micrograms of protein were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore). The membranes were incubated respectively with BRCA1
 25 antibody (1:2000 diluted), Bcl-2 antibody (1:2000 diluted), Rb antibody (1:2000 diluted), Bax (1:2000 diluted) and β -actin antibody (Epitomics) (1:4000 diluted) for overnight, followed by 1:10000 anti-mouse secondary antibody conjugated with HRP (Epitomics) for 2 h. Membranes were blotted by Westar ECL kits (Cyanagen Srl) and exposed to film for autoradiography.

30 Effects of RNA nanoparticles on growth and apoptosis of MGC803 cells

Effects of prepared RNA nanoprobe on viability of MGC803 cells and GES-1 cells were analyzed using Cell Counting Kit-8 (CCK8) assay (23). MGC803 cells and GES-1 cells were cultured in the 96-well microplate at the concentration of 5000 cells per well and incubated in a humidified 5% CO₂ balanced air incubator at 37°C for 24 h. Except for control

wells, the remaining wells were added into medium with prepared RNA nanoparticles, final concentrations were, respectively, 10, 20, 40 and 80 $\mu\text{g/ml}$, then those cells were continued to culture for 24h, 48h and 72 h, respectively, then, the ODs were measured using the thermomultiskan MK3 ELISA plate reader according to the protocol of CCK8 assay kit, and
 5 calculated the survival rate of cells. The survival rate of cells can be calculated by the following equation:

$$\text{Cell viability (\%)} = \text{optical density (OD) of the treated cells} / \text{OD of the non-treated cells} \times 100$$

The prepared RNA nanoparticles were incubated with MGC803 cells for 48 h, cell
 10 apoptosis and necrosis were determined by Annexin V-FITC/PI double staining and quantified by flow cytometry. Briefly, 1×10^5 MGC 803 cells were harvested 48 h after transfection and resuspended in 100 μL binding buffer containing 5 μL annexin V-FITC and 5 μL PI provided with the Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen) for 15 min at room temperature in the dark. Samples were then analyzed with a FACSCalibur (BD
 15 Biosciences). The live cells were identified as Annexin V-FITC⁻/PI⁻ (lower left quadrant), early apoptotic cells as Annexin V-FITC⁺/PI⁻ (upper left quadrant), late-stage apoptotic cells as Annexin V-FITC⁺/PI⁺ (upper right quadrant), and necrotic cells as Annexin V-FITC⁻/PI⁺ (upper left quadrant). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Yeasen Corporation (Shanghai, China).

20 RNA nanoparticles for NIR fluorescent imaging of *in vivo* gastric cancer

Female athymic nude mice (18-22 g) were purchased from Shanghai Slac Laboratoty Animal Co. Ltd (Shanghai, China). For the establishment of tumor model, MGC803 cells were resuspended in PBS and 2×10^6 cells/site was subcutaneously injected in the right flank. When the tumor nodules had reached a volume of 0.1-0.3 cm^3 after approximately 3 weeks
 25 post-injection, mice were used for biodistribution and imaging studies. For tumor imaging, FA-Alexa Fluor 647-labeled pRNA-3WJ nanoparticle (about 20 nmol in PBS buffer, equal 32 mg/kg) was administrated intravenously into the MGC-803-tumour-bearing mice. Time-course fluorescent images (excitation: 630/20 nm, emission: 700/30 nm, integration time: 15 s) were acquired on a Bruker In-Vivo F PRO imaging system (Billerica, MA). All the post
 30 injection images were captured at the same parameter setting and are scaled to the same maximum values. For the *ex vivo* imaging, the mice (3 mice per time point) were then sacrificed and collected tumors and the major organs after 3, 24, 48, 96 h and 7 day intravenously (iv) injection. Excised tumor and organs were imaged by the Bruker In-Vivo F PRO imaging system with the same parameters as mentioned above.

RNA nanoparticles for targeted therapy of *in vivo* gastric cancer

Nude mice loaded with gastric cancer MGC803 cells were prepared according to the previous reports (12-15), and were randomly divided into three groups: test group (10 mice) (FA-pRNA-3WJ-BRCAA1siRNA, 1mg/kg body weight); control group (10 mice) (FA-pRNA-3WJ-Scram siRNA, 1mg/kg body weight) and blank control (10 mice) (untreated). When the tumor sizes reached about 5 mm in diameter, the nude mice were injected with prepared RNA nanoparticles in PBS via tail vein (1mg/kg body weight). Every two days, the tumor volume was measured, up to 15 days. Then, these mice were sacrificed.

Effects of RNA nanoparticles on important organs

The mice in testing group were sacrificed after being raised for 15 days. For histological evaluation, excised important organs including heart, liver, spleen, lung and kidney were frozen and embedded by medium at -20°C , and then were sectioned into 8 μm slices, then were stained by hematoxylin and eosin (HE) stain method, and were observed by microscopy to confirm whether there is pathological lesion in important organs existed.

Statistical Analysis

Each experiment was repeated three times in duplicate. The results were presented as mean $\pm$ SD. Statistical differences were evaluated using the t-test and considered significance at $P < 0.05$.

RESULTS

Construction and characterization of triple-functional pRNA-3WJ nanoparticles

The pRNA-3WJ nanoparticles were prepared by mixing the three strands $a_{3\text{WJ}}$, $b_{3\text{WJ}}$, and $c_{3\text{WJ}}$ respectively, at equal molar ratio (Fig.1a). Atomic force microscopy (AFM) image highly suggested the formation of homogeneous triangular branched architectures, confirming that pRNA-3WJ nanoparticles were successfully prepared (Fig. 1b). Regarding the RNA nanoparticles' size and surface charge, the dynamic light scattering experiments showed that the size of the nanoparticle is 5.20 ± 0.83 nm in diameter, and the zeta potential is -16.57 ± 0.75 mv, as shown in Figure 12A and 12B.

In order to evaluate the effects of a wide pH range on the stability of RNA nanoparticles, the prepared RNA nanoparticles were dispersed in varied pH buffers (RNA nanoparticles / buffer = 1:1(v/v)) for 12 h. The details of the preparation of a series of buffer solutions with pH 2 to 13 were shown in Table 1. The effects of pH on the fluorescent intensity of RNA nanoparticles is investigated. As shown in Figure 13A, in the range of pH 2

to 13, RNA nanoparticles exhibited different fluorescent intensity, and in the range of pH 5-9, RNA nanoparticles displayed more than 90% strong fluorescent signals. The study used 1.2% agarose gel electrophoresis to characterize the stability of prepared RNA nanoparticles. As shown in Figure 13B, tested RNA nanoparticles displayed identical position and similar brightness on the gel, no degradation, suggesting that the RNA nanoparticles are stable in the range of pH 2 to 13.

The melting temperature of the 3WJ-BRCAA1 siRNA nanoparticle was determined as 69.2 ± 0.9 °C by real-time PCR, as shown in Figure 14. Melting experiments were conducted by monitoring the fluorescence of the RNA nanoparticles or the assembly intermediates using the LightCycler 480 Real-Time PCR System (Roche). 1× SYBR Green dye was used for all the experiments. The RNA samples were dissolved in 1 × TMS buffer and slowly cooled from 95 to 20 °C. The melting temperature of the 3WJ-BRCAA1 siRNA nanoparticle was determined as 69.2 ± 0.9 °C. The data represents the mean and standard deviation of three independent experiments.

Effects of RNAase A on the stability of RNA nanoparticles were also investigated. RNase A-free purified water was used to dilute RNase A (Sigma Company). The resulting solutions were respectively exhibited different concentration of RNase A (10U, 50 U, 100U, 500U, 1000U, 10000U). Then each tube was respectively added 1 μg RNA nanoparticles, incubated at 37°C for 12h, and finally 10% SDS-PAGE gel electrophoresis was used to examine the effects of RNase A on the stability of RNA nanoparticles. As shown in Figure 15, RNA nanoparticles on different lanes exhibited identical position, similar brightness, no obvious degradation, which highly suggests that prepared RNA nanoparticles own good stability against RNase A (less than 10000U) degradation.

The resultant pRNA-3WJ nanoparticles are thermodynamically and chemically stable, which makes them an attractive candidate for *in vivo* nano-delivery for the purpose of cancer detection or treatment. In this study, folate is incorporated, as targeting ligand; Alexa₆₄₇, as imaging module; and BRCAA1 siRNA (or scrambled control) into the pRNA-3WJ scaffold.

Binding efficiency of pRNA nanoparticles to gastric cancer cell

Flow cytometry data in Figure 2 showed that the prepared 3WJ-FA-A647 nanoparticles can bind with the MGC803 cells with almost 100% binding efficiency, while the GES-1 cells display a weak signal, which highly suggested that the prepared RNA nanoparticles did not bind with GES-1 cells. Results also demonstrate that folic acid receptor exhibits over-

expression on the surface of MGC803 cells, no expression on the surface of GES-1 cells, similar to a previous report (15).

Effects of RNA nanoparticles on the silence of BRCA1 gene in MGC803 cells

The qRT-PCR results in Figure 3a showed that, prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles could silence expression of BRCA1 gene in MGC803 cells after incubating with MGC803 cells for 48 h, in contrast, prepared FA-pRNA-3WJ-Scram siRNA nanoparticles could not silence expression of BRCA1 gene in MGC803 cells after incubation for 48 h, between two groups, there existed statistical difference ($P < 0.01$). Compared with BRCA1 siRNA, prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles achieved similar silencing efficiency of BRCA1 gene in MGC803 cells. The Ct, Delta Ct, and Delta Delta Ct values for the qRT-PCR assay are shown in Table 2. Additionally, as shown in Figure 3b, Western blotting results further confirmed that prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles and BRCA1 siRNA could down-regulate BRCA1 expression in MGC803 cells, while prepared FA-pRNA-3WJ-Scram siRNA nanoparticles had little down-regulation of BRCA1 protein expression in MGC803 cells, thus showing prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles can specifically silence expression of BRCA1 protein in MGC803 cells. Importantly, the silencing potency was comparable to the Lipofectamine 2000 carried BRCA1 siRNA group.

Table S2. The expression levels of BRCA1 normalized to the exogenous GAPDH mRNA in the treated group and control group (the Ct, Delta Ct, and Delta Delta Ct values).

group	Ct	ΔCt	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$	P value
GAPDH	6.943				
BRCA1-siRNA		14.931	7.988	1.435	0.485
FA-pRNA-3WJ-Scram-siRNA		20.566	13.623	4.427	0.051
GAPDH		6.944	1		
FA-pRNA-3WJ-BRCA1-siRNA		15.194	8.252	1.507	0.404
GAPDH		6.942	1		

Effects of RNA nanoparticles on growth and apoptosis of gastric cancer cell MGC803

As shown in Figure 4, MGC 803 cells were treated with 400 μ g/mL FA-pRNA-3WJ-BRCA1 siRNA nanoparticles for 24 h, 48 h and 72 h, the inhibition rate of MGC 803 cells increased as the incubation time increased, at 48 h, maximal inhibition rate of MGC803 cells is 44.5 $\pm$ 2.6 %, compared with FA-pRNA-3WJ-Scram siRNA group, inhibition rate is 12.5 $\pm$ 1.9 %, there existed statistical difference between two groups, $P < 0.01$.

Previous study shows that BRCA1 can inhibit MGC803 cell apoptosis and improve the proliferation of MGC803 cells, it is hypothesized that the performed RNA interference (RNAi) by RNA nanoparticles could induce MGC803 cell apoptosis. As shown in Fig. 5, the transfection with 25nM FA-pRNA-3WJ-BRCA1 siRNA in MGC803 cells induced 2.51% of early apoptotic cells and 15.0% of late apoptotic cells, respectively, in the normal control, MGC803 cells exhibited 0.085% of early apoptotic cells, there existed statistical difference between treated group with FA-pRNA-3WJ-BRCA1 siRNA and control group, $P < 0.05$. The light scattering plot of MGC 803 cells treated with FA-pRNA-3WJ-BRCA1 siRNA nanoparticles for 48h is shown in Figure 16. These results show that prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles can induce apoptosis of MGC803 cells.

Fluorescent RNA nanoparticles for *in vivo* imaging of gastric cancer

It has been reported that unmodified siRNA ribonucleic acid sequences have extremely poor pharmacokinetic properties due to short *in vivo* half-life and fast kidney clearance caused by their small size (hydrodynamic diameters, HDs; typically < 5 nm, which is smaller than the kidney filtration threshold (KFT) of 5.5nm). Tumor targeting efficiency by RNA nanoparticles was investigated by collecting and analyzing *in situ* fluorescence images of MGC803 xenografts in nude mice at different post-injection (p.i.) time points (Fig.6a and c). Tumor area was hardly distinguished in the mouse in the first 30 min p.i. because of the strong fluorescence background in normal tissues. However, as the time increased, the decrease in the fluorescence background of normal tissues and the accumulations at the tumor site caused the tumor area became readily defined 5 h p.i.. *Ex vivo* images of normal tissues, organs, and tumors taken from the RNA nanoparticles-injected mice showed that the tumors taken at 5 and 24 h p.i. exhibited the strongest signal (Fig. 6b). In terms of tumor accumulation kinetics, RNA nanoparticles reached their highest accumulation within 5 h and remained in the tumor site 96 h p.i., which indicted the high tumor targeting efficiency and tumor retention capability of the constructed RNA nanoparticles.

RNA nanoparticles for *in vivo* targeted therapy of siRNA to gastric cancer

As shown in Figure 7 and 8, the tumor in the mouse without treatment grew very rapidly, the size of tumor enlarged as a control. In contrast, the tumor in mice with treatment showed regressed growth and the size of tumor is smaller comparing to controls. The difference between FA-pRNA-3WJ-BRCA1siRNA treated group and FA-pRNA-3WJ-Scram-siRNA treated group was statistically different ($P < 0.01$). The result fully demonstrated that prepared FA-pRNA-3WJ-BRCA1 siRNA nanoparticles can specifically inhibit the growth of gastric cancer cells *in vivo*.

Undetectable of organ damage by RNA nanoparticles after systemic injection.

Harris Hematoxylin and Eosin (HE) staining are used to check the potential damage to important organs including the heart, liver, spleen, lung and kidney by the RNA nanoparticles. As shown in Figure 9, no obvious tissue damages were observed, which indirectly suggested that the prepared RNA nanoparticles displayed good biocompatibility and no negative effects on important organs in the body was observed.

In this study, in order to investigate the feasibility of applying RNA nanoparticles as theranostic agents for gastric cancer diagnosis and therapy, the pRNA-3WJ nanoparticle is designed consisting of three fragments, a_{3WJ}, b_{3WJ} and c_{3WJ}, and functionalized with folate, as targeting ligand; Alexa₆₄₇, as imaging module; and BRCAA1 siRNA (or scrambled control), as therapeutic module, respectively. FA-pRNA-3WJ-BRCAA1 siRNA nanoparticles is prepared and the resulting RNA nanoparticles showed good pH and thermodynamic stability, good stability against RNase A (less than 10000U) degradation, and exhibited stability of fluorescent intensity. These results demonstrated that the prepared RNA nanoparticles should be very stable in the blood circulation and can act as high efficient theranostic agent for targeted imaging and siRNA therapy of gastric cancer *in vivo*, which lay foundation for RNA nanoparticles' further clinical application.

Nanotoxicity of nanotheranostic agents has caused broad attention. In this study, prepared RNA nanoparticles did not exhibit obvious toxicity. After being injected into *in vivo* blood circulation *via* tail vein, RNA nanoparticles gradually accumulated in the site of *in vivo* tumor within 6h p.i., clearly displayed the imaging of tumor tissues, and exhibited specific targeting ability. The RNA nanoparticles were also proved to be able to retention in the tumor for long time and generate tumor regression effects.

In addition, the alteration of biochemical parameters in the mice after treating with FA-pRNA-3WJ-BRCAA1 siRNA nanoparticles was investigated as shown in Table 3 and obvious tissue damages were observed for liver and kidneys. To study the effects of RNA nanoparticles on blood biochemical parameters, blood samples were centrifuged twice at 3000rpm for 10 min. Liver function was evaluated with serum levels of alanine aminotransferase(ALT), aspartate aminotransferase(AST). Nephrotoxicity was determined by blood urea nitrogen(BUN) and creatinine(Cr). These parameters were all assayed using a Hitachi 7600 Automatic Biochemical Autoanalyzer.

Table S3. Blood biochemical examination results

WBC	AST	ALT	Cr	UN
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FA-pRNA-3WJ-BRCAA1-siRNA NPs						
20μg	4.3x10 ⁵	32	35	0.005	0.041	
1day						
7day	4.2x10 ⁵	33	35	0.005	0.042	
14 day	4.4x10 ⁵	33	35	0.006	0.043	
60μg	5.2 x10 ⁵	35	42	0.007	0.038	
1 day						
7 day	4.9 x10 ⁵	33	42	0.004	0.039	
14day	4.5 x10 ⁵	34	42	0.005	0.039	
FA-pRNA-3WJ-Scramb-siRNA						
20μg	4.2x10 ⁵	32	36	0.004	0.052	
1day						
7day	4.2x10 ⁵	34	37	0.002	0.054	
14 day	4.2x10 ⁵	35	38			
60μg	4.2x10 ⁵	32	34	0.031	0.045	
1day						
7 day	4.2x10 ⁵	33	35	0.036	0.037	
14 day	4.2x10 ⁵	35	37	0.034	0.038	
Control						
0	3.9x10 ⁵	28	32	0.001	0.008	

Further HE staining results also confirmed that prepared RNA nanoparticles did not damage important organs such as brain, heart, lungs, liver and kidneys. Therefore, it can be confirmed that the prepared RNA nanoparticles should be safe for *in vivo* application.

5 In this study, the results of *in vivo* evaluation of therapeutic efficacy also showed that the prepared RNA nanoparticles can actively target *in vivo* gastric cancer tissues and inhibited tumor growth significantly. However, the concrete molecular mechanism is not well understood. In order to investigate the potential molecular mechanism, Western blotting is used to detect the expression level of BRCAA1, Bcl-2, Rb and Bax in MGC803 cells treated with prepared RNA nanoparticles for 24h and 48 h. As shown in Figure 10, RNA nanoparticles can down-regulate or silence the expression of BRCAA1 gene, down-regulate expression of Bcl-2 gene, adversely up-regulate expression of Rb and Bax genes in MGC803 cells. Based on these results, a molecular mechanism of RNA nanoparticle induced MGC803 growth inhibition is studied: the prepared RNA nanoparticles (FA-pRNA-3WJ-BRCAA1 siRNA) actively bind to the folic acid receptor on the surface of MGC803 cells *via* folic acids conjugated on the RNA nanoparticles, and then induce the endocytosis of RNA nanoparticles into tumor cytoplasm. The double-stranded BRCAA1 siRNA region on the RNA nanoparticle can be recognized by RNA-induced silencing complex (RISC) in the cytoplasm and processed. The released siRNA antisense strand can further recognize target BRCAA1 mRNA, degrade it, and result in silence of BRCAA1 gene in MGC80 3 cells. The down regulate or silencing of the BRCAA1 gene will cause subsequent down-regulation of Bcl-2

gene, and further up-regulation of Rb and Bax gene, which will end up with inducing cell apoptosis and inhibiting the cell growth. The proposed mechanism is summarized in Figure 11 and the concrete study of the regulation signal pathway is under way.

In recent years, BRCAA1 gene, as an important member of ARID family, called as ARID4B, has been found to involve in the regulation of the male fertility and stem cells, ARID4B protein can regulate Rb binding protein 1, which highly suggest that ARID4B may be a tumor suppressor.

Throughout this document, various references are mentioned. All such references are incorporated herein by reference, including the references set forth in the following list.

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CLAIMS

What is claimed is:

1. An artificial RNA nanostructure molecule, comprising: a multiple branched RNA
5 junction motif comprising at least one RNA oligonucleotides, and a gastric cancer targeting
module coupled to the RNA junction motif.
2. The molecule of claim 1, further comprising at least one bioactive agent coupled to
the RNA junction motif.
- 10 3. The molecule of claim 1, wherein the at least one bioactive agent is a therapeutic
agent.
4. The molecule of claim 1, wherein the nanostructure comprises at least one chemical
15 modification at 2' position.
5. The molecule of claim 4, wherein the chemical modification comprises 2'Fluoro,
2'Amine, and 2'O-Methyl.
- 20 6. The molecule of claim 1, wherein the multiple branched RNA comprises sequence 5'
CCACAUAAGGGCCCACUAuuCCCACAUAUUUGUUGAUCC 3'
7. The molecule of claim 1, wherein the multiple branched RNA comprises sequence 5'
UAGUGGGCCCUUUAUGUGG 3'.
- 25 8. The molecule of claim 1, wherein the multiple branched RNA junction motif is a
three-branched RNA junction motif.
9. The molecule of claim 1, wherein the diameter of the molecule is at least about 40 nm
30 or less.
10. The molecule of claim 9, wherein the diameter of the molecule is at least about 20
nm or less.

11. The molecule of claim 10, wherein the diameter of the molecule is at least about 10 nm or less.
12. The molecule of claim 1, wherein the molecule has zeta potential ranging from about
5 -150 mV to about 150 mV.
13. The molecule of claim 1, wherein the molecule has zeta potential ranging from about -125 mv to about 75 mV.
- 10 14. The molecule of claim 1, wherein the molecule is substantially stable in a pH value ranges from about 2 to about 13.
15. The molecule of claim 8, wherein a branch of the three-branched RNA junction motif comprises an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); or
15 a c3WJ RNA module (SEQ ID NO: 3).
16. The molecule of claim 8, wherein the three-branched RNA junction motif comprises an a3WJ RNA module (SEQ ID NO: 1); a b3WJ RNA module (SEQ ID NO: 2); and a c3WJ RNA module (SEQ ID NO: 3).
20
17. The molecule of claim 15 or 16, wherein SEQ ID NO: 1 comprises nucleotide sequence 5'-UUG CCA UGU GUA UGU GGG-3'.
18. The molecule of claim 15 or 16, wherein SEQ ID NO: 2 comprises nucleotide
25 sequence 5'-CCC ACA UAC UUU GUU GAUCC-3'.
19. The molecule of claim 15 or 16, wherein SEQ ID NO: 3 comprises nucleotide sequence 5'-GGA UCA AUC AUG GCA A-3'.
- 30 20. The molecule of claim 1, wherein the gastric cancer targeting module comprises a ligand that binds to at least one gastric cancer cell surface marker.

21. The molecule of claim 20, wherein the ligand binds to a folate receptor, an epidermal growth factor receptor 2 (ErbB-2/HER2), an epidermal growth factor receptor (EGFR), a HER2 or a combination thereof.
- 5 22. The molecule of claim 20, wherein the ligand is an aptamer.
23. The molecule of claim 22, wherein the aptamer binds to EGFR, PDGFR, folate receptor, or a combination thereof.
- 10 24. The molecule of claim 23, wherein the ligand is a EGFR targeting aptamer.
25. The molecule of claim 23, wherein the ligand has sequence 5'- G CCU UAG UAA CGU GCU UUG AUG UCG AUU CGA CAG GAG GC -3'.
- 15 25. The molecule of claim 21, wherein the targeting module is a folate.
26. The molecule of claim 25, wherein the folate is folic acid, 5 -methyltetrahydro folate, 5-formyltetrahydrofolate, dihydrofolate, tetrahydrofolate, or a combination thereof.
- 20 27. The molecule of claim 2, wherein the bioactive agent is a drug, a fluorescent dye, a chemical, or a combination thereof.
28. The molecule of claim 2, wherein the bioactive agent is a siRNA, a miRNA, an anti-mRNA, a ribozyme RNAs, or an antisense RNAs.
- 25 29. The molecule of claim 2, wherein the bioactive agent is directed a gastric cancer marker.
30. The molecule of claim 2, wherein the bioactive agent is a siRNA sequence.
- 30 31. The molecule of claim 30, wherein the siRNA is directed to Survivin, Bcl-2, XIAP, BCL-XL, or BRCA1.
31. The molecule of claim 28, wherein the bioactive agent comprises siRNA sequence 5' GGACCACCGCAUCUCUACAdTdT 3', 5' dTdTCCUGGUGGCGUAGAGAUGU 3', 5'-

AAGCUG_uCACAGAGGGGCUAC-3', 5'GUAGCCCCUCUGUGACAGCUU-3, 5'-
 CCAUGUGCUAUACAGUCAUUACUUU-3', 5'-
 AAAGUAAUGACUGUAUAGCACAUGG-3', 5'-UUGGACAAUGGACUGGUUGA-3', or
 5'-UCAACCAGUCCAUUGUCCAA-3.

5

32. The molecule of claim 31, wherein the siRNA is a BRCAA1 siRNA.

33. The molecule of claim 32, wherein the siRNA is 5'-CCACAUAAGGGCCCACUA-
 3'.

10

34. The molecule of claim 32, wherein the siRNA is 5'-UAGUGGGCCCUUUAUGUGG-
 3'.

35. The molecule of claim 2, wherein the bioactive agent is a microRNA sequence.

15

36. The molecule of claims 35, wherein the bioactive agent is an anti-miRNA molecule
 for a miRNA comprising miR-9, miR-10b, miR-21, or miR-26.

37. The molecule of claim 35, wherein the bioactive agent is a miRNA molecule for a
 20 miRNA comprising let-7a, miR-10b, miR-25, miR-34a, miR-124, miR-145, or miR-181b.

38. A composition, comprising a therapeutically effective amount of the RNA
 nanostructure molecule of any one of the claims 1-37.

25 39. The composition of claim 38, further comprising a pharmaceutically acceptable
 carrier.

40. A nanoparticle delivery system, comprising a RNA nanostructure of any one of
 claims 1-37.

30

41. The nanoparticle delivery system of claim 40, further comprising a pharmaceutically
 acceptable carrier.

42. A method of treating gastric cancer in a subject having or at risk of having gastric cancer, comprising administering to the subject a therapeutically effective amount of a composition comprising a molecule of any one of claims 1-37.

5 43. The method of claim 30, the composition further comprises a pharmaceutically acceptable carrier.

44. The method of claim 42, wherein the subject is a mammal or a non-mammal vertebrate.

10

45. The method of claim 42, wherein the subject is a human.

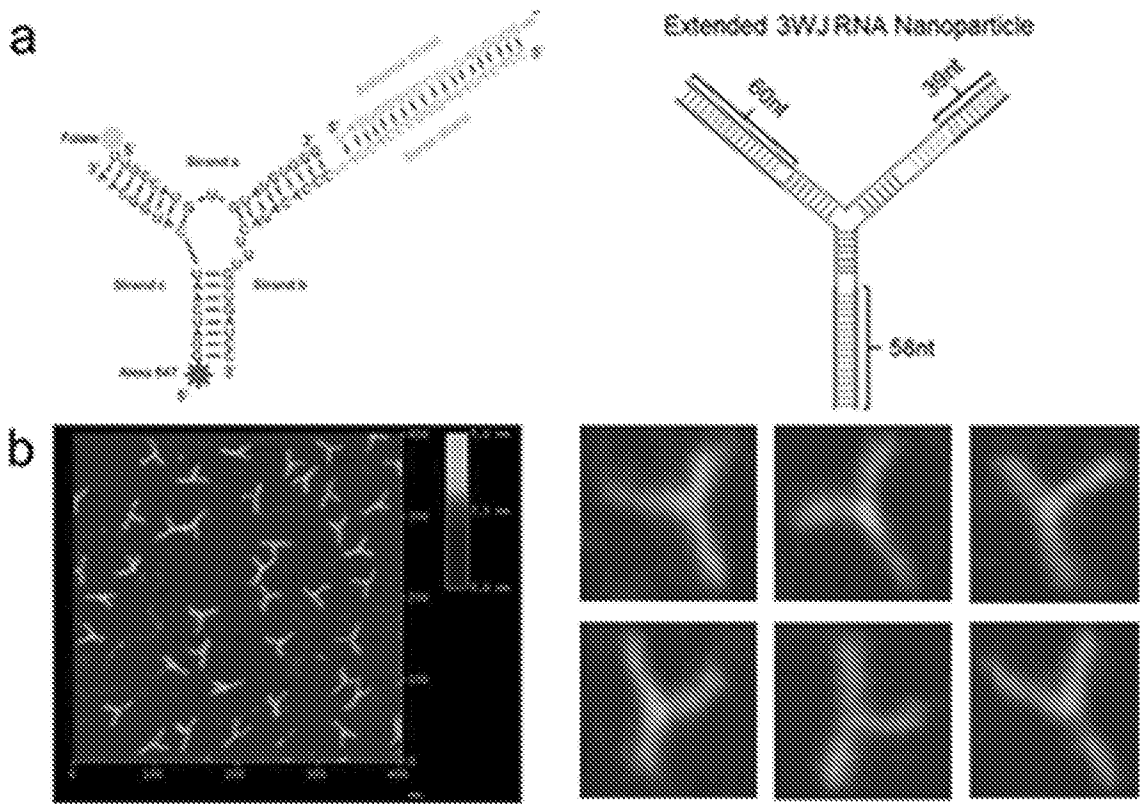


FIG. 1

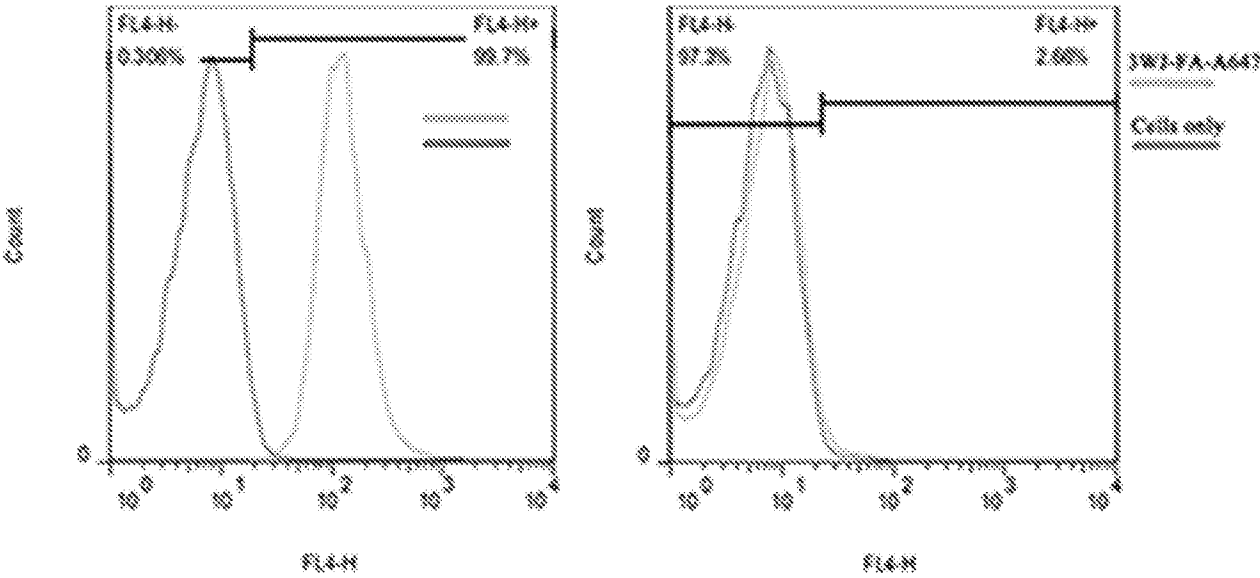


FIG. 2

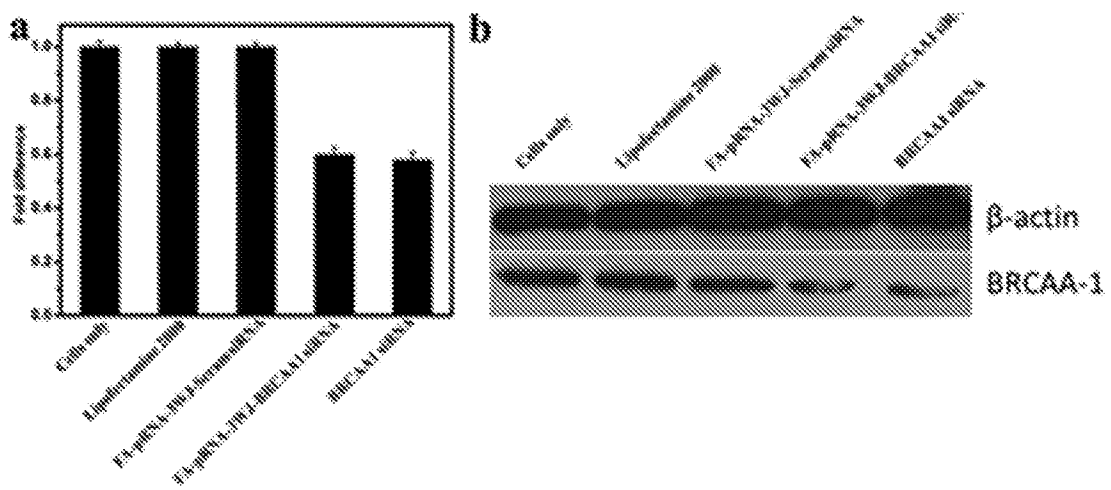


FIG. 3

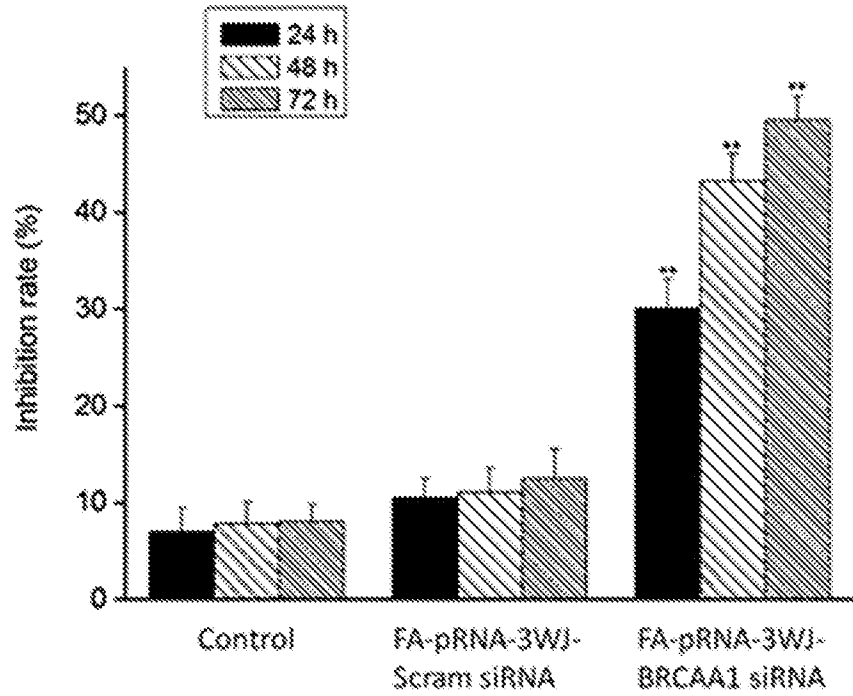


FIG. 4

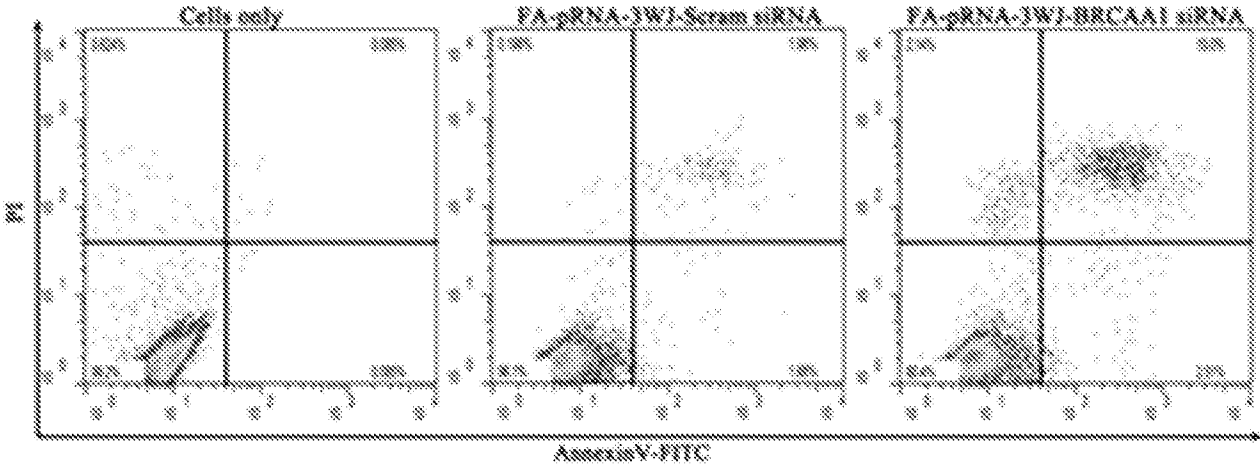


FIG. 5

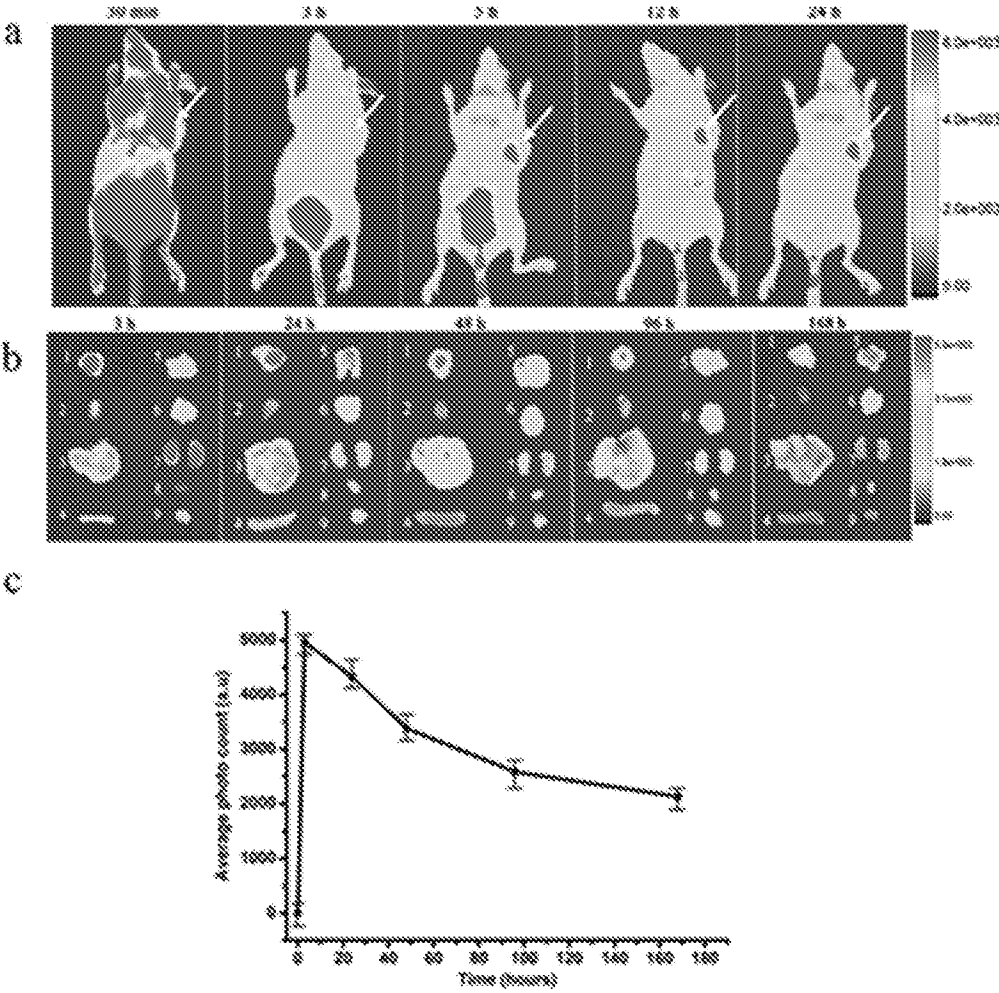


FIG. 6

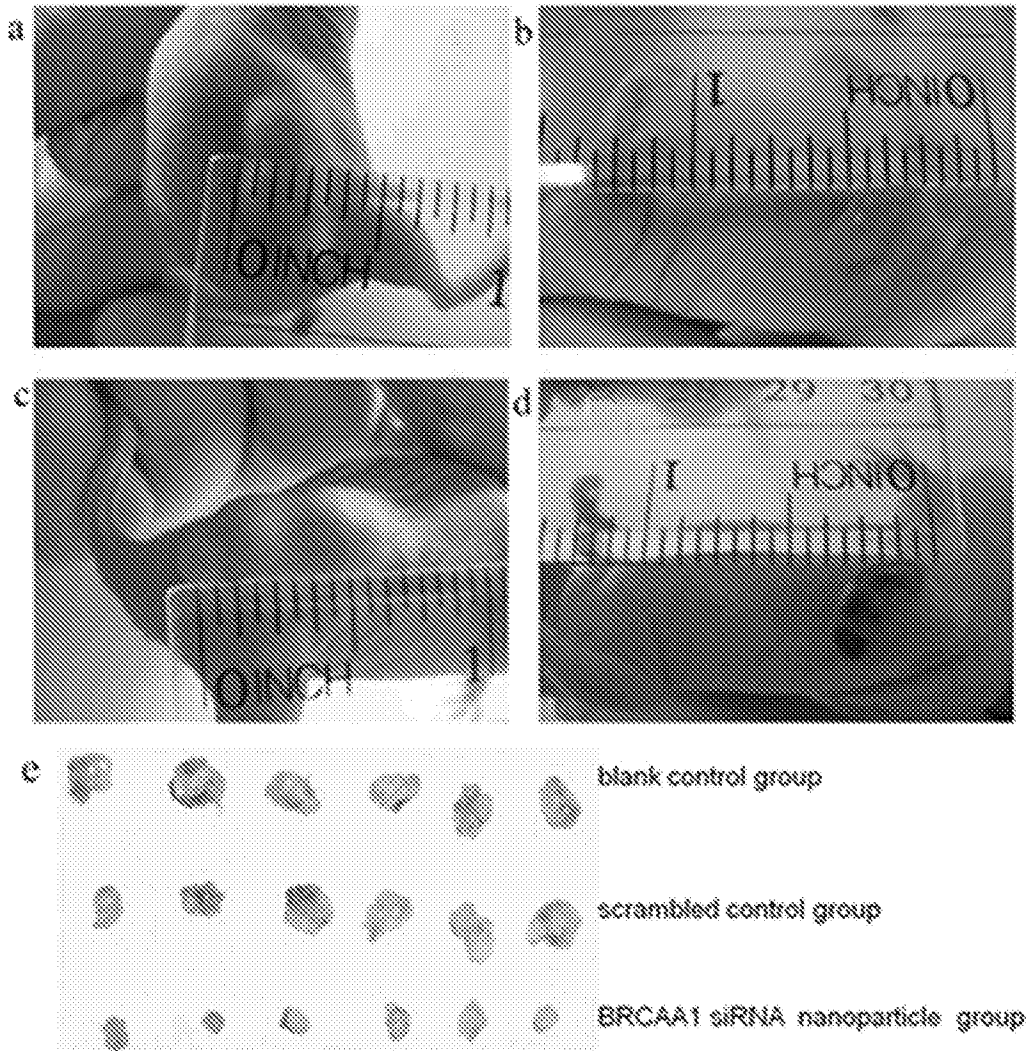


FIG. 7

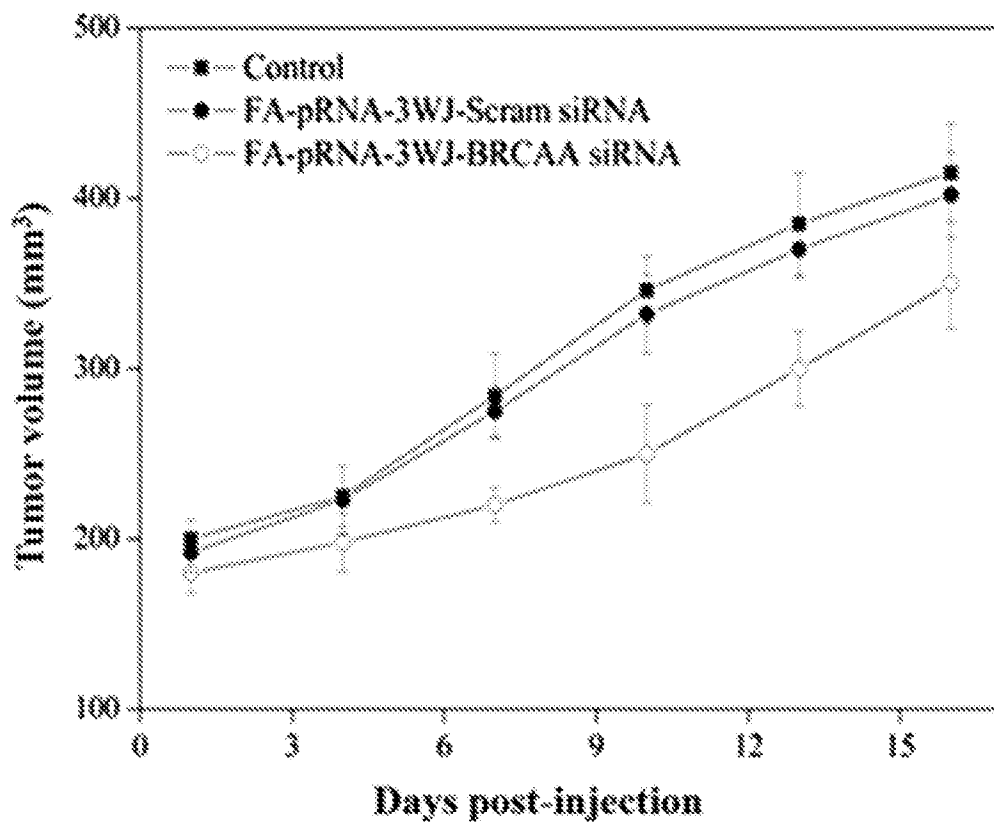


FIG. 8

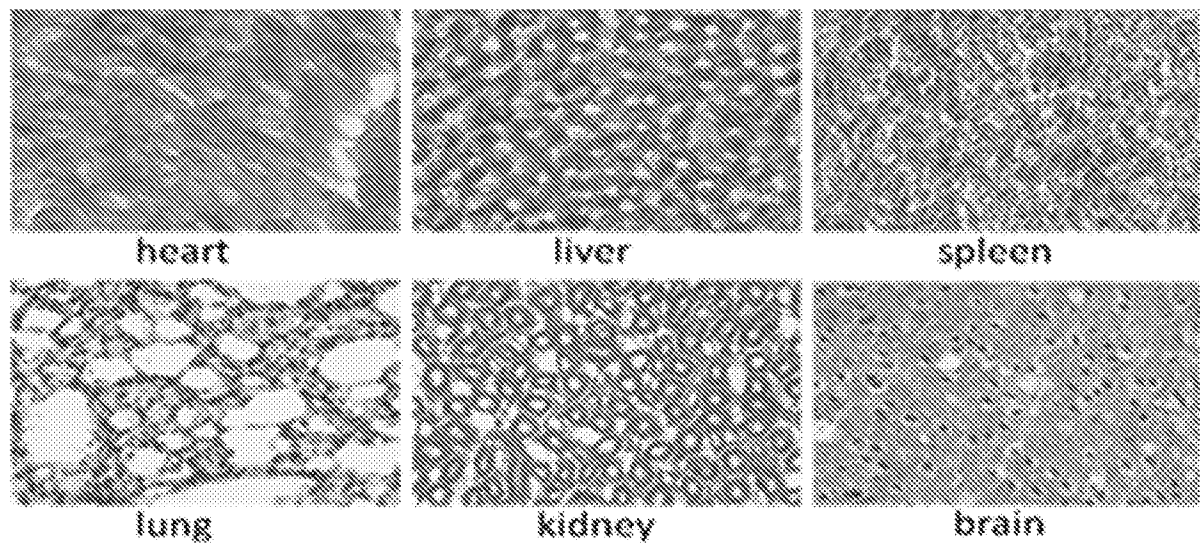


FIG. 9

6/9

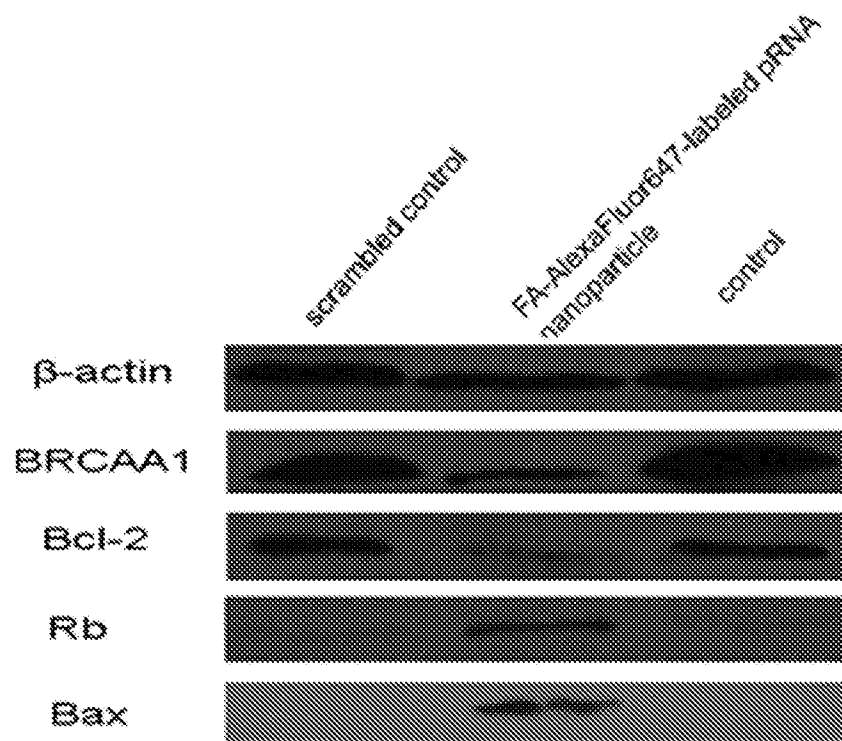


FIG. 10

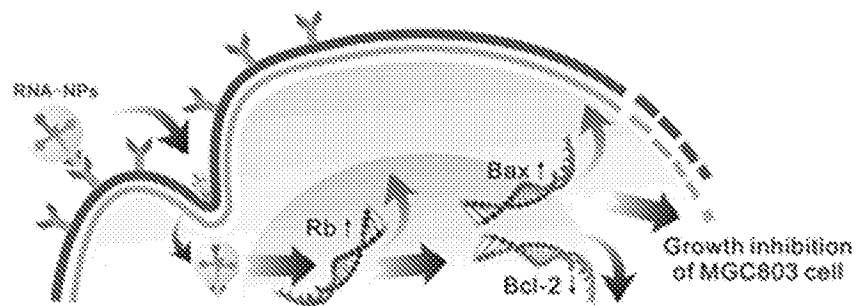


FIG. 11

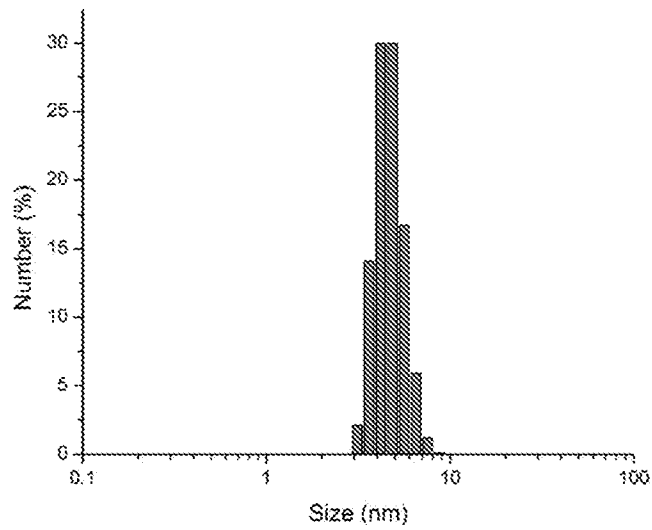


FIG. 12A

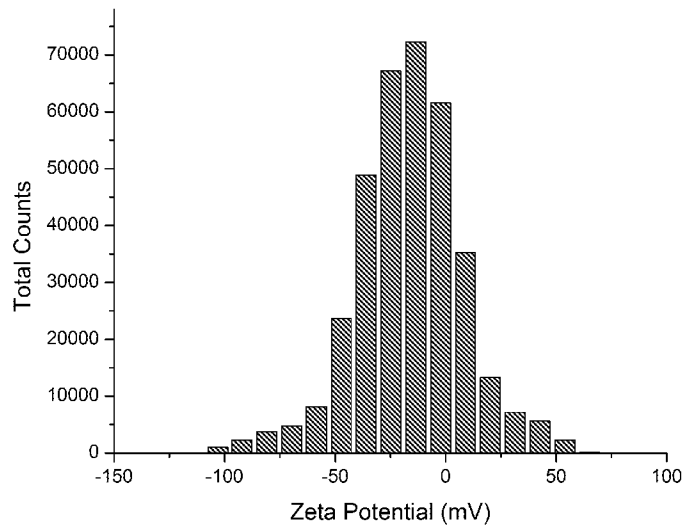


FIG. 12B

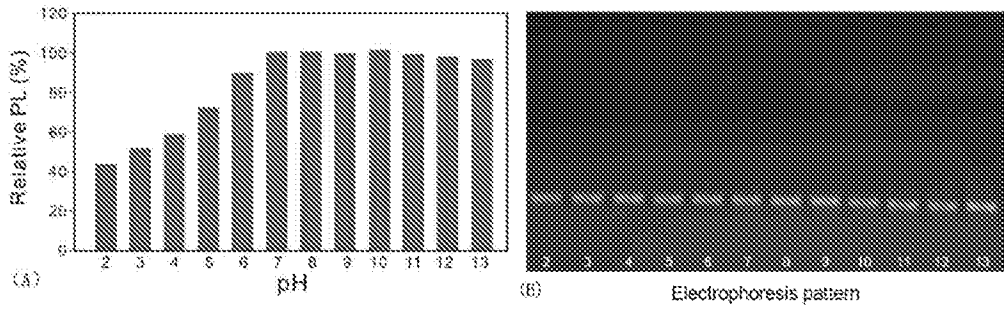


FIG. 13

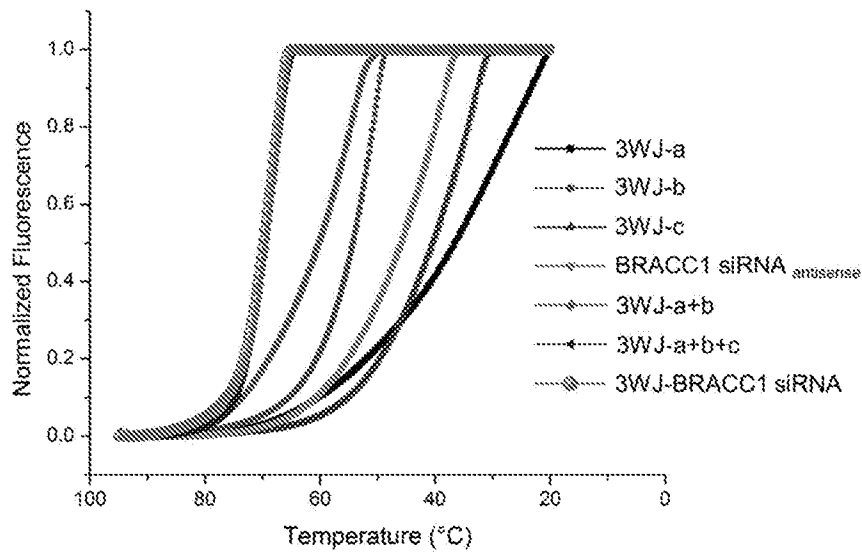


FIG. 14

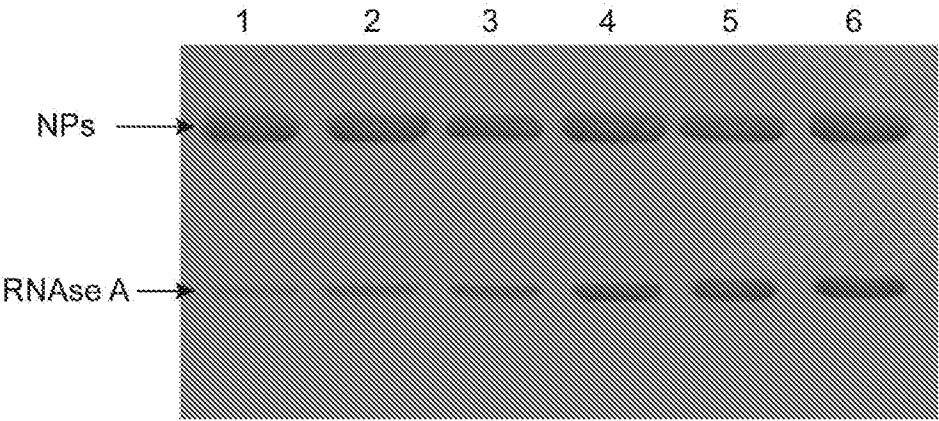


FIG. 15

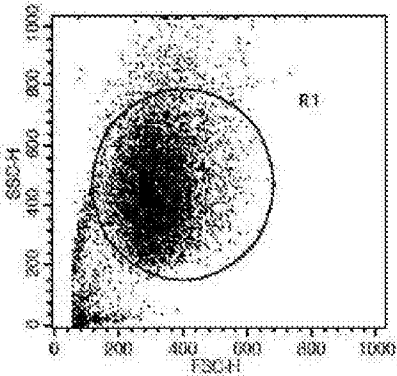


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/021444

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07H 21/04; C12N 15/11; C12N 15/113; C12N 15/115 (2016.01)

CPC - C12N 15/11; C12N 15/113; C12N 15/115; C12N 2310/16 (2016.05)

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)

IPC(8) - C07H 21/04; C12N 15/11; C12N 15/113; C12N 15/115 (2016.01)

CPC - C12N 15/11; C12N 15/113; C12N 15/115; C12N 2310/16 (2016.05)

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

USPC - 514044A; 514044R; 536/23.1; 536/24.5 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar.

Search terms used: branched rna sirna gastric cancer (drug OR fluorescent) target* egfr brcaa1 "zeta potential" mir-10b

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0179758 A1 (GUO) 26 June 2014 (26.06.2014) entire document	1-5, 8-11, 14-23, 25b, 27-30, 31a, 35, 43
Y		6, 7, 12, 13, 24, 25a, 26, 31b, 32-34, 36, 37
Y	CN 104327141 A (SHANGHAI JIAOTONG UNIVERSITY) 04 February 2015 (04.02.2015) entire document; see machine translation	6
Y	US 2005/0221371 A1 (BOUGUELERET) 06 October 2005 (06.10.2005) entire document	7, 33, 34
Y	US 2011/0275785 A1 (MIXSON) 10 November 2011 (10.11.2011) entire document	12, 13
Y	US 2013/0177556 A1 (DE FRANCISCIS et al) 11 July 2013 (11.07.2013) entire document	24, 25a, 26
Y	US 2011/0053862 A1 (XIE et al) 03 March 2011 (03.03.2011) entire document	31b, 32-34
Y	CUI et al. "Characterization of BRCA1 and its Novel Antigen Epitope Identification," Cancer Epidemiology Biomarkers & Prevention, 01 July 2004 (01.07.2004), Vol. 13, No. 7, Pgs. 1136-1145, entire document	32-34
Y	US 2012/0277163 A1 (GAUR ARTI B et al) 01 November 2012 (01.11.2012) entire document	36, 37
P, X	CUI et al. "Regression of Gastric Cancer by Systemic Injection of RNA Nanoparticles Carrying Both Ligand and siRNA," Scientific Reports, 03 July 2015 (03.07.2015), Vol. 5, Pgs. 1-14, entire document	1-24, 25a, 25b, 26-30, 31a, 31b, 32-37, 43

☒ 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

"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

01 June 2016

Date of mailing of the international search report

12 JUL 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, VA 22313-1450

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/021444

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010/0323018 A1 (IRVINE et al) 23 December 2010 (23.12.2010) entire document	1-24, 25a, 25b, 26-30, 31a, 31b, 32-37, 43
A	US 2014/0186430 A1 (RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY) 03 July 2014 (03.07.2014) entire document	1-24, 25a, 25b, 26-30, 31a, 31b, 32-37, 43

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/021444

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:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs:1-20 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/021444

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.: 38-42, 44, 45
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:

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.:

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.