MRNA TARGETING MOLECULE COMPRISING N-ACETYLGALACTOSAMINE BINDING POLYPEPTIDE AND PREPARATION METHOD THEREFOR

[0001] The present application claims the priority of Chinese Patent Application No. 201911300610.2, entitled "MRNA TARGETING MOLECULE COMPRISING N-ACETYLGALACTOSAMINE BINDING POLYPEPTIDE AND PREPARATION METHOD THEREFOR", filed with the China National Intellectual Property Administration on December 17, 2019, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

[0002] The present invention belongs to the field of biotechnology, and in particular relates to an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide and a preparation method therefor.

BACKGROUND OF THE INVENTION

[0003] An asialoglycoprotein receptor (ASGPR) is an abundant hetero-oligomer endocytic receptor, mainly exists on a surface of cell membrane of liver parenchymal cells facing the sinusoid, has specificity to sugar and is an endocytic receptor specifically expressed by hepatocytes. In recent years, the use of ASGPR's high-affinity ligand N-acetylgalactosamine (GalNAc) as a targeting molecule has made breakthroughs in the liver-targeting delivery of nucleic acid drugs, such as small interfering RNAs (siRNAs). Although the receptor has been discovered for many years, the messenger RNA (mRNA) delivery system based on the receptor and its ligands has failed to achieve a breakthrough because the existing technical means cannot achieve effective coupling of mRNA and GalNAc.

[0004] At present, the delivery of mRNAs into cells can be achieved through different methods, such as electroporation, sonoporation, microinjection, or cell transfection based on macromolecular compounds, but these methods are relatively toxic to cells and have certain difficulties in clinical transformations.

SUMMARY OF THE INVENTION

[0005] In view of the above technical problems, the present invention discloses an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide and a preparation method therefor. Through a brand-new mRNA synthesis and modification strategy, GalNAc

modification of mature mRNA molecules is realized, thereby realizing the liver-targeting delivery of mRNA drugs, which is of great significance for innovative basic research, new drug design and development.

[0006] To achieve the above objects of the invention, the present invention provides the following technical solutions:

[0007] a DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide. The DNA fragment comprises a promoter, a target gene, a specific protease cleavage sequence, and a polypeptide GlaNAc Binding Domain (GBD) sequence capable of binding to N-acetylgalactosamine, that are sequentially connected.

[0008] With this technical solution, the DNA fragment can be used to construct an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide.

[0009] As a further improvement of the present invention, the GBD sequence is one or a combination of more than one of SEQ ID NOs.1-5.

[0010] As a further improvement of the present invention, the target gene sequence is set forth in SEQ ID NO. 6 or 7, wherein the target gene can be replaced with other genes, and the corresponding target genes can be selected according to different diseases to be treated.

[0011] As a further improvement of the present invention, the specific protease cleavage sequence is one or more of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with a GBD sequence.

[0012] As a further improvement of the present invention, the promoter is T3, T7 or SP6 promoter.

[0013] The present invention also discloses an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, which comprises an mRNA molecule obtained by *in vitro* transcription using a plasmid containing the above DNA fragment. The sequence of the mRNA molecule sequentially comprises a 5' cap, a target gene sequence, a specific protease cleavage sequence and a polypeptide GBD protein; the polypeptide GBD protein is obtained by ribosomal translation of the GBD sequence; and the GBD sequence end of the mRNA molecule is connected to the GBD protein through puromycin, and the GBD protein is connected to N-acetylgalactosamine through an enzymatic reaction.

[0014] With this technical solution, an mRNA molecule comprising a 5' cap, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence is synthesized by *in vitro*

transcription using the above DNA fragment as a template; under the action of T4 ligase, the mRNA molecule binds to the DNA-puromycin linker to form an mRNA-puromycin complex; through an *in vitro* translation system, the puromycin is connected to the tail of the antibody through the A-site of ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex; and this product is subjected to specific protease cleavage to obtain an mRNA-puromycin-GBD complex.

[0015] Puromycin is an analog of transfer RNA (tRNA), which can bind to the A-site of ribosome during the transcription process and form a peptide bond with the polypeptide fragment being synthesized to block the elongation of the peptide fragment. In addition, puromycin can also bind to the 3' end of RNA or DNA. Based on these properties, by binding a special peptide fragment with puromycin to the 3' end of an RNA molecule, a peptide fragment-RNA fusion molecule (peptide-RNA fusion product) can be formed. In the technical solution of the present invention, based on this principle, an mRNA-peptide fragment fusion molecule is designed and synthesized, and then the coupling of the mRNA molecule and GalNAc is realized through GalNAc modification on the special peptide fragment. Under the action of N-acetylgalactosamine transferase, the N-acetylgalactosamine specifically binds to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex, so that the mRNA molecule can be targeted to liver cells, thereby achieving specific delivery of mRNA drug molecules.

[0016] The present invention also discloses a method for preparing the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide described above, comprising the following steps:

[0017] step S1, selecting a specific cell surface receptor according to the tissue, organ or cell to which the mRNA is delivered, designing a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, and cloning a combination of a promoter sequence, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence into a plasmid vector to obtain a plasmid DNA;

[0018] step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template to obtain an mRNA sequence comprising a 5' cap, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence;

[0019] step S3, under the action of T4 ligase, binding the mRNA molecule to the DNA-puromycin linker to form an mRNA-puromycin complex;

[0020] step S4, *in vitro* translating the mRNA-puromycin complex obtained in step S3, wherein the mRNA-puromycin complex is translated by a ribosome into a fusion protein sequence of gene function protein-specific protease cleavage sequence-GBD;

[0021] step S5, at the end of translation, connecting the puromycin to the tail of the antibody through the A-site of the ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex;

[0022] step S6, cleaving the product obtained in step S5 by a specific protease, wherein the part of the specific protease cleavage sequence-gene function protein in the mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex is cleaved to obtain an mRNA-puromycin-GBD complex; and

[0023] step S7, under the action of N-acetylgalactosamine transferase, specifically binding N-acetylgalactosamine to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex.

[0024] As a further improvement of the present invention, the GBD sequence is set forth in SEQ ID NOS. 1-5.

SEQID No. 1

GGCGGCGGCGGCAGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGC

SEQ ID No. 2

GGCGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGC

SEQ ID No. 3

GGCGGCAGCGGCGGCAGCGGCGGCAGC

SEQ ID No. 4

GGCAGCGGCAGCGGCAGC

SEQ ID No. 5

AGCAGCAGC

[0025] As a further improvement of the present invention, the DNA sequence of the DNA-puromycin linker is set forth in SEQ ID No. 8.

SEQ ID No. 8

AAAAAAAAAAAAAAAAAAAAAAAAACC

[0026] As a further improvement of the present invention, in step S1, the plasmid vector is modified from pCDNA3.1.

[0027] The present invention also discloses a use of the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide described above in the preparation of an mRNA drug for specific drug delivery using the mRNA tissue-specific delivery material targeting N-acetylgalactosamine, wherein N-acetylgalactosamine is connected to the 3' end, and by specifically binding to the asialoglycoprotein receptor on a surface of liver cells through the N-acetylgalactosamine, endocytosis is induced, which allows an mRNA to enter the cell for expression.

[0028] Compared with the prior art, the present invention has the following beneficial effects:

[0029] With the technical solution of the present invention, by connecting the 3' end of the mRNA drug molecule to a fragment of polypeptide sequence capable of coupling with GalNAc, and therefore connecting GalNAc to the mRNA-polypeptide complex, the GalNAc modification on the mature mRNA molecule is realized; the problem that the existing GalNAc conjugation technology can only achieve the direct coupling of GalNAc with a short fragment of RNA is solved. Further, since N-acetylgalactosamine can connect and specifically bind to specific target cells in liver cells, the efficacy of mRNA drug molecules is improved, solving the technical problem of targeted delivery of nucleic acid drugs in the drug delivery process, and achieving the purpose of tissue-specific delivery through GalNAc modification on mRNA without using physical methods and chemical transfection reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is a schematic flow chart of a method for preparing the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide of the present invention; wherein a) is a schematic diagram of DNA fragment in a plasmid DNA; b) is a schematic diagram of an mRNA obtained by *in vitro* transcription using the plasmid DNA as a template and DNA-puromycin linker in a to-be-bond configuration; c) is a schematic diagram of the mRNA-puromycin complex; d) is a schematic diagram of the *in vitro* translation of the mRNA-puromycin complex; e) is a schematic diagram of cleavage of the mRNA-puromycin-GBD-specific protease obtained after translation to obtain an mRNA-puromycin-GBD complex; f) is a schematic diagram of the coupling of the mRNA-puromycin-GBD complex with GalNAc; and g) is a schematic diagram of the finally obtained mRNA-puromycin-GBD-GalNAc complex;

[0031] Figure 2 is a schematic diagram of the principle of the GalNAc-mediated mRNA liver cell delivery system of the present invention, in which the GalNAc-mRNA conjugate causes endocytosis by binding to ASGPR on the liver cell surface, thereby allowing the mRNA to enter the cell;

[0032] Figure 3 is a schematic diagram of the optimization of the GalNAc-mRNA liver cell delivery system of the present invention, in which, the triple GalNAc-mRNA conjugate has the highest transfection efficiency for liver cells;

[0033] Figure 4 is a schematic diagram of the comparison of expression of green fluorescent protein (GFP) in liver cells between the GalNAc-mRNA liver cell delivery system of the present invention and controls; and

[0034] Figure 5 is a schematic diagram of the results of delivering luciferase (Luc) to the liver tissue *in vivo* using the GalNAc-mRNA liver cell delivery system of the present invention and controls.

DETAILED DESCRIPTION OF THE INVENTION

[0035] In order to better understand the present invention, the specific embodiments of the present invention will be further described in detail below in conjunction with the accompanying drawings.

[0036] A DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, the DNA fragment comprises a promoter, a target gene, a specific protease cleavage sequence, and a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, that are sequentially connected.

[0037] Further, the GBD sequence is one or a combination of more than one of SEQ ID NOs.1-5.

[0038] The target gene sequence is set forth in SEQ ID NO. 6 or 7.

[0039] The specific protease cleavage sequence is one or more of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with the GBD sequence.

[0040] The promoter is T3, T7 or SP6 promoter.

[0041] Based on the DNA fragment constructed above, the present invention discloses an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, which comprises an mRNA molecule obtained by *in vitro* transcription using a plasmid containing the above DNA fragment. The sequence of the mRNA molecule sequentially comprises a 5' cap, a

target gene sequence, a specific protease cleavage sequence and a polypeptide GBD protein; the polypeptide GBD protein is obtained by ribosomal translation of the GBD sequence; and the GBD sequence end of the mRNA molecule is connected to the GBD protein through puromycin, and the GBD protein is connected to N-acetylgalactosamine through an enzymatic reaction.

[0042] The mRNA tissue-specific delivery material targeting N-acetylgalactosamine is prepared by the following steps:

[0043] step S1, as shown in Figure 1, selecting a specific cell surface receptor according to the tissue, organ or cell to which the mRNA is delivered, designing a fragment of polypeptide sequence (GBD) capable of binding to N-acetylgalactosamine (GalNAc), and cloning a combination of the relevant cloning elements into a pCDNA3.1 plasmid vector;

[0044] step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template, an mRNA sequence generated by the *in vitro* transcription comprising a 5' cap, a target gene sequence, and a specific protease cleavage sequence with a GBD sequence; and

[0045] the specific protease cleavage sequence is one or more of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO;

[0046] step S3, under the action of T4 ligase, binding the mRNA molecule to a DNA-puromycin linker to form an mRNA-puromycin complex;

[0047] step S4, *in vitro* translating the mRNA-puromycin complex obtained in step S3, wherein the mRNA-puromycin complex is translated by a ribosome into a fusion protein sequence of gene function protein-specific protease cleavage sequence-GBD;

[0048] step S5, at the end of translation, connecting the puromycin to the tail of the antibody through the A-site of the ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex;

[0049] step S6, cleaving the product obtained in step S5 by a specific protease, wherein under the action of 2A peptide self-cleavage or TEV, VLP1, and SUMO specific proteases, the part of the specific protease cleavage sequence-gene function protein in the mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex is cleaved to obtain an mRNA-puromycin-GBD complex; and

[0050] step S7, under the action of N-acetylgalactosamine transferase, specifically binding N-acetylgalactosamine to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex.

[0051] Wherein, the sequence of the DNA-puromycin linker is set forth in SEQ ID NO. 8; and the GBD sequence is set forth in SEQ ID NOs. 1-5.

[0052] Further, in step S1, the plasmid vector is modified from pCDNA3.1.

[0053] In the preparation of an mRNA drug for specific drug delivery using the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide described above, a GalNAc-mediated mRNA liver cell delivery system is formed. In this system, N-acetylgalactosamine is connected to the 3' end, and by specifically binding to the asialoglycoprotein receptor on a surface of liver cells through the N-acetylgalactosamine, endocytosis is induced, which allows an mRNA to enter the cell for expression, as shown in Figure 2.

[0054] There are multiple design schemes for the GBD in the GBD-GalNAc sequence based on different designs. According to the needs, a GBD bond with only one GalNAc, a GBD bond with two GalNAcs, a GBD bond with three GalNAcs or a GBD bond with n GalNAcs can be generated. Further preferably, the use of the triple GalNAc-mRNA conjugate has the highest transfection efficiency for liver cells, and the comparison results are shown in Figure 3.

[0055] Specific operation procedures:

[0056] Cell transfection

[0057] About 24 hours after seeding 293T cells (purchased from the Cell Bank of the Chinese Academy of Sciences), the status of the cells in a 6-well plate was observed, until the confluence reached 88% - 92%. In the biological safety cabinet, 90% (volume percentage) DMEM + 10% (volume percentage) FBS medium was prepared. 30 minutes before transfection, the medium in the plate was discarded, and 1 mL of fresh medium, that is, 90% (volume percentage) DMEM + 10% (volume percentage) FBS medium was added to each well.

[0058] Preparation of the transfection system: 200 μL opti-MEM was taken, and 10 μg of the test product (including mRNA-GalNAc1, mRNA-GalNAc2, mRNA-GalNAc3, mRNA/lipo2000, mRNA/lipo3000, mRNA/LNP, mRNA/TransIT, mRNA/lipo RNAiMAX, and mRNA/In vivo-jetPEI, at a concentration of 2 μg/μL, 5 μL) or a negative control of vector-free GFP-mRNA was added. The prepared transfection system was directly and evenly added dropwise into the cultured cells, followed by shaking well on all sides to make the transfection system evenly distributed on the cells. The medium was changed 6 hours after transfection, the old medium was aspirated, and each well was replaced with 2 mL of fresh medium (90% DMEM + 10% FBS). The fluorescence intensity was measured under a fluorescence microscope 36 hours after

transfection. The experimental results are shown in Figure 3, in which the mRNA expression intensity of the mRNA-GalNAc group is significantly higher than that of other vector groups, and the triple GalNAc-mRNA conjugate achieves the highest transfection efficiency.

[0059] The present invention will be further exemplified below through specific examples, and the examples are only used to explain the present invention, instead of limiting the scope of the present invention.

Example 1

[0060] Provided herein is an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, which is a novel mRNA drug capable of specific binding to liver cells. Wherein, the GalNAc modification of the mRNA molecule was bound to the GBD protein sequence of the mRNA-puromycin-GBD molecule by an N-acetylgalactosamine transferase, to form an mRNA-puromycin-GBD-GalNAc molecule. Puromycin was connected to the GBD polypeptide sequence; the mRNA molecule was obtained by *in vitro* transcription using a plasmid containing the above DNA fragment, the sequence of the mRNA molecule sequentially comprised a 5' cap, a target gene sequence, a specific protease cleavage sequence, and a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, and the GBD polypeptide was obtained by ribosomal translation of the GBD sequence. The mRNA targeting molecule was prepared by the following steps:

[0061] step S1, on the basis that the liver cell was the tissue to which the mRNA is delivered, selecting the green fluorescent protein mWasabi as the target gene, and designing a fragment of polypeptide sequence (GBD) capable of binding to N-acetylgalactosamine (GalNAc). The combination of the promoter sequence, the target gene sequence, the specific protease cleavage sequence, and the GBD sequence was cloned into the pCDNA3.1 plasmid vector to obtain the plasmid DNA.

[0062] In this example, the GBD sequence was one or a combination of more than one of SEQ ID NOs.1-5.

SEQID No. 1

GGCGGCGGCGGCAGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGC

SEQ ID No. 2

GGCGGCGGCAGCGGCGGCGGCAGCGGCGGCGGCAGC

SEQ ID No. 3

GGCGGCAGCGGCGGCAGCGGCGGCAGC

SEQ ID No. 4

GGCAGCGGCAGCGGCAGC

SEQ ID No. 5

AGCAGCAGC

[0063] In this example, the GBD as set forth in SEQ ID No. 2 was used.

[0064] The target gene sequence was set forth in SEQ ID NO. 6.

SEQ ID No. 6

GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC
CTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTG
TCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
AAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC
TCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCC
CGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAA
GGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACT
ACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGA
ACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACA
TCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTA
TATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAG
ATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACT
ACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCG
ACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA
ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGC
CGGGATCACTCTCGGCATGGACGAGCTGTACAAGAAGCTTAGCCAT
GGCTTCCCGCCGGCGGTGGCGGCGCAGGATGATGGCACGCTGCCCA
TGTCTTGTGCCCAGGAGAGCGGGATGGACCGTCACCCTGCAGCCTG
TGCTTCTGCTAGGATCAATGTG

[0065] The specific protease cleavage sequence was one or more of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with a GBD sequence. In this example, the specific protease cleavage sequence used was Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser), as set forth in SEQ ID No. 9 and SEQ ID No. 10.

[0066] The promoter was T3, T7 or SP6 promoter. In this example, the T7 promoter was used, and the sequence was set forth in SEQ ID No. 11:

SEQ ID No.11: TAATACGACTCACTATAGG

[0067] The DNA sequence of the DNA-puromycin linker was set forth in SEQ ID No. 8;

SEQ ID No.8: AAAAAAAAAAAAAAAAAAAAAAAAAACC

[0068] step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template, an mRNA sequence generated by the *in vitro* transcription comprising a 5' cap, a gene sequence, and one or more sequences of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with a GBD sequence.

[0069] step S3, under the action of T4 ligase, binding the mRNA molecule to the DNA-puromycin linker to form an mRNA-puromycin complex;

[0070] step S4, *in vitro* translating the mRNA-puromycin complex obtained in step S3, wherein the mRNA-puromycin complex was translated by a ribosome into a fusion protein sequence of gene function protein-specific protease cleavage polypeptide sequence-GBD polypeptide;

[0071] step S5, at the end of translation, connecting the puromycin to the tail of the antibody through the A-site of the ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex;

[0072] step S6, cleaving the product obtained in step S5 by a specific protease, wherein under the action of 2A peptide self-cleavage or TEV, VLP1, and SUMO specific proteases, the part of the specific protease cleavage sequence-gene function protein in the mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex was cleaved to obtain an mRNA-puromycin-GBD polypeptide complex; and

[0073] step S7, under the action of N-acetylgalactosamine transferase, specifically binding the N-acetylgalactosamine to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex.

[0074] The mRNA-puromycin-GBD-GalNAc complex can be used to specifically bind to the ASGPR receptor on a surface of liver cells to achieve specific liver delivery of an mRNA.

[0075] The above mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide was used to prepare an mRNA drug for specific drug delivery. Accordingly, a GalNAc-mediated mRNA liver cell delivery system was formed, with its 3' end connected to N-acetylgalactosamine. By specifically binding to the asialoglycoprotein receptor on a surface of

liver cells through the N-acetylgalactosamine, endocytosis is induced, which allows an mRNA to enter the cell for expression. Comparative experiments showed that, as shown in Figure 4, the delivery system comprising the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide was more efficient in expression of green fluorescent protein (GFP) in liver cells than the existing mRNA and mRNA/LNP delivery systems.

[0076] Specific operation procedures:

[0077] Cell transfection

[0078] About 24 hours after seeding 293T cells (purchased from the Cell Bank of the Chinese Academy of Sciences), the status of the cells in a 6-well plate was observed, until the confluence reached 88% - 92%. In the biological safety cabinet, 90% (volume percentage) DMEM + 10% (volume percentage) FBS medium was prepared. 30 minutes before transfection, the medium in the plate was discarded, and 1 mL of fresh medium, that is, 90% (volume percentage) DMEM + 10% (volume percentage) FBS medium was added to each well.

[0079] Preparation of the transfection system: 200 μL opti-MEM was taken, and 10 μg of the test product (GFP mRNA-GalNAc and GFP mRNA/LNP, at a concentration of 2 μg/μL, 5 μL) or a negative control of vector-free GFP-mRNA (at a concentration of 2 μg/μL, 5 μL) was added. The prepared transfection system was directly and evenly added dropwise into the cultured cells, followed by shaking well on all sides to make the transfection system evenly distributed on the cells. The medium was changed 6 hours after transfection, the old medium was aspirated, and each well was replaced with 2 mL of fresh medium (90% DMEM + 10% FBS). The fluorescence intensity was measured under a fluorescence microscope 36 hours after transfection. The experimental results were shown in Figure 4, in which the mRNA expression intensity of the mRNA-GalNAc group is significantly higher than that of the LNP vector group.

Example 2

[0080] Provided herein is an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, which was prepared by the following steps:

[0081] step S1, on the basis that the liver cell was the tissue to which the mRNA is delivered, selecting the luciferase (Luc) as the target gene, and designing a fragment of polypeptide sequence (GBD) capable of binding to N-acetylgalactosamine (GalNAc), and cloning a combination of the relevant cloning elements into the pCDNA3.1 plasmid vector, wherein, the DNA fragment in the plasmid DNA included a promoter, a target gene, a specific protease

cleavage sequence, and a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, that were sequentially connected.

[0082] In this example, the GBD as set forth in SEQ ID No. 2 was used as the GBD sequence.

[0083] The target gene sequence was set forth in SEQ ID NO. 7.

SEQ ID No.7

GTGAGCAAGGGCGAGGAGACCACAATGGGCGTAATCAAGCCC
GACATGAAGATCAAGCTGAAGATGGAGGGCAACGTGAATGGCCAC
GCCTTCGTGATCGAGGGCGAGGGCGAGGGCAAGCCCTACGACGGC
ACCAACACCATCAACCTGGAGGTGAAGGAGGGAGCCCCCCTGCCC
TTCTCCTACGACATTCTGACCACCGCGTTCAGTTACGGCAACAGGG
CCTTCACCAAGTACCCCGACGACATCCCCAACTACTTCAAGCAGTC
CTTCCCCGAGGGCTACTCTTGGGAGCGCACCATGACCTTCGAGGAC
AAGGGCATCGTGAAGGTGAAGTCCGACATCTCCATGGAGGAGGAC
TCCTTCATCTACGAGATACACCTCAAGGGCGAGAACTTCCCCCCCA
ACGGCCCCGTGATGCAGAAGGAGACCACCGGCTGGGACGCCTCCA
CCGAGAGGATGTACGTGCGCGACGGCGTGCTGAAGGGCGACGTCA
AGATGAAGCTGCTGCTGGAGGGCGGCGGCCACCACCGCGTTGACT
TCAAGACCATCTACAGGGCCAAGAAGGCGGTGAAGCTGCCCGACTA
TCACTTTGTGGACCACCGCATCGAGATCCTGAACCACGACAAGGAC
TACAACAAGGTGACCGTTTACGAGATCGCCGTGGCCCGCAACTCCA
CCGACGGCATGGACGAGCTGTACAAG

[0084] In this example, the specific protease cleavage sequence used was Glu-Asn-Leu-Tyr-Phe-Gln-(Gly/Ser), as set forth in SEQ ID No. 9 and SEQ ID No. 10. The promoter was T3, T7 or SP6 promoter.

[0085] In this example, the T7 promoter was used, and the sequence was set forth in SEQ ID No. 11:

SEQ ID No.11: TAATACGACTCACTATAGG

[0086] The DNA sequence in the DNA-puromycin linker was set forth in SEQ ID No. 8;

SEQ ID No.8

AAAAAAAAAAAAAAAAAAAAAAAAAACC

[0087] step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template, an mRNA sequence generated by the *in vitro* transcription comprising a 5' cap, a gene sequence, and one or more sequences of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with a GBD sequence.

[0088] step S3, under the action of T4 ligase, binding the mRNA molecule to the DNA-puromycin linker to form an mRNA-puromycin complex;

[0089] step S4, *in vitro* translating the mRNA-puromycin complex obtained in step S3, wherein the mRNA-puromycin complex was translated by a ribosome into a fusion protein sequence of gene functional protein-specific protease cleavage sequence-GBD.

[0090] step S5, at the end of translation, connecting the puromycin to the tail of the antibody through the A-site of the ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex.

[0091] step S6, cleaving the product obtained in step S5 by a specific protease, wherein under the action of 2A peptide self-cleavage or TEV, VLP1, and SUMO specific proteases, the part of the specific protease cleavage sequence-gene function protein in the mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex was cleaved to obtain the mRNA-puromycin-GBD complex.

[0092] step S7, under the action of N-acetylgalactosamine transferase, specifically binding the N-acetylgalactosamine to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex.

[0093] The above mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide was used to prepare an mRNA drug for specific drug delivery. Accordingly, a GalNAc-mRNA delivery system was formed.

[0094] Specific operation procedures:

[0095] The luciferase modified Luc mRNA-GalNAc, Luc mRNA/LNP and Luc mRNA prepared in the above example were directly introduced into the systemic circulation of mice via tail vein administration, and the expression intensity of the modified mRNA *in vivo* was characterized via the *in vivo* biofluorescence signals.

[0096] Tail vein injection

[0097] Balb/c mice were fixed on the platform for tail vein injection, and 200 μg of the above three mRNA drugs (1 μg/μg, 200 μL) were injected, respectively. Fluorescence imaging observation was performed 24 hours later.

[0098] Small animal imaging

[0099] D-fluorescein substrate was dissolved in a physiological saline to obtain a solution at a concentration of 15 mg/mL, and 100 μL of the solution was injected into the mice through the tail vein. 10 minutes later, the IVIS small animal imaging system was used to quantitatively analyze the signal intensity in the lung.

[00100] Comparative experiments showed that, as shown in Figure 5, the GalNAc-mRNA delivery system could be more efficient in delivering luciferase (Luc) to the liver tissue than the existing mRNA and mRNA/LNP delivery systems.

[00101] The foregoing description is only the preferred embodiments of the present invention. It should be noted that for those of ordinary skill in the art, several improvements and embellishments can be made without departing from the principle of the present invention, and these improvements and embellishments are also deemed to be within the scope of protection of the present invention.

CLAIMS

WHAT IS CLAIMED IS:

1. A DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, comprising a promoter, a target gene, a specific protease cleavage sequence, and a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, that are sequentially connected.

2. The DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to claim 1, wherein the polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine is one or a combination of more than one of SEQ ID NOs.1-5.

3. The DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to claim 2, wherein the sequence of the target gene is set forth in SEQ ID NO. 6 or SEQ ID NO. 7.

4. The DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to any one of claims 1 to 3, wherein the specific protease cleavage sequence is one or more of T2A, P2A, E2A, F2A, TEV, VLP1 and SUMO specific protease cleavage sequences with a GBD sequence.

5. The DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to claim 4, wherein the specific protease cleavage sequence is set forth in SEQ ID NO. 9 or SEQ ID NO. 10.

6. The DNA fragment for constructing an mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to any one of claims 1 to 4, wherein the promoter is T3, T7 or SP6 promoter.

7. An mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide, comprising an mRNA molecule, wherein the mRNA molecule is obtained by *in vitro* transcription using a plasmid containing the DNA fragment according to any one of claims 1 to 5;

the sequence of the mRNA molecule sequentially comprises a 5' cap, a target gene sequence, a specific protease cleavage sequence and a polypeptide GBD protein;

the polypeptide GBD protein is obtained by ribosomal translation of the GBD sequence; and

the GBD sequence end of the mRNA molecule is connected to the GBD protein through puromycin, and the GBD protein is connected to N-acetylgalactosamine through an enzymatic reaction.

8. A method for preparing the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to claim 7, comprising the following steps:

step S1, selecting a specific cell surface receptor according to the tissue, organ or cell to which the mRNA is delivered, designing a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, and cloning a combination of a promoter sequence, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence into a plasmid vector to obtain a plasmid DNA; and

step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template to obtain an mRNA sequence comprising a 5' cap, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence.

9. Use of the mRNA targeting molecule comprising an N-acetylgalactosamine binding polypeptide according to claim 7 in the preparation of an mRNA drug for specific drug delivery, wherein N-acetylgalactosamine is connected to the 3' end, and by specifically binding to the asialoglycoprotein receptor on a surface of liver cells through the N-acetylgalactosamine, endocytosis is induced, which allows an mRNA to enter the cell for expression.

10. A method for preparing an mRNA-puromycin-GBD-GalNAc complex, comprising the following steps:

step S1, selecting a specific cell surface receptor according to the tissue, organ or cell to which the mRNA is delivered, designing a polynulcleotide sequence encoding a GBD capable of binding to N-acetylgalactosamine, and cloning a combination of a promoter sequence, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence into a plasmid vector to obtain a plasmid DNA;

step S2, performing *in vitro* transcription using the plasmid DNA of step S1 as a template to obtain an mRNA sequence comprising a 5' cap, a target gene sequence, a specific protease cleavage sequence, and a GBD sequence;

step S3, under the action of T4 ligase, binding the mRNA molecule to a DNA-puromycin linker to form an mRNA-puromycin complex;

step S4, *in vitro* translating the mRNA-puromycin complex obtained in step S3, wherein the mRNA-puromycin complex is translated by a ribosome into a fusion protein sequence of gene function protein-specific protease cleavage sequence-GBD;

step S5, at the end of translation, connecting the puromycin to the tail of the antibody through the A-site of the ribosome to form an mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex;

step S6, cleaving the product obtained in step S5 by a specific protease, wherein the part of the specific protease cleavage sequence-gene function protein in the mRNA-puromycin-GBD-specific protease cleavage sequence-gene function protein complex is cleaved to obtain an mRNA-puromycin-GBD complex; and

step S7, under the action of N-acetylgalactosamine transferase, specifically binding N-acetylgalactosamine to the GBD protein sequence to form an mRNA-puromycin-GBD-GalNAc complex.

11. The method for preparing an mRNA-puromycin-GBD-GalNAc complex according to claim 10, wherein the sequence of the DNA-puromycin linker is set forth in SEQ ID NO. 8; and the GBD sequence is set forth in any one of SEQ ID NOs. 1-5.

12. The method for preparing an mRNA-puromycin-GBD-GalNAc complex according to claim 10, wherein in step S1, the plasmid vector is modified from pCDNA3.1.

13. The method for preparing an mRNA-puromycin-GBD-GalNAc complex according to claim 10, wherein in step S7, the GBD protein sequence binds to one N-acetylgalactosamine, to two N-acetylgalactosamines, to three N-acetylgalactosamines, or to n N-acetylgalactosamines.

14. A GalNAc-mRNA delivery system, comprising the mRNA-puromycin-GBD-GalNAc complex prepared by the method of any one of claims 10 to 13.

15. Use of the GalNAc-mRNA delivery system according to claim 14 in targeted treatment of a liver disease.

16. The use according to claim 15, wherein the GalNAc-mRNA delivery system is introduced into the body.