ANTI-VEGFA FUSION CONSTRUCT AS WELL AS PREPARATION METHOD THEREFOR AND USE THEREOF

TECHNICAL FIELD

The present disclosure relates to the technical field of biological medicines, and particularly relates to an anti-VEGFA fusion construct, a preparation method therefor, and use thereof.

BACKGROUND

Vascular endothelial growth factor A (VEGFA), an important member of the VEGF family, is a major angiogenic factor that can promote vascular endothelial cell differentiation, proliferation, migration, and invasion, and regulate the formation and development of blood vessels. VEGFA promotes angiogenesis mainly by binding to and activating the receptor VEGFR2 (VEGF Receptor 2) and downstream pathways thereof. The binding of VEGFA to the extracellular domain of VEGFR2 results in receptor dimerization, protein kinase activation, tyrosine phosphorylation, and activation of downstream signaling pathways, stimulates the proliferation of endothelial cells, and increases vascular permeability. Activation of the VEGF signaling pathways occurs in a variety of diseases including cancer, autoimmune diseases, retinopathy, and the like. Inhibiting the VEGF signaling pathways can treat diseases associated with abnormal vascular proliferation, such as wet age-related macular degeneration (wAMD). At present, a number of anti-VEGF protein drugs are available on the market for the treatment of tumors and diseases associated with ocular neovascularization.

The first anti-VEGFA protein drug is the monoclonal antibody bevacizumab (Avastin). In addition, anti-VEGFA protein drugs also include ranibizumab (Lucentis), aflibercept, brolucizumab (Beovu), and the like. Among these, free aflibercept has a half-life of about 11 days in human aqueous humor, which is longer than that of ranibizumab (7 days) and bevacizumab (9 days); the administration interval for aflibercept is once every 2 months. Currently, increasing the administration dose of aflibercept from 2 mg to 8 mg has been proven in clinical trials to further extend the administration interval to once every 3 or 4 months. Brolucizumab (Beovu) can be prepared into a 120 mg/mL protein solution, and the quantity of the protein in a single intravitreal injection can reach 6 mg, which is currently the maximum dose for a single injection. In the administration regimen of brolucizumab, patients receive an injection once a month for the first three months, and subsequently once every 3 months, yielding a visual acuity improvement that is non-inferior to the control group receiving aflibercept (injected once every 2 months). The route of administration of these anti-VEGF protein drugs is intravitreal injection, with an administration volume not exceeding 100 μL. The longest administration interval for brolucizumab is 3 months, while the administration interval for other drugs is 1 to 2 months between two injections. Frequent intraocular injections increase the risk of injection-induced complications such as elevated intraocular pressure, intraocular inflammation, and retinal detachment. Therefore, there is an urgent clinical need for long-acting anti-VEGFA therapeutic drugs to reduce the number of injections in patients.

Methods in the prior art for reducing the frequency of administration include increasing the concentration of the protein drug to increase the dose of the drug per single injection; other means also include enhancing the biological activity of the anti-VEGFA antibody to lower the minimum effective concentration, or prolonging the half-life of the drug to achieve the purpose. Currently, the development of long-acting anti-VEGFA therapeutic drugs has achieved certain success in clinical trials, but some problems still exist. For example, high-concentration protein formulations often suffer from technical problems such as increased viscosity, susceptibility to forming protein aggregates, or poor stability, which increases the risk of intravitreal injection; in addition, problems such as insufficient biological efficacy or insignificant half-life enhancement still exist. Therefore, the design and development of anti-VEGFA drugs require further research and improvement.

Heavy-chain antibodies are IgG2 and IgG3 antibodies from the serum of camelids (camels, dromedaries, llamas, and the like) that contain only heavy chains and lack light chains. The variable domain of the heavy chain (VHH) of a heavy-chain antibody possesses independent antigen-binding activity and is currently the functional fragment with the smallest molecular weight among natural antibodies, referred to as a nanobody or a single domain antibody. A nanobody has a molecular weight of about 13 kDa, high thermal stability, and good water solubility, demonstrating relatively great potential for development into relatively high-concentration protein formulations. Albumin is the most abundant protein in plasma (with a concentration of about 40 mg/mL) and is critical for maintaining plasma osmotic pressure; meanwhile, albumin is an important transport carrier for endogenous ligands (e.g., fatty acids, metal ions, and hormones) and exogenous ligands (e.g., drugs).

Albumin has a molecular weight of 66.5 kDa and can bind to the cell surface receptor FcRn (neonatal Fc receptor) under weakly acidic conditions, whereas the binding ability is relatively weak under neutral conditions. Therefore, after binding to FcRn, albumin is endocytosed into the endosome. As the endosomal environment acidifies, the binding ability between albumin and FcRn increases, and albumin is recycled back to the cell surface by FcRn, or undergoes transcytosis. Consequently, albumin can achieve a long half-life by virtue of the FcRn recycling mechanism. Human serum albumin has a half-life of about 3 weeks in plasma. Therefore, small molecule compounds bound to albumin, or proteins expressed as albumin fusions (e.g., the marketed drugs Tanzeum® and Idelvion®), possess significantly improved pharmacokinetic properties and achieve a longer half-life, potentially extending the administration interval for patients and providing better administration regimens for patients.

An anti-albumin antibody or an antigen-binding fragment thereof can bind to albumin of different species with high affinity. At the same time, the anti-albumin antibody can be linked to a biologically active effector molecule, such as an anti-VEGFA antibody or an antigen-binding fragment, to form a fusion construct. The fusion construct can achieve improved pharmacokinetic properties and a longer half-life through binding to albumin. However, extensive creative work is still required regarding the structural design of the fusion construct, the linkage modes between individual domains, and whether the activity of the biologically active effector molecule will be affected after binding to albumin. The present application aims to utilize nanobody technology in the development of anti-albumin antibodies or antigen-binding fragments, as well as in the development and design of anti-VEGFA fusion constructs, thereby providing superior anti-VEGFA fusion constructs.

SUMMARY

To overcome the deficiencies of the prior art, the present application utilizes a VEGFA-binding nanobody as a basic active unit, performs monovalent and bivalent designs of the nanobody, and prepares anti-VEGFA fusion proteins with good stability and strong biological activity. Furthermore, the anti-VEGFA fusion protein is linked via a linker to an antibody or an antigen-binding fragment against another antigen, such as an anti-albumin antibody, to prepare a fusion construct of the anti-VEGFA antibody or the antigen-binding fragment and the anti-albumin antibody or the antigen-binding fragment. The fusion construct possesses strong binding affinity for VEGFA and biological activity in inhibiting VEGFA-stimulated HUVEC proliferation; in addition, the functions between individual domains in the fusion construct do not interfere with one another. Specifically,

in a first aspect of the present disclosure, provided is an anti-VEGFA fusion construct comprising an anti-albumin antibody or an antigen-binding fragment thereof and an anti-VEGFA antibody or an antigen-binding fragment thereof, wherein the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the anti-albumin antibody or the antigen-binding fragment thereof.

Preferably, the anti-albumin antibody or the antigen-binding fragment thereof comprises CDR-H1, CDR-H2, and CDR-H3 of a variable domain of a heavy chain.

An amino acid sequence of the CDR-H1 comprises SEQ ID NO: 1 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 1;

an amino acid sequence of the CDR-H2 comprises SEQ ID NO: 41 (GISVX$_1$X$_2$SFLDYADAVKG) or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 41;

an amino acid sequence of the CDR-H3 comprises SEQ ID NO: 3 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 3.

X in SEQ ID NO: 41 (GISVX$_1$X$_2$SFLDYADAVKG) may be any natural amino acid residue, such as alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), threonine (T), proline (P), serine (S), tryptophan (W), tyrosine (Y), or valine (V).

In one specific embodiment of the present disclosure, X$_1$X$_2$ in SEQ ID NO: 41 (GISVX$_1$X$_2$SFLDYADAVKG) represents DS, DA, EG, or DG.

In one specific embodiment of the present disclosure, the amino acid sequences of the CDR-H1, the CDR-H2, and the CDR-H3 comprise any one of the following groups (see Table 42 for details):

A) SEQ ID NOs: 1, 2, and 3;

B) SEQ ID NOs: 1, 10, and 3;

C) SEQ ID NOs: 1, 11, and 3;

D) SEQ ID NOs: 1, 12, and 3.

Table 42. CDR sequences of anti-albumin candidate antibodies

Candidate	SEQ	CDR-H1	SEQ	CDR-H2	SEQ ID	CDR-H3

antibody number	ID NO		ID NO		NO	
3005	1	NYYMS	2	GISVDGSFLDYADAVKG	3	ASGPQGLRLGAP
3005Hz6 (DA)	1	NYYMS	10	GISVDASFLDYADAVKG	3	ASGPQGLRLGAP
3005Hz6 (DS)	1	NYYMS	11	GISVDSSFLDYADAVKG	3	ASGPQGLRLGAP
3005Hz6 (EG)	1	NYYMS	12	GISVEGSFLDYADAVKG	3	ASGPQGLRLGAP

Preferably, the amino acid sequences of the CDR-H1, the CDR-H2, and the CDR-H3 are arranged in the order from the N terminus to the C terminus.

The anti-albumin antibody or the antigen-binding fragment thereof comprises a variable domain of a heavy chain. Amino acids in the CDR regions of the antibodies in the present application are defined using the Kabat numbering system.

Preferably, the anti-albumin antibody or the antigen-binding fragment thereof comprises a humanized sequence, wherein an engineered site of the humanized sequence is located in a non-CDR region, and further preferably, the engineered site of the humanized sequence is located in a framework region of the variable domain and/or a constant region.

Preferably, a structure of the anti-albumin antibody or the antigen-binding fragment thereof comprises a nanobody, a chimeric antibody, a Fab fragment, a Fab' fragment, an Fd fragment, an Fv fragment, a dAb fragment, a F(ab')2 fragment, a single chain antibody fragment (scFv), or a linear antibody.

The anti-albumin antibody or the antigen-binding fragment thereof may be a single domain antibody or a nanobody.

In one specific embodiment of the present disclosure, the anti-albumin antibody or the antigen-binding fragment thereof is a nanobody, which, at the same mass, has a higher molar concentration and can bind to more antigen molecules as compared to a full-length IgG antibody, a Fab, or an scFv.

The anti-albumin antibody or the antigen-binding fragment thereof can bind to mammalian albumin.

Preferably, the mammal includes a human or a non-human mammal, wherein the non-human mammal may be a wild animal, a zoo animal, an economic animal, a companion animal, a laboratory animal, or the like.

Preferably, the non-human mammal includes, but is not limited to, a pig, a cow, a sheep, a horse, a donkey, a fox, a raccoon dog, a mink, a camel, a dog, a cat, a rabbit, a rodent (e.g., a rat, a mouse, a guinea pig, a hamster, a gerbil, a chinchilla, and a squirrel), a monkey, etc.

Preferably, an amino acid sequence of the anti-albumin antibody or the antigen-binding fragment thereof comprises any one of the amino acid sequences of SEQ ID NOs: 4, 6-9, and 13-15, or has at least 80% identity to any one of the amino acid sequences of SEQ ID NOs: 4, 6-9, and 13-15.

In one specific embodiment of the present disclosure, the amino acid sequence of the anti-albumin antibody or the antigen-binding fragment thereof is set forth in any one of the amino acid sequences of SEQ ID NOs: 4, 6-9, and 13-15.

The anti-albumin antibody or the antigen-binding fragment thereof can be constructed using any conventional method in the prior art, such as artificial synthesis or eukaryotic expression or prokaryotic expression.

Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof comprises CDR-H1, CDR-H2, and CDR-H3 of a variable domain of a heavy chain,

wherein an amino acid sequence of the CDR-H1 comprises SEQ ID NO: 16 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 16;

an amino acid sequence of the CDR-H2 comprises SEQ ID NO: 17 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 17;

an amino acid sequence of the CDR-H3 comprises SEQ ID NO: 18 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 18.

The anti-VEGFA antibody or the antigen-binding fragment thereof comprises a variable domain of a heavy chain.

A structure of the anti-VEGFA antibody or the antigen-binding fragment thereof comprises a nanobody, a chimeric antibody, a Fab fragment, a Fab' fragment, an Fd fragment, an Fv fragment, a dAb fragment, a F(ab')2 fragment, a single chain antibody fragment (scFv), or a linear antibody.

The anti-VEGFA antibody or the antigen-binding fragment thereof may be a single domain antibody or a nanobody.

The anti-VEGFA antibody or the antigen-binding fragment thereof may be a humanized antibody or a fully

human antibody.
Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof comprises a humanized sequence, wherein an engineered site of the humanized sequence is located in a non-CDR region, and further preferably, the engineered site of the humanized sequence is located in a framework region of the variable domain and/or a constant region.
In one specific embodiment of the present disclosure, the anti-VEGFA antibody or the antigen-binding fragment thereof is a nanobody, which, at the same mass, has a higher molar concentration and can bind to more antigen molecules as compared to a full-length IgG antibody, a Fab, or an scFv.
Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof can bind to a human or monkey VEGFA protein.
Preferably, an amino acid sequence of the anti-VEGFA antibody or the antigen-binding fragment thereof comprises any one of the amino acid sequences of SEQ ID NOs: 19-28, or has at least 80% identity to any one of the amino acid sequences of SEQ ID NOs: 19-28.
Preferably, the fusion construct comprises one or more anti-VEGFA antibodies or antigen-binding fragments thereof.
Preferably, the fusion construct comprises one or more anti-albumin antibodies or antigen-binding fragments thereof.
Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the anti-VEGFA antibody or the antigen-binding fragment thereof directly or indirectly.
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Preferably, the anti-albumin antibody or the antigen-binding fragment thereof is linked to the anti-albumin antibody or the antigen-binding fragment thereof directly or indirectly.
Preferably, the indirect linkage may be a linkage via a linker, a functional domain, and/or a linker for conjugation,
wherein the linker is selected from a linker peptide, an oligopeptide, an oligopeptide polymer, a polypeptide, a polypeptide polymer, PEG, a nucleic acid, a polysaccharide, a fatty chain, biotin, streptavidin, and avidin.
The functional domain is one or a combination of two or more of an Fc fragment, serum albumin, a cytokine, transferrin, or a scaffold protein.
The linker for conjugation comprises a functional group linker, and preferably, the functional group linker comprises sulfhydryl, amino, hydroxy, and/or carboxyl reactive groups, which can covalently conjugate the anti-albumin antibody or the antigen-binding fragment thereof with the anti-VEGFA antibody or the antigen-binding fragment thereof.
Preferably, the N terminus and/or the C terminus of the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the C terminus and/or the N terminus of another anti-VEGFA antibody or the antigen-binding fragment thereof via a linker peptide.
In one specific embodiment of the present disclosure, the fusion construct comprises, sequentially from the N terminus to the C terminus, a first anti-VEGFA antibody or an antigen-binding fragment thereof, a linker peptide, and a second anti-VEGFA antibody or an antigen-binding fragment thereof.
Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the N terminus, the C terminus, and/or an internal residue of the anti-albumin antibody or the antigen-binding fragment thereof directly or indirectly.
In one specific embodiment of the present disclosure, the fusion construct comprises two anti-VEGFA antibodies or antigen-binding fragments thereof, wherein the two anti-VEGFA antibodies or the antigen-binding fragments thereof are linked to the N terminus, the C terminus, and/or an internal residue of the anti-albumin antibody or the antigen-binding fragment thereof directly or indirectly.
For example, in the fusion construct, from the N terminus to the C terminus, the anti-albumin antibody or the antigen-binding fragment thereof and the anti-VEGFA antibody or the antigen-binding fragment thereof are linked in the following order:

1) the anti-albumin antibody or the antigen-binding fragment thereof, the linker peptide, and the anti-VEGFA antibody or the antigen-binding fragment thereof;
2) the anti-VEGFA antibody or the antigen-binding fragment thereof, the linker peptide, and the anti-albumin antibody or the antigen-binding fragment thereof;
3) the anti-albumin antibody or the antigen-binding fragment thereof, the linker peptide, the first anti-VEGFA antibody or the antigen-binding fragment thereof, the linker peptide, and the second anti-VEGFA antibody or the antigen-binding fragment thereof; or
4) the first anti-VEGFA antibody or the antigen-binding fragment thereof, the linker peptide, the

anti-albumin antibody or the antigen-binding fragment thereof, the linker peptide, and the second anti-VEGFA antibody or the antigen-binding fragment thereof, and the like;

the linkage may not comprise a linker peptide.

Preferably, the two or more anti-VEGFA antibodies or antigen-binding fragments thereof are anti-VEGFA antibodies or antigen-binding fragments thereof with completely identical sequences, anti-VEGFA antibodies or antigen-binding fragments thereof with partially identical sequences, or anti-VEGFA antibodies or antigen-binding fragments thereof with completely different sequences.

In one specific embodiment, the fusion construct comprises any one of the amino acid sequences of SEQ ID NOs: 29-35, or has at least 80% identity to any one of the amino acid sequences of SEQ ID NOs: 29-35.

Preferably, the fusion construct comprises an Fc fragment.

Further preferably, the Fc fragment comprises SEQ ID NO: 38, or has at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 38.

Preferably, the fusion construct further comprises a secretion peptide. The secretion peptide is linked to the N terminus of the fusion construct. Further preferably, an amino acid sequence of the secretion peptide may be SEQ ID NO: 36.

Preferably, the fusion construct further comprises a tag.

Preferably, the tag is linked to the C terminus of any one of the antibodies or the antigen-binding fragments thereof or the fusion construct.

Preferably, the fusion construct can inhibit or compete with binding of other anti-VEGFA antibodies (preferably antibodies that bind to the same or overlapping epitopes as the anti-VEGFA antibody of the present disclosure) to VEGFA.

Preferably, the fusion construct can also inhibit or compete with binding of other anti-albumin antibodies (preferably antibodies that bind to the same or overlapping epitopes as the anti-albumin antibody of the present disclosure) to albumin.

In a second aspect of the present disclosure, provided is a nucleic acid encoding the antibody or the antigen-binding fragment described in the present application, or the fusion construct described above. For example, the nucleic acid comprises DNA and/or mRNA. For example, the nucleic acid is a therapeutic nucleic acid.

In some embodiments, the nucleic acid is DNA encoding the antibody or the antigen-binding fragment described in the present application, or the fusion construct described above.

Preferably, a nucleotide sequence encoding the CDR-H1 in the anti-albumin antibody or the antigen-binding fragment thereof comprises the nucleotide sequence of SEQ ID NO: 42 or the nucleotide sequence of SEQ ID NO: 51 or a degenerate sequence thereof, or has at least 80% identity to the nucleotide sequence of SEQ ID NO: 42 or the nucleotide sequence of SEQ ID NO: 51.

Preferably, a nucleotide sequence encoding the CDR-H2 in the anti-albumin antibody or the antigen-binding fragment thereof comprises any one of the nucleotide sequences of SEQ ID NOs: 43 and 52-54 or a degenerate sequence thereof, or has at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 43 and 52-54.

Preferably, a nucleotide sequence encoding the CDR-H3 in the anti-albumin antibody or the antigen-binding fragment thereof comprises the nucleotide sequence of SEQ ID NO: 44 or the nucleotide sequence of SEQ ID NO: 55 or a degenerate sequence thereof, or has at least 80% identity to the nucleotide sequence of SEQ ID NO: 44 or the nucleotide sequence of SEQ ID NO: 55.

Preferably, a nucleotide sequence encoding the CDR-H1 in the anti-VEGFA antibody or the antigen-binding fragment thereof comprises the nucleotide sequence of SEQ ID NO: 59 or a degenerate sequence thereof, or has at least 80% identity to the nucleotide sequence of SEQ ID NO: 59.

Preferably, a nucleotide sequence encoding the CDR-H2 in the anti-VEGFA antibody or the antigen-binding fragment thereof comprises the nucleotide sequence of SEQ ID NO: 60 or a degenerate sequence thereof, or has at least 80% identity to the nucleotide sequence of SEQ ID NO: 60.

Preferably, a nucleotide sequence encoding the CDR-H3 in the anti-VEGFA antibody or the antigen-binding fragment thereof comprises the nucleotide sequence of SEQ ID NO: 61 or a degenerate sequence thereof, or has at least 80% identity to the nucleotide sequence of SEQ ID NO: 61.

More preferably, a nucleotide sequence encoding the anti-VEGFA antibody or the antigen-binding fragment thereof comprises any one of the nucleotide sequences of SEQ ID NOs: 62-71 or a degenerate sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 62-71 and having a function of encoding an anti-VEGFA antibody or an antigen-binding fragment thereof.

More preferably, a nucleotide sequence encoding the anti-albumin antibody or the antigen-binding fragment thereof comprises any one of the nucleotide sequences of SEQ ID NOs: 45, 47-50, and 56-58 or a degenerate

sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 45, 47-50, and 56-58 and having a function of encoding an anti-albumin antibody or an antigen-binding fragment thereof.

More preferably, a nucleotide sequence encoding the fusion construct described above comprises any one of the nucleotide sequences of SEQ ID NOs: 72-78 or a degenerate sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 72-78 and having a function of encoding the fusion construct described above.

In some embodiments, the nucleic acid is mRNA. One or more modification techniques may be used to produce more stable mRNA. The known mRNA modification techniques can be roughly divided into three categories: replacing a natural ribonucleic acid with an artificially synthesized non-natural ribonucleic acid to synthesize mRNA; adding a 5' cap, a 3' poly(A) "tail", and a UTR (untranslated region) sequence; adopting a special novel formulation technique to effectively protect mRNA. Among them, the preferred mRNA modification technique can replace a natural ribonucleic acid with an artificially synthesized non-natural ribonucleic acid to synthesize mRNA. Chemical modifications on eukaryotic mRNA can be roughly divided into three categories: methylation, pseudouridine (Ψ), and hypoxanthine. For example, the chemical modification can be selected from: pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methoxyuridine, and 2'-O-methyluridine.

In a third aspect of the present disclosure, provided is a vector comprising the nucleic acid described above. The vector is capable of expression *in vivo* or *in vitro* or *ex vivo*. Preferably, the vector is a recombinant vector such as a prokaryotic expression vector, a viral expression vector, or a eukaryotic expression vector.

For example, a viral expression vector can be used. The viral expression vector may comprise a virus-derived DNA or RNA sequence for packaging into a virus (e.g., a retrovirus, a replication-defective retrovirus, an adenovirus, a replication-defective adenovirus, and an adeno-associated virus (AAV)). The virus and viral expression vector can be used for *in vitro*, *ex vivo*, and/or *in vivo* delivery. For example, the recombinant vector may be comprised in a delivery vector. In certain embodiments, the delivery vector comprises the antibody or the antigen-binding fragment thereof described in the present application, the nucleic acid described in the present application, and/or the recombinant vector described in the present application, and optionally a liposome and/or a lipid nanoparticle (LNP). For example, the delivery vector can be introduced into a cell by physical delivery methods. Examples of physical methods include microinjection, electroporation, and hydrodynamic delivery. For example, LNPs can encapsulate the nucleic acid described in the present application within cationic lipid particles (e.g., liposomes), thereby enabling relatively easy intracellular delivery.

In a fourth aspect of the present disclosure, provided is a host cell comprising the nucleic acid described above or the vector described above.

The host cell may be a eukaryotic cell or a prokaryotic cell.

The eukaryotic cell includes animal and plant cells, fungi, and the like, such as T cells, yeast cells, HEK293 cells, or CHO cells.

The prokaryotic cell includes, for example, *Escherichia coli*.

In a fifth aspect of the present disclosure, provided is a preparation method for a host cell comprising the nucleic acid described above or the vector described above, which comprises introducing the nucleic acid or the vector described above into a host cell.

In a sixth aspect of the present disclosure, provided is a preparation method for the fusion construct described above, which comprises culturing the host cell described above and expressing the fusion construct.

In a seventh aspect of the present disclosure, provided is a product for treating and/or diagnosing a disease, which comprises any one of the following:

A) the fusion construct described above;

B) the nucleic acid described above;

C) the vector described above; or

D) the host cell described above.

Preferably, the anti-VEGFA antibody or the antigen-binding fragment thereof has activity for treating and/or diagnosing a disease.

Preferably, the product may be a diagnostic kit, a medicament, a diagnostic chip, or the like.

The disease is a disease associated with the VEGFA signaling pathway. Further preferably, it may be a

tumor, abnormal vascular proliferation, an ophthalmic disease involving angiogenesis (e.g., a fundus vascular disease), or the like.
Preferably, the fusion construct blocks VEGFA-mediated vascular endothelial cell proliferation or inhibits angiogenesis.
In an eighth aspect of the present disclosure, provided is a method for detecting VEGFA, which comprises contacting a sample to be tested with the fusion construct described above, and subsequently, detecting a content of a complex formed by VEGFA and the fusion construct described above.
The detection method is to detect the presence or the content of VEGFA. The presence indicates presence or absence, and the content may be an expression level, a protein concentration, or the like.
Preferably, the VEGFA is from a mammal; more preferably, the mammal is a human, a murine, or a monkey.
In a ninth aspect of the present disclosure, provided is a method for blocking VEGFA-mediated vascular endothelial cell proliferation or inhibiting angiogenesis, which comprises contacting a vascular endothelial cell with the fusion construct described above, the nucleic acid described above, the vector described above, the host cell described above, or the product for treating and/or diagnosing a disease described above.
Preferably, the method comprises diluting the fusion construct described above, the nucleic acid described above, the vector described above, the host cell described above, or the product for treating and/or diagnosing a disease described above with a serum-free medium of a vascular endothelial cell, followed by incubation with an antigen (e.g., VEGFA165).
Preferably, a step of discarding the vascular endothelial cell complete medium in the culture plate after the incubation is comprised.
Preferably, an antigen and the fusion construct described above, the nucleic acid described above, the vector described above, the host cell described above, or the product for treating and/or diagnosing a disease described above are mixed with a vascular endothelial cell, added to a vascular endothelial cell culture plate, and cultured on the culture plate.
Preferably, determination is performed after the culture.
Preferably, the incubation is performed for 0.5-5 h, e.g., 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 4.5 h, or 5 h.
Preferably, the incubation is performed at a temperature of room temperature to 45 °C, preferably 30-40 °C, e.g., 25 °C, 30 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 45 °C, etc.
Preferably, the culture is performed at a temperature of room temperature to 45 °C, preferably 30-40 °C, e.g., 25 °C, 30 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, 40 °C, 45 °C, etc. Preferably, the culture is performed in an incubator with 5% CO_2.
Preferably, the culture is performed for 24-120 h, preferably 48-96 h, e.g., 24 h, 36 h, 48 h, 68 h, 72 h, 96 h, 120 h, etc.
Preferably, the determination is to determine the number of viable vascular endothelial cells.
In a tenth aspect of the present disclosure, provided is a method for treating and/or preventing a disease, which comprises administering to an individual the fusion construct described above, the nucleic acid described above, the vector described above, the host cell described above, or the product for treating and/or diagnosing a disease described above.
The disease includes a disease associated with the VEGFA signaling pathway. Further preferably, it may be a tumor, abnormal vascular proliferation, an ophthalmic disease involving angiogenesis (e.g., a fundus vascular disease), or the like.
Preferably, the target cell is selected from cells expressing VEGFA, such as cardiomyocytes, renal proximal tubule cells, hepatocytes, vascular endothelial cells, granulosa cells, specialized epithelial cells, mesenchymal cells, macrophages, platelets, dendritic cells, activated T cells, retinal pigment epithelial cells, Muller cells in the retina, astrocytes, osteoblasts, bronchial and alveolar epithelial cells, pericytes, vascular smooth muscle cells, myofibroblasts, keratinocytes, renal mesangial cells, tumor cells, etc.
In an eleventh aspect of the present disclosure, provided is use of the fusion construct described above, the nucleic acid described above, the vector described above, or the host cell described above in preparing a product for treating and/or preventing a disease, wherein the anti-VEGFA antibody or the antigen-binding fragment thereof has activity for treating and/or diagnosing a disease.
The disease includes a disease associated with the VEGFA signaling pathway. Further preferably, it may be a tumor, abnormal vascular proliferation, an ophthalmic disease involving angiogenesis (e.g., a fundus vascular disease), or the like.
Preferably, the product includes a product for blocking VEGFA-mediated vascular endothelial cell proliferation or inhibiting angiogenesis.
The product described in the present application may be a kit or a medicament or a chip or an antibody-drug

conjugate, or the like.
The “fusion construct” as used herein defines a fusion of the anti-albumin antibody or the antigen-binding fragment thereof of the present disclosure with an additional compound. The fusion construct may comprise one or a plurality of anti-albumin antibodies or antigen-binding fragments thereof, and the plurality of anti-albumin antibodies or antigen-binding fragments thereof may be identical or different. The fusion construct may comprise one or a plurality of additional compounds, and the plurality of additional compounds may also be identical or different. The compound may be a protein compound or a non-protein compound. Where the compound is a protein compound or the fusion construct comprises only a plurality of anti-albumin antibodies or antigen-binding fragments thereof, the fusion construct may also be referred to as a fusion protein. Where the compound is fused to the anti-albumin antibody or the antigen-binding fragment thereof in a conjugated form, the fusion construct may also be referred to as a conjugate.
The “medicament” described in the present disclosure may be used for treating a human or non-human animal, such as a non-human mammal. The medicament may comprise pharmaceutically acceptable carriers, auxiliary materials, or salts common in the prior art. The medicament may be administered by any suitable route of administration, such as gastrointestinal routes (e.g., oral administration) or parenteral routes (e.g., intravenous, intramuscular, subcutaneous, intradermal, intraorgan, intranasal, intraocular, instillation, intracerebral, intrathecal, transdermal, and intrarectal administrations). The medicament may be in any suitable dosage form, such as dosage forms for gastrointestinal or parenteral administration. The dosage forms preferably include, but are not limited to, tablets, pills, powders, granules, capsules, lozenges, syrups, liquids, emulsions, microemulsions, suspensions, injections, sprays, aerosols, powder aerosols, lotions, ointments, plasters, pastes, patches, eye drops, nose drops, sublingual tablets, suppositories, aerosols, effervescent tablets, dripping pills, gels, and the like. The various dosage forms of the medicament can be prepared according to conventional production methods in the field of pharmaceuticals.
The medicament may comprise the fusion construct, the nucleic acid, the vector, or the host cell, and the like in a weight ratio of 0.01%-99.5% (specifically, 0.01%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or 99.5%). The medicament may be prepared as a reagent with a protein concentration of 1-300 mg/mL (e.g., 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 25 mg/mL, 30 mg/mL, 35 mg/mL, 40 mg/mL, 45 mg/mL, 50 mg/mL, 55 mg/mL, 60 mg/mL, 65 mg/mL, 70 mg/mL, 75 mg/mL, 80 mg/mL, 85 mg/mL, 90 mg/mL, 95 mg/mL, 100 mg/mL, 105 mg/mL, 110 mg/mL, 115 mg/mL, 120 mg/mL, 130 mg/mL, 140 mg/mL, 150 mg/mL, 160 mg/mL, 170 mg/mL, 180 mg/mL, 190 mg/mL, 200 mg/mL, 210 mg/mL, 220 mg/mL, 230 mg/mL, 240 mg/mL, 250 mg/mL, 260 mg/mL, 270 mg/mL, 280 mg/mL, 290 mg/mL, or 300 mg/mL).
A single administration dose of the medicament may be 0.1-1000 mg, e.g., 0.1 mg, 0.2 mg, 0.5 mg, 0.75 mg, 1 mg, 1.25 mg, 1.5 mg, 1.75 mg, 2 mg, 2.25 mg, 2.5 mg, 3 mg, 5 mg, 10 mg, 20 mg, 50 mg, 80 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 550 mg, 600 mg, 650 mg, 700 mg, 750 mg, 800 mg, 850 mg, 900 mg, 950 mg, or 1000 mg.
The “pharmaceutically acceptable” described in the present disclosure means that it neither significantly stimulates an organism nor inhibits the biological activity and properties of the active substance in the administered product.
The “method for...” described in the present disclosure can be used for the purposes of diagnosing and treating diseases or non-disease conditions.
The “antigen-binding fragment” described in the present disclosure is a portion of an antibody that retains the specific binding activity of the antibody, i.e., any portion of the antibody that is capable of specifically binding to an epitope on a target molecule of the antibody. It includes, for example, Fab, Fab', F(ab')2, Fv, Fd, and variants of these fragments, such as the heavy chain and/or the light chain of an antibody, the variable domain of the heavy chain and/or the variable domain of the light chain of an antibody, or a single CDR or 2 or more CDRs from the heavy chain or the light chain of an antibody. Among these,
a nanobody or single domain antibody refers to the variable domain of the heavy chain (VHH) of an antibody, which has independent antigen-binding activity.
A chimeric antibody refers to an antibody in which a portion of the heavy chain and/or light chain is derived from a specific source or species, while the remaining portion of the heavy chain and/or light chain is derived from a different source or species.
A single chain antibody fragment is an antibody formed by linking the variable domain of the heavy chain and the variable domain of the light chain via a linker peptide (linker).
Fab is a monovalent fragment consisting of VL, VH, CL, and CH1 domains.
Fab' is a Fab fragment with one or more cysteine residues at the C terminus of the CH1 domain.
F(ab')2 is a bivalent fragment comprising two Fab fragments linked via disulfide bonds at the hinge region.

Fd is an Fd fragment consisting of VH and CH1 domains.
Fv is an Fv fragment consisting of VL and VH domains of a single arm of an antibody.
A dAb fragment is an antibody fragment consisting of a VH domain.
The “linear antibody” described in the present disclosure comprises one or more pairs of tandem antibody fragments, wherein the antibody fragment may be an Fd segment (VH-CH1), a single chain antibody fragment (scFv), an antibody fragment (Fab), or a single domain antibody (VHH). These fragments are linked together in tandem via a linker peptide to form a continuous antibody structure.
Among these, VH represents the variable domain of the heavy chain, VL represents the variable domain of the light chain, CL represents the constant region of the light chain, and CH represents the constant region of the heavy chain.
The “Fc” region described in the present disclosure comprises two heavy chain fragments containing the CH2 and CH3 domains of the antibody. The two heavy chain fragments form a dimer through two or more disulfide bonds in the hinge region and are held together by the hydrophobic interactions in the CH3 domains.
The “comprise”, “contain”, or “include” described in the present disclosure is an open-ended expression. When the term is used to describe a sequence of a protein or nucleic acid, the protein or the nucleic acid may consist of the sequence, or may have additional amino acids or nucleotides at one or both ends of the protein or the nucleic acid, while still retaining the same or similar activity as the original sequence.
The “homology” or “identity” described in the present disclosure means that, in the case of using protein sequences or nucleotide sequences, those skilled in the art can adjust the sequence according to the actual work needs, such that the sequences used have (including but not limited to) 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% homology/identity to the sequence obtained from the prior art.
The "humanized antibody" described in the present disclosure refers to an antibody in which all or part of the framework region and/or all or part of the constant region (e.g., a CH region), or all or part of the antibody, is encoded by a human antibody gene. In one specific embodiment of the present disclosure, the CDR regions of the antibody are not humanization-engineered.
The “individual” described in the present disclosure may be a human or a non-human mammal, and the non-human mammal may be a wild animal, a zoo animal, an economic animal, a companion animal, a laboratory animal, or the like. Preferably, the non-human mammal includes, but is not limited to, a pig, a cow, a sheep, a horse, a donkey, a fox, a raccoon dog, a mink, a camel, a dog, a cat, a rabbit, a rodent (e.g., a rat, a mouse, a guinea pig, a hamster, a gerbil, a chinchilla, and a squirrel), a monkey, etc.
The “treat”, “treating”, or “treatment” described in the present disclosure refers to slowing, interrupting, arresting, controlling, stopping, reducing, or reversing the progression or severity of a sign, symptom, disorder, condition, or disease after the disease has begun to progress, but it does not necessarily involve the complete elimination of all disease-related signs, symptoms, conditions, or disorders.
The “prevent”, “preventing”, or “prevention” described in the present disclosure refers to the practice of preventing or delaying the onset of a disease or condition or symptom in the body.
The “diagnose”, “diagnosing”, or “diagnosis” described in the present disclosure refers to the determination of whether a patient has suffered from a disease or condition in the past, is suffering from a disease or condition at the time of diagnosis, or will suffer from a disease or condition in the future, or the determination of the progression or likely progression in the future of a disease.
The “tumor” described in the present disclosure may be any adverse cell proliferation (or any disease that manifests itself as adverse cell proliferation) or neoplasm, or increased predisposition or risk for adverse cell proliferation, neoplasm, or a tumor. The tumor may be benign or malignant, and may also be primary or secondary (metastatic). The neoplasm may be any abnormal growth or proliferation of cells and may be located in any tissue. Examples of the tissue include the adrenal gland, adrenal medulla, anus, appendix, bladder, blood, bone, bone marrow, brain, mammary gland, cecum, central nervous system (including or excluding the brain), cerebellum, cervix, colon, duodenum, endometrium, epithelial cells (e.g., renal epithelial cells), gallbladder, esophagus, glial cells, heart, ileum, jejunum, kidney, lacrimal gland, larynx, liver, lung, lymph, lymph nodes, lymphoblasts, maxilla, mediastinum, mesenterium, myometrium, nasopharynx, omentum, oral cavity, ovary, pancreas, parotid gland, peripheral nervous system, peritoneum, pleura, prostate, salivary glands, sigmoid colon, skin, small intestine, soft tissue, spleen, stomach, testis,

thymus, thyroid, tongue, tonsils, trachea, uterus, vulva, and white blood cells. Further preferably, the tumor is selected from prostate cancer, breast cancer, liver cancer, glioma (e.g., neuroglioma), intestinal cancer, cervical cancer, non-small cell lung cancer, lung cancer, pancreatic cancer, gastric cancer, bladder cancer, skin cancer, rhabdomyosarcoma, tongue squamous cell carcinoma, nasopharyngeal cancer, ovarian cancer, placental choriocarcinoma, lymphoma (e.g., non-Hodgkin lymphoma, Hodgkin lymphoma, and cutaneous T-cell lymphoma), leukemia, rectal adenocarcinoma, medulloblastoma, meningioma, neurofibroma (e.g., neurofibrosarcoma), ependymoma, schwannoma, astrocytoma, melanoma, mesothelioma, myeloma, chronic myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, chronic lymphocytic leukemia, epidermoid cancer, colon cancer, thymus cancer, blood cancer, head and neck cancer, and oropharyngeal cancer.

The "fundus vascular disease" described in the present disclosure refers to the general term for diseases occurring in the retinal arteries or veins or diseases associated with choroidal neovascularization, including but not limited to age-related macular degeneration (AMD), diabetic macular edema (DME), diabetic retinopathy, central retinal vein occlusion, pathological myopia, neovascular glaucoma, and the like.

In summary, the beneficial technical effects of the present disclosure include:

1) The anti-VEGFA fusion construct obtained by the technology of the present application has strong binding affinity for VEGFA (K_D = 2.4-5.4 pM as determined by SPR), which is much higher than that of existing anti-VEGFA antibody drugs, and it can compete with VEGFR2 for binding to VEGFA.

2) The fusion construct can inhibit VEGFA-stimulated HUVEC proliferation, and has an IC50 value similar to that of the receptor drug aflibercept. In VEGFA-stimulated reporter gene cells, the fusion construct can inhibit luciferase expression and luminescence. In HUVEC cells, the fusion construct can inhibit the VEGFA-activated VEGFR2 phosphorylation.

3) The functions between individual domains in the fusion construct do not interfere with one another. The fusion construct of the present disclosure links the anti-albumin antibody to the anti-VEGFA antibody and still has relatively strong binding affinity for human albumin (K_D = 35-69 nM as determined by SPR) and VEGFA (K_D = 2.4-5.4 pM as determined by SPR).

4) *In vivo*, the fusion construct has the potential to inhibit VEGFA-stimulated vascular proliferation and through binding to albumin, is carried by the albumin into a specific albumin-enriched tissue microenvironment, potentially improving selectivity of delivery of the fusion construct to a specific tissue. Moreover, due to an increase in the molecular weight of the fusion construct after binding to albumin, the fusion construct can further participate in a binding and recycling utilization mechanism of the albumin with FcRn to extend a half-life; therefore, the fusion construct of the anti-VEGFA antibody and the anti-albumin antibody has the potential to achieve a long-lasting effect *in vivo*.

BRIEF DESCRIPTION OF THE DRAWINGS

Hereinafter, examples of the present disclosure will be described in detail with reference to the drawings, where:

FIG. 1 shows the Coomassie-stained gel images of the His-tagged antibody Alb Nb and antibody 3005 after Ni column purification, where (A) shows the Coomassie-stained gel image of the antibody Alb Nb, and (B) shows the Coomassie-stained gel image of the antibody 3005; the cell medium supernatant from transient transfection of the antibody Alb Nb is labeled as Input, the flow-through fraction that fails to bind to the Ni column is labeled as FT, the antibody Alb Nb that binds to and is eluted from the Ni column is labeled as E, and Marker is labeled as M.

FIG. 2 shows the ELISA assays of the binding of antibodies 3005-Fc and Alb Nb-Fc to albumins of different species, where (A) shows the HSA binding assay, (B) shows the MSA binding assay, and (C) shows the rat albumin binding assay.

FIG. 3 shows the Coomassie-stained gel image of the His-tagged 3005 humanized antibody after Ni column purification.

FIG. 4 shows the ELISA binding activity assay of the antibody 3005 and its humanized antibodies to MSA.

FIG. 5 shows the ELISA binding activity assays of the antibody 3005 and its humanized antibody 3005Hz6 and the control antibody Alb Nb to HSA and rat albumin, where (A) shows the HSA binding assay and (B) shows the rat albumin binding assay.

FIG. 6 shows the binding and dissociation curves of humanized antibody 3005Hz6 to HSA and MSA (SPR method), where (A) shows the binding and dissociation curves of the antibody 3005Hz6 to HSA, and (B) shows the binding and dissociation curves of the antibody 3005Hz6 to MSA.

FIG. 7 shows the SDS-PAGE Coomassie-stained gel images of the humanized antibody 3005Hz6 mutant proteins 3005Hz6 (EG), 3005Hz6 (DS), and 3005Hz6 (DA) after purification, where (A) shows the SDS-

PAGE Coomassie-stained gel image of 3005Hz6 (EG) and 3005Hz6 (DS), where the cell medium supernatants from transient expression of 3005Hz6 (EG) and 3005Hz6 (DS) are labeled as Input, the flow-through fractions that fail to bind to the Ni column are labeled as FT, and the products that are eluted and purified are labeled as E; and (B) shows the SDS-PAGE Coomassie-stained gel image of 3005Hz6 (DA).

FIG. 8 shows the ELISA binding activity assay of humanized antibody 3005Hz6 and its mutant proteins 3005Hz6 (DA), 3005Hz6 (DS), and 3005Hz6 (EG) to HSA.

FIG. 9 shows the SDS-PAGE gel images of fusion constructs V1SA-3005Hz6, 3005Hz6-V1SA, and V1DP-3005Hz6 after Ni column purification, where (A) shows the SDS-PAGE Coomassie-stained gel image of V1SA-3005Hz6 and 3005Hz6-V1SA, and (B) shows an SDS-PAGE Coomassie-stained gel image of V1DP-3005Hz6; the cell medium supernatant from transient expression of each protein is labeled as Input, the flow-through fractions that fail to bind to the Ni column are labeled as FT, and the products that are eluted and purified are labeled as E.

FIG. 10 shows the ELISA binding activity assay of the fusion constructs of the humanized antibody 3005Hz6 and the monovalent anti-VEGFA nanobody, and the humanized antibody 3005Hz6 to HSA.

FIG. 11 shows the VEGFR2 competition ELISA activity assay of fusion constructs and monovalent nanobody V1-SA1.

FIG. 12 shows the SDS-PAGE images of fusion constructs of humanized antibody 3005Hz6 and bivalent anti-VEGFA nanobody after purification, where (A) shows the Coomassie-stained gel image of V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA, where the flow-through fractions are labeled as FT, the eluted products of V1SA-3005Hz6-V1SA are labeled as components A1 and A2, and the eluted products of 3005Hz6-2V1SA are labeled as components A5 and A6; (B) shows the Coomassie-stained gel image of the tag-free protein product 3005Hz6-2V1SA; (C) shows the Coomassie-stained gel image of V1DP-3005Hz6-V1DP; and (D) shows the Coomassie-stained gel image of 3005Hz6-2V1DP.

FIG. 13 shows the ELISA assay of fusion constructs V1SA-3005Hz6-V1SA, 3005Hz6-2V1SA, and 3005Hz6-2V1DP binding to HSA.

FIG. 14 shows the affinity of proteins for HSA determined by the SPR method, where FIG. (A) shows the binding and dissociation curves of the fusion construct V1SA-3005Hz6-V1SA with HSA, FIG. (B) shows the binding and dissociation curves of the fusion construct 3005Hz6-2V1SA with HSA, and FIG. (C) shows the binding and dissociation curves of the antibody 3005Hz6 with HSA.

FIG. 15 shows the affinity of proteins for VEGFA determined by the SPR method, where FIG. (A) shows the binding and dissociation curves of V1SA-3005Hz6-V1SA with VEGFA, FIG. (B) shows the binding and dissociation curves of 3005Hz6-2V1SA with VEGFA, and FIG. (C) shows the binding and dissociation curves of the positive control aflibercept with VEGFA.

FIG. 16 shows the inhibition experiment of VEGFA-induced HUVEC proliferation by fusion constructs, where FIG. (A) shows determination results of V1SA-3005Hz6-V1SA and a premix of V1SA-3005Hz6-V1SA and HSA (V1SA-3005Hz6-V1SA + HSA), and FIG. (B) shows determination results of 3005Hz6-2V1SA and a premix of 3005Hz6-2V1SA and HSA (3005Hz6-2V1SA + HSA).

FIG. 17 shows the cell experiment of the inhibition of VEGFA induced reporter gene expression by fusion constructs, where FIG. (A) shows the determination results of V1SA-3005Hz6-V1SA and V1SA-3005Hz6-V1SA + HSA, and FIG. (B) shows the determination results of 3005Hz6-2V1SA and 3005Hz6-2V1SA + HSA.

DETAILED DESCRIPTION

The technical solutions in the examples of the present disclosure will be described clearly and completely below with reference to the drawings. It is obvious that the described examples are only a part of the examples of the present disclosure, rather than all of them. Based on the examples of the present disclosure, all other examples obtained by those of ordinary skill in the art without creative work shall fall within the protection scope of the present disclosure.

Example 1. Alpaca Immunization and Antibody Library Construction

1. Alpaca immunization and titer determination

Alpaca immunization was performed using human serum albumin HSA (purchased from Sigma-Aldrich, Cat. No. A3782) after emulsification. The alpaca was immunized with 1 mg of human serum albumin per immunization once every 2 weeks for a total of three immunizations. Starting from the second immunization, serum was collected one week post-immunization to determine the titer.

The method for serum titer determination was as follows:

1) The HSA antigen was diluted to 1 μg/mL with a CBS buffer, and the dilution was added to a microplate at 100 μL/well. The plate was incubated at 4 °C overnight.

2) The plate was washed 3 times with PBST (PBS containing 0.1% Tween 20, pH = 7.4). 200 μL of the skim milk powder was added to each well, and the plate was left to stand at room temperature for 1 h and washed 3 times with PBST.
3) Sample addition: The immunized serum and the negative serum were each diluted 1000-fold, 3000-fold, 9000-fold, 27000-fold, 81000-fold, and 243000-fold, and 100 μL of each dilution was added to the blocked well. The plate was left to stand at room temperature for 1 h and washed 3 times with PBST.
4) Secondary antibody: HRP-labeled Goat Anti-Alpaca IgG was added at 100 μL/well, and the plate was left to stand at room temperature for 1 h and washed 3 times with PBST.
5) TMB color development: The TMB color development solution was added to the microplate at 100 μL/well, and color development was performed for 15 min; the stopping solution was added at 50 μL/well, and the microplate was placed in a microplate reader to read the light absorption value at 450 nm (OD450). The serum titer determination after the second and third alpaca immunizations is shown in Table 1. It can be seen that after the serum was diluted 243000-fold, the signal values for binding to human serum albumin were both above 0.59, both meeting the library construction standard. Peripheral blood was collected from the alpaca after the third immunization for phage library construction.

Table 1. Serum titer determination after second and third immunizations of alpaca with albumin

Antigen coating	Human serum albumin HSA (1 μg/mL)			
	OD450			
Serum dilution factor	Serum after second immunization	Negative serum	Serum after third immunization	Negative serum
1000	2.1439	0.0734	2.3233	0.0945
3000	1.9308	0.0553	2.3931	0.054
9000	1.5265	0.0541	2.1041	0.0526
27000	1.3957	0.0442	1.8906	0.0502
81000	1.029	0.0404	1.4248	0.0407
243000	0.592	0.035	0.8564	0.035
Blank well	0.0418	0.0538	0.0073	0.0538

2. Phage library construction

Peripheral blood was collected from the alpaca, and total RNA was extracted from the isolated alpaca PBMCs using Trizol, followed by reverse transcription to synthesize cDNA. Nanobody-specific primers were employed to amplify the variable region VH fragment (about 700 bp in length) via two rounds of amplification. The fragments obtained above and the pcomb3X vector were digested with the SfiI enzyme, and after digestion, they were mixed in an appropriate proportion and ligated using a T4 ligase. After ligation, the constructs were used for electroporation of XL1-Blue competent cells. Based on the colony growth of the dilution of competent cells on the resistant plate, the capacity of the transformation library obtained from the alpaca was calculated to be 6.24×10^8.

Example 2. Acquisition of Antibodies Cross-Binding to Human, Mouse, and Cynomolgus Monkey Albumins by Antibody Library Screening

1. Phage library screening

The screening process was as follows:
1) The microplate was coated separately with human serum albumin HSA and mouse albumin MSA (purchased from Sigma-Aldrich, Cat. No. A3559). A negative control was set up by coating the microplate with the 3% skim milk powder. The microplate was incubated at room temperature for 1 h. The blocking buffer was discarded, and the plate was washed with PBST.
2) About 5×10^{12} pfu of the library was added to the antigen plate and incubated with the antigens at 37 °C for 2 h. The plate was washed with 0.1% PBST.
3) Elution was performed with low-pH glycine-HCl, followed by neutralization with Tris-HCl until the pH was 7.4.
4) The eluate was mixed with *Escherichia coli* XL1-Blue, followed by incubation and shaking at 37 °C.
5) After the addition of phages, culturing was performed at 30 °C overnight, and the supernatant was collected.

6) 1)-5) were repeated to obtain a second-round library.

7) For the second-round amplified library obtained under the condition of coating with MSA, an antigen-binding ELISA assay was performed. In the ELISA experiment, the microplate was separately coated with HSA and MSA using a CBS buffer, and the ELISA assay results are shown in Table 2. It can be seen that relatively strong phages capable of binding to HSA and MSA were also obtained under this enrichment condition.

Table 2. Test results of second-round phage library enriched under MSA coating conditions for binding to HSA and MSA

Library enrichment mode	Dilution factor of amplified library	OD450 (coated with 5 μg/mL HSA)	OD450 (coated with 5 μg/mL MSA)	OD450 (coated with 3% milk powder)
Enrichment through MSA coating	10	1.9721	1.7435	0.0826

Summary: The antigen-binding ELISA of the second-round phage library enriched through MSA coating showed positive binding to both HSA and MSA, indicating that phages binding to HSA and MSA were enriched, and binding screening at the phage monoclonal level could be performed.

2. Screening and sequencing of phage monoclonals

The experimental process was as follows:

1) 96 monoclonal colonies were selected from the second-round phage library enriched through MSA coating for phage expression, and a microplate was separately coated with HSA, MSA, and monkey albumin for ELISA binding activity determination.

2) The microplate was coated with antigens HSA, MSA, and monkey albumin at 4 °C overnight, and the protein concentration during coating was 0.5 μg/mL.

3) Blocking: The blocking was performed with the 3% milk powder (200 μL/well) at room temperature for 1 h.

4) The 10-fold diluted phage expression supernatant was added at 100 μL/well at room temperature for 1 h.

5) The detection antibody Anti-M13 Antibody (HRP-labeled) (at a concentration of 0.2 μg/mL) was added at 100 μL/well, and the mixture was incubated at room temperature for 1 h.

6) The TMB color development solution was added at 200 μL/well for color development for 20 min, and the stopping solution was added at 50 μL/well. OD450 was measured using a microplate reader.

From the 96 phage clones of the second-round phage library enriched through MSA coating, the clone numbered 3005 was selected, and the ELISA assay results are shown in Table 3.

Table 3. Determination for activity of phage monoclonal 3005 binding to HSA, MSA, and monkey albumin

Phage clone number	OD450			
	Coated with MSA at 0.5 μg/mL	Coated with HSA at 0.5 μg/mL	Coated with monkey albumin at 0.5 μg/mL	Coated with 3% milk powder control
3005	2.9804	4.3634	2.7835	0.0556

7) After DNA sequencing and codon translation, the amino acid sequences of the candidate nanobody were obtained. The amino acid sequences of the CDR regions (CDR-H1, CDR-H2, and CDR-H3) of the preferred anti-albumin nanobody 3005 are shown in Table 4, the nucleic acid sequences of the CDR regions are shown in Table 5, the amino acid sequence of the variable region of the heavy chain (VH) of the antibody is shown in Table 6, and the nucleic acid sequence is shown in Table 7.

Table 4. Amino acid sequences of CDR regions of anti-albumin nanobody 3005

Antibody number	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
3005	1	NYYMS	2	GISVDGSFLDYADAVKG	3	ASGPQGLRLGAP

Table 5. Nucleic acid sequences encoding CDR regions of anti-albumin nanobody 3005

Antibody number	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
3005	42	AACTATTAC ATGAGT	43	GGTATTAGTGTTGATG GTAGTTTCCTTGACTAT GCAGACGCCGTGAAG GGC	44	GCCTCTGGGC CCCAGGGAC TACGCCTTGG GGCCCCC

Table 6. Amino acid sequence of anti-albumin nanobody 3005

SEQ ID NO	Antibody number	Antibody amino acid sequence
4	3005	QVQLVESGGGLAQPGGSLRLSCAASGFTFS**NYYMS**WARQAPGK GLEWVS**GISVDGSFLDADAVKG**RFTISRDNAKNTVYLQMNSLK PEDTALYYCAR**ASGPQGLRLGAP**RGQGTQVTVSS

Table 7. Nucleic acid sequence of anti-albumin nanobody 3005

SEQ ID NO	Antibody number	Antibody nucleic acid sequence
45	3005	CAGGTGCAGCTGGTGGAGTCTGGAGGAGGCTTGGCGCAGCC TGGGGGGTCTCTGAGACTCTCCTGTGCAGCCTCTGGATTCAC CTTCAGTAACTATTACATGAGTTGGGCTCGCCAGGCTCCAGG AAAGGGACTCGAGTGGGTGTCCGGTATTAGTGTTGATGGTAG TTTCCTTGACTATGCAGACGCCGTGAAGGGCCGATTCACCAT CTCCAGAGACAACGCCAAGAACACGGTGTATCTGCAAATGA ACAGCCTGAAACCTGAGGACACGGCACTGTATTACTGTGCA AGAGCCTCTGGGCCCCAGGGACTACGCCTTGGGGCCCCCCG GGGCCAGGGGACCCAGGTCACCGTCTCCTCA

Example 3. Detection of Activity of Anti-Albumin Candidate Antibody Binding to Albumins of Different Species

1. Expression of anti-albumin candidate antibody

The candidate antibody was expressed in mammalian cells. After protein purification, antigen-binding activity determination was performed to verify the albumin-binding activity of the candidate antibody at the protein level.

Through gene synthesis, expression plasmids for the His-tagged antibody 3005 and the positive control antibody Alb Nb were constructed. The positive control Alb Nb antibody is a nanobody against human serum albumin, and the antibody amino acid sequence is from SEQ ID NO: 62 in patent US 2007/0269422 A1.

A tPA secretion signal peptide MDAMKRGLCCVLLLCGAVFVSPS (SEQ ID NO: 36) was added to the N terminus of the antibody described above, and a linker GGGGS (SEQ ID NO: 37) and a 6xHis tag were added to the C terminus of the antibody. The gene sequence was inserted into the expression vector pCDNA3.1 (+) via the enzyme cutting sites NheI and XbaI. After the plasmid was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells was performed, followed by affinity purification using a Ni column.

After purification via the Ni column, the SDS-PAGE staining results of the His-tagged antibody Alb Nb and antibody 3005 are shown in FIGs. 1(A) and 1(B). Their molecular weights were consistent with the expected value (about 15 kDa). In FIG. 1(A), the cell medium supernatant from transient transfection of the antibody Alb Nb was labeled as Input, the flow-through fraction that failed to bind to the Ni column was labeled as FT, and the antibody Alb Nb that bound to and was eluted from the Ni column is labeled as E.

Meanwhile, fusion proteins of the antibody 3005 and the positive control antibody Alb Nb with Fc were constructed. Through primer design, the tPA secretion signal peptide MDAMKRGLCCVLLLCGAVFVSPS (SEQ ID NO: 36) was added to the N terminus of the antibody coding sequence. The amino acid sequence of human IgG1 Fc was added to the C terminus of the antibody sequence by homologous recombination.

Amino acid sequence of IgG1 Fc:
PPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD
ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV

FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 38)

The anti-albumin nanobody-Fc construct gene fragment was inserted into the mammalian cell expression vector pCDNA3.1 (+). The amino acid sequence of the anti-albumin nanobody-Fc fusion protein is shown in Table 8, and the nucleic acid sequence is shown in Table 9.

Table 8. Amino acid sequence of anti-albumin nanobody-Fc fusion protein

SEQ ID NO	Name of antibody	Antibody amino acid sequence
5	3005-Fc	QVQLVESGGGLAQPGGSLRLSCAASGFTFS**NYYMS**WARQAPGKGLEWVS **GISVDGSFLDYADAVKG**RFTISRDNAKNTVYLQMNSLKPEDTALYYCAR **ASGPQGLRLGAP**RGQGTQVTVSSPPCPAPELLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Table 9. Nucleic acid sequence encoding anti-albumin nanobody-Fc fusion protein

SEQ ID NO	Name of antibody	Antibody nucleic acid sequence
46	3005-Fc	CAGGTGCAGCTGGTGGAGTCTGGAGGAGGCTTGGCGCAGCCTGGGGG GTCTCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAACTATT ACATGAGTTGGGCTCGCCAGGCTCCAGGAAAGGGACTCGAGTGGGTGT CCGGTATTAGTGTTGATGGTAGTTTCCTTGACTATGCAGACGCCGTGAA GGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACACGGTGTATCT GCAAATGAACAGCCTGAAACCTGAGGACACGGCACTGTATTACTGTGC AAGAGCCTCTGGGCCCCAGGGACTACGCCTTGGGGCCCCCCGGGGCCA GGGGACCCAGGTCACCGTCTCCTCACCTCCTTGTCCTGCCCCCGAGCTG CTGGGCGGACCTTCTGTGTTCCTGTTCCCTCCCAAGCCCAAGGACACCC TGATGATCAGCAGGACCCCCGAGGTGACCTGCGTGGTGGTGGACGTGT CCCACGAGGACCCTGAGGTGAAGTTCAATTGGTACGTGGATGGCGTGG AGGTGCACAATGCCAAGACAAAGCCCAGAGAGGAGCAGTACAATTCC ACCTACAGAGTGGTGAGCGTGCTGACAGTGCTGCACCAGGACTGGCTG AATGGCAAGGAGTACAAGTGTAAGGTGTCCAACAAGGCCCTGCCTGCC CCCATCGAGAAGACCATCAGCAAGGCCAAGGGCCAGCCTAGGGAGCCC CAGGTGTACACCCTGCCTCCTTCCAGGGACGAGCTGACAAAGAACCAG GTGAGCCTGACCTGCCTGGTGAAGGGCTTCTACCCCAGCGACATCGCC GTGGAGTGGGAGTCCAATGGCCAGCCTGAGAACAACTACAAGACAAC ACCCCCTGTGCTGGATAGCGACGGCTCCTTCTTCCTGTACTCCAAGCTG ACAGTGGACAAGTCCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCTCC GTGATGCACGAGGCCCTGCACAATCACTACACACAGAAGAGCCTGAGC CTGAGCCCCGGCAAG

After the expression plasmid of the Fc fusion protein was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells and Protein A affinity purification were performed to obtain the target protein.

2. Detection of activity of anti-albumin candidate antibody binding to albumins

Bio-layer interferometry (BLI) was used to determine the affinity of the monovalent nanobody binding to albumins of different species. 8 NTA sensors were used to bind the same His-tagged monovalent nanobody (at a concentration of 1 μg/mL). The antigens were human serum albumin HSA (Baxter AG), mouse albumin MSA (purchased from Equitech-bio, Cat. No. MSA62-1000), and rat albumin (purchased from abcam, Cat. No. ab198656). In the BLI experiment, the antigen concentration gradient was set at 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, and 3.125 nM, and the antigen and antibody were allowed to associate for 180 s and dissociate for 360 s.

Table 10 shows the binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of the His-tagged monovalent nanobody 3005 binding to albumins of different species after fitting using the "1:1" model.

Table 10. Binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of nanobody 3005 binding to albumins of different species

Name of antibody	Antigen	K_D (M)	ka (1/Ms)	kd (1/s)	Full R^2
3005	Human serum albumin HSA	4.05E-09	1.87E+05	7.57E-04	0.9983
	Mouse albumin MSA	5.78E-09	2.71E+05	1.57E-03	0.9858
	Rat albumin	5.32E-09	1.55E+05	8.26E-04	0.9982

The data showed that 3005 can bind to HSA and MSA with high affinity, and can also bind to rat albumin.
The ELISA method was used again to determine the activity of 3005 binding to albumins of different species. The experimental method was as follows:
The antigens were diluted with the ELISA coating buffer. The final concentration of the human serum albumin HSA (Baxter AG) was 1 μg/mL, the final concentration of the mouse albumin MSA (purchased from Equitech-bio, Cat. No. MSA62-1000) was 2 μg/mL, and the final concentration of the rat albumin (purchased from abcam, Cat. No. ab198656) was 2 μg/mL.The antigen dilution was added to a microplate at 100 μL/well, and the microplate was coated at 4 °C overnight. After blocking with the 5% skim milk powder, PBST (PBS containing 0.1% Tween 20, pH = 7.4) was added to serial dilutions of the candidate antibody 3005-Fc and the positive control antibody Alb Nb-Fc (0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM), and the mixture was incubated at 37 °C for 1 h. After the plate was washed, the HRP-labeled goat anti-human IgG Fc antibody (purchased from Abbkine, Cat. No. A21050) was added, and the mixture was incubated at room temperature for 45 min. After the plate was washed, 100 μL of TMB (purchased from Tiangen Biotech, Cat. No. PA107-01) was added to each well for color development at 37 °C for 15 min, followed by the addition of 50 μL of the stopping solution. The absorbance at 450 nm (OD450) was measured using a microplate reader.
The ELISA assay results of antibodies 3005-Fc and Alb Nb-Fc binding to albumins of different species are shown in FIG. 2, and the summary of EC50 from the ELISA experiment is shown in Table 11.

Table 11. Summary of EC50 from ELISA experiment of antibody 3005-Fc and positive control Alb Nb-Fc binding to albumins of different species

Protein	EC50 (nM) for binding to HSA	EC50 (nM) for binding to MSA	EC50 (nM) for binding to rat albumin
3005-Fc	0.24	0.23	0.40
Alb Nb-Fc	0.19	0.27	Weak binding

The results showed that the antibody 3005-Fc can efficiently bind to HSA and MSA, and the binding activity was similar to that of the positive control Alb Nb-Fc. The positive control antibody Alb Nb-Fc showed very weak binding activity to rat albumin, whereas antibody 3005-Fc showed relatively good rat serum albumin binding activity.

Example 4. Humanization Engineering of Anti-Albumin Candidate Antibody

1. Humanization engineering, expression, and purification of candidate antibody

Humanization engineering was performed through antibody CDR region grafting. The candidate nanobody 3005 (SEQ ID NO: 4) was used as a starting antibody for humanization engineering of the antibody. The homology between the sequence of the candidate antibody 3005 and human antibody sequences was first aligned through the antibody databases IGBLAST and IMGT. Subsequently, the human antibody framework region sequence with high homology was combined with 3 CDR region sequences (SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3) of the candidate antibody 3005 to construct a humanized antibody.
A secretion signal peptide was added to the N terminus of the antibody, and a linker GGGGS (SEQ ID NO: 37) and 6xHis were added to the C terminus of the antibody. The coding DNA sequence was obtained through gene synthesis, and subsequently inserted into the expression vector pCDNA3.1 (+) via the enzyme cutting sites NheI and XbaI. After the plasmid was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells was performed, followed by affinity purification using a Ni column.
The amino acid sequences of the humanization-engineered antibodies of the nanobody 3005 are shown in Table 12, and the nucleic acid sequences are shown in Table 13.

Table 12. Amino acid sequences of 3005 humanization-engineered nanobodies

SEQ ID NO	Name of antibody	Antibody amino acid sequence
6	3005Hz3	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWVRQAPGKGLEW VSGISVDGSFLDYADAVKGRFTISRDNAKNTLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS
7	3005Hz4	QVQLVESGGGLVKPGGSLRLSCAASGFTFSNYYMSWIRQAPGKGLEW VSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYY CARASGPQGLRLGAPRGQGTQVTVSS
8	3005Hz5	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWIRQAPGKGLEW VSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS
9	3005Hz6	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEW VSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS

Table 13. Nucleic acid sequences encoding 3005 humanization-engineered nanobodies

SEQ ID NO	Name of antibody	Antibody nucleic acid sequence
47	3005Hz3	GAGGTGCAGCTGGTGGAAAGCGGCGGCGGACTGGTTCAACCTGGA GGATCTCTGCGGCTGAGCTGTGCTGCTTCTGGCTTTACCTTCAGCAA CTACTATATGAGCTGGGTCAGACAGGCCCCTGGCAAAGGCCTGGAAT GGGTGTCCGGCATCAGCGTGGACGGCAGCTTCCTGGACTACGCCGA TGCCGTGAAGGGCAGATTCACAATCAGCAGAGATAATGCCAAGAAC ACCCTGTACCTGCAGATGAACAGCCTGCGCCCCGAGGACACCGCCG TGTACTACTGCGCCAGAGCCTCTGGACCTCAGGGCCTCCGGCTGGG CGCCCCAAGAGGCCAGGGCACACTGGTGACCGTGTCCAGC
48	3005Hz4	CAAGTGCAGCTGGTTGAGAGCGGCGGAGGCCTGGTGAAACCTGGA GGATCTCTGAGACTGTCTTGTGCTGCTTCTGGCTTTACATTCAGCAAC TACTATATGAGCTGGATCAGACAGGCCCCAGGCAAGGGCCTGGAATG GGTCAGCGGCATCAGCGTGGACGGCAGCTTCCTGGACTACGCCGAT GCCGTGAAGGGCAGATTCACAATCAGCAGAGATAATGCCAAGAACT CCCTGTACCTGCAGATGAACAGCCTGCGGGCCGAGGACACCGCCGT GTACTACTGCGCCAGAGCCAGCGGACCTCAGGGCCTCCGGCTGGGC GCCCCCAGAGGCCAGGGCACCCAGGTGACCGTGTCCAGC
49	3005Hz5	GAGGTGCAGCTGGTGGAAAGCGGCGGAGGACTGGTCCAGCCCGGC GGAAGCCTGCGGCTCTCTTGTGCTGCTTCTGGCTTTACCTTCAGCAA CTACTATATGAGCTGGATCAGACAGGCCCCTGGCAAAGGCCTGGAAT GGGTGTCCGGCATCAGCGTGGACGGCAGCTTCCTGGACTACGCCGA TGCCGTGAAGGGCAGATTCACAATCAGCAGAGACAATGCCAAGAAC AGCCTGTACCTGCAGATGAACAGCCTGCGGCCTGAGGACACCGCCG TGTACTACTGCGCCAGAGCCAGCGGACCTCAAGGCCTGAGACTGGG CGCCCCAAGAGGCCAGGGCACCCTGGTGACAGTGTCCAGC
50	3005Hz6	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGACTGGTGCAACCTGGC GGCAGCCTCAGACTGAGCTGTGCTGCTTCTGGCTTCACCTTCAGCAA CTACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGCCTGGAA TGGGTTTCCGGCATCAGCGTGGACGGCTCTTTTCTGGACTACGCCGA TGCCGTGAAGGGCAGATTCACAATCTCTCGGGATAATGCCAAGAACA GCCTGTACCTGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGT GTACTACTGCGCCCGGGCCAGCGGCCCCCAGGGCCTGCGGCTGGGA GCCCCTAGAGGCCAGGGTACACTGGTGACCGTGTCCAGC

After the His-tagged humanized proteins were purified via the Ni column, they were separated by SDS-

PAGE electrophoresis. Their molecular weights were consistent with the expected value (about 13 kDa), and the Coomassie staining results are shown in FIG. 3.

2. Detection of binding activity of humanized antibodies

In this example, the ELISA method was used to determine the activity of the original candidate antibody 3005 and the humanization-engineered antibodies binding to the mouse albumin MSA.

In the experiment, a microplate was coated with the mouse albumin MSA at a protein concentration of 5 μg/mL at 100 μL/well at 4 °C overnight. After blocking with the 5% skim milk powder, dilutions of the His-tagged proteins to be tested (0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM) were prepared with PBST (PBS containing 0.1% Tween 20, pH = 7.4). The proteins to be tested were the original antibody 3005 and four humanization-engineered antibodies. The protein dilution was added to the microplate, and the microplate was incubated at 37 °C for 1 h. After the plate was washed, an HRP-labeled mouse anti-His tag monoclonal antibody (purchased from Proteintech, Cat. No. HRP-66005) was added, and the mixture was incubated at room temperature for 45 min. After the plate was washed, 100 μL of TMB (purchased from Tiangen Biotech, Cat. No. PA107-01) was added to each well for color development at 37 °C for 15 min, followed by the addition of 50 μL of the stopping solution. The absorbance at 450 nm (OD450) was measured using a microplate reader.

The ELISA assay results of the binding of the antibody 3005 and its humanized antibodies to MSA are shown in FIG. 4. The summary of EC50 from the ELISA experiments is shown in Table 14.

Table 14. Summary of EC50 and maximum binding values from ELISA experiment of antibody 3005 and its humanized antibodies binding to MSA

Antibody	EC50 (nM) for binding to MSA	Maximum binding value OD450
3005	2.3	1.38
3005Hz3	22.0	0.89
3005Hz4	49.8	0.79
3005Hz5	31.6	0.80
3005Hz6	2.9	1.34

The results showed that the activity of the humanized antibody 3005Hz6 binding to MSA was similar to that of the original antibody 3005. The activity of the other humanized antibodies binding to MSA was inferior to that of 3005Hz6.

The ELISA method was used to determine the activity of the original candidate antibody 3005 and 3005Hz6 obtained by humanization binding to HSA and rat albumin.

In the experiment, a microplate was coated with HSA or rat albumin at a protein concentration of 2 μg/mL at 100 μL/well at 4 °C overnight. After blocking with the 5% skim milk powder, dilutions of the His-tagged proteins to be tested (0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, 0.3 nM, 1 nM, 10 nM, and 100 nM) were prepared with PBST (PBS containing 0.1% Tween20, pH = 7.4). The proteins to be tested were the positive control antibody Alb Nb, the original antibody 3005, and the humanized antibody 3005Hz6. The protein dilution was added to the microplate, and the microplate was incubated at 37 °C for 1 h. After the plate was washed, an HRP-labeled mouse anti-His tag monoclonal antibody (purchased from Proteintech, Cat. No. HRP-66005) was added, and the mixture was incubated at room temperature for 45 min. After the plate was washed, 100 μL of TMB (purchased from Tiangen Biotech, Cat. No. PA107-01) was added to each well for color development at 37 °C for 15 min, followed by the addition of 50 μL of the stopping solution. The absorbance at 450 nm (OD450) was measured using a microplate reader.

The ELISA results are shown in FIGs. 5(A) and 5(B). The EC50 values fitted from the binding ELISA curves of FIG. 5 are shown in Table 15.

Table 15. Summary of EC50 for antibody 3005 and its humanized antibody 3005Hz6 binding to HSA and rat albumin

Name of antibody	EC50 (nM) for binding to HSA	EC50 (nM) for binding to rat albumin
3005	0.31	2.74
3005Hz6	0.26	1.64
Alb Nb	0.66	Weak binding

As can be seen from FIG. 5 and Table 15, the binding activity of the humanized antibody 3005Hz6 to HSA and rat albumin is comparable to that of the original antibody 3005, both of which are stronger than that of the positive control antibody Alb Nb.

The surface plasmon resonance (SPR) method was used to determine the binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of the humanized antibody 3005Hz6 binding to HSA and MSA. In the experiment, a Biacore 8K instrument (Cytiva) was used, and Series S Sensor Chip NTA was utilized to capture the His-tagged humanized antibody 3005Hz6. The analytes were serially diluted HSA or MSA and were diluted with a running buffer (composition: 10 mM HEPES, 150 mM NaCl, 0.05% v/v Tween-20, pH = 7.4). The binding time was 150 s, and the dissociation time was 900 s. The binding and dissociation curves of the antibody 3005Hz6 with HSA are shown in FIG. 6(A), and the binding and dissociation curves of the antibody 3005Hz6 with MSA are shown in FIG. 6(B). A "Two state reaction" model was used to fit the binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of 3005Hz6 binding to HSA and MSA, and the results are shown in Table 16.

Table 16. Binding rates (ka), dissociation rate (kd), and equilibrium dissociation constant (K_D) of humanized antibody 3005Hz6 with HSA and MSA

Ligand	Analyte	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/s)	kd2 (1/s)	K_D (M)
3005Hz6	HSA	9.04E+04	1.56E-03	3.67E-04	8.08E-04	1.19E-08
3005Hz6	MSA	7.57E+04	1.51E-02	5.01E-03	3.02E-04	1.14E-08

As can be seen from Table 16, the humanized antibody 3005Hz6 exhibited similar affinity for HSA and MSA. The K_D determined by the SPR method was about 11 nM.

With reference to Table 10 of Example 3, it was determined that the K_D value of the original antibody 3005 for HSA and MSA was about 5 nM as determined by the BLI method, and the value differed from the K_D value in Table 16 by less than 3 times. Therefore, it is considered that the humanized antibody 3005Hz6 exhibited affinity for HSA and MSA similar to that of the original antibody 3005.

Example 5. Risk Site Engineering and Activity Determination of Humanized Antibody 3005Hz6

1. Risk site mutation, expression, and purification of humanized antibody 3005Hz6

Through sequence analysis of the antibody 3005Hz6, its CDR-H2 region (SEQ ID NO: 2) was found to contain an amino acid motif DG, which is prone to aspartic acid (D) isomerization. Therefore, the DG amino acid motif in the CDR-H2 region of 3005Hz6 was engineered and mutated to DA, DS, or EG, separately, to reduce the risk of structural and activity alterations in the protein. The names of the engineered antibodies and the amino acid sequences of their CDR regions are shown in Table 17, the nucleic acid sequences are shown in Table 18, the amino acid sequences of the variable regions of the heavy chains (VHs) of the antibodies are shown in Table 19, and the nucleic acid sequences are shown in Table 20.

Table 17. Amino acid sequences of CDR regions of humanized antibody 3005Hz6 mutants

Name of antibody	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
3005Hz6 (DA)	1	NYYMS	10	GISV**DA**SFLDYADAVKG	3	ASGPQGLRLGAP
3005Hz6 (DS)	1	NYYMS	11	GISV**DS**SFLDYADAVKG	3	ASGPQGLRLGAP
3005Hz6 (EG)	1	NYYMS	12	GISV**EG**SFLDYADAVKG	3	ASGPQGLRLGAP

Table 18. Nucleic acid sequences encoding CDR regions of humanized antibody 3005Hz6 mutants

Name of antibody	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
3005Hz6 (DA)	51	AACTACTA TATGAGC	52	GGCATCAGCGTGGACG CTAGCTTTCTGGACTAC GCCGATGCCGTGAAGG GC	55	GCTTCCGGTC CTCAGGGCC TGCGGCTGG GAGCTCCC
3005Hz6 (DS)	51	AACTACTA TATGAGC	53	GGCATCAGCGTGGACT CTAGCTTTCTGGACTAC GCCGATGCCGTGAAGG GC	55	GCTTCCGGTC CTCAGGGCC TGCGGCTGG GAGCTCCC

3005Hz6 (EG)	51	AACTACTA TATGAGC	54	GGCATCAGCGTGGAGG GCAGCTTTCTGGACTAC GCCGATGCCGTGAAGG GC	55	GCTTCCGGTC CTCAGGGCC TGCGGCTGG GAGCTCCC

Table 19. Amino acid sequences of humanized nanobody 3005Hz6 mutants

SEQ ID NO	Name of antibody	Amino acid sequence of 3005Hz6 mutant
13	3005Hz6 (DA)	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEW VSGISVDASFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS
14	3005Hz6 (DS)	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEW VSGISVDSSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS
15	3005Hz6 (EG)	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEW VSGISVEGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYC ARASGPQGLRLGAPRGQGTLVTVSS

Table 20. Nucleic acid sequences encoding humanized nanobody 3005Hz6 mutants

SEQ ID NO	Name of antibody	Nucleic acid sequence of 3005Hz6 mutant
56	3005Hz6 (DA)	GAAGTGCAGCTGGTCGAGAGCGGTGGAGGACTGGTGCAACCTGGCG GAAGCCTCAGACTGAGCTGTGCTGCCTCTGGCTTCACCTTCAGCAAC TACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGCCTGGAATG GGTTTCCGGCATCAGCGTGGACGCTAGCTTTCTGGACTACGCCGATGC CGTGAAGGGCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGCC TGTACCTGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTAC TACTGCGCAAGGGCTTCCGGTCCTCAGGGCCTGCGGCTGGGAGCTCC CAGAGGTCAGGGTACACTGGTGACCGTGTCCAGC
57	3005Hz6 (DS)	GAAGTGCAGCTGGTCGAGAGCGGTGGAGGACTGGTGCAACCTGGCG GAAGCCTCAGACTGAGCTGTGCTGCCTCTGGCTTCACCTTCAGCAAC TACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGCCTGGAATG GGTTTCCGGCATCAGCGTGGACTCTAGCTTTCTGGACTACGCCGATGC CGTGAAGGGCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGCC TGTACCTGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTAC TACTGCGCAAGGGCTTCCGGTCCTCAGGGCCTGCGGCTGGGAGCTCC CAGAGGTCAGGGTACACTGGTGACCGTGTCCAGC
58	3005Hz6 (EG)	GAAGTGCAGCTGGTCGAGAGCGGTGGAGGACTGGTGCAACCTGGCG GAAGCCTCAGACTGAGCTGTGCTGCCTCTGGCTTCACCTTCAGCAAC TACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGCCTGGAATG GGTTTCCGGCATCAGCGTGGAGGGCAGCTTTCTGGACTACGCCGATG CCGTGAAGGGCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGC CTGTACCTGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTA CTACTGCGCAAGGGCTTCCGGTCCTCAGGGCCTGCGGCTGGGAGCTC CCAGAGGTCAGGGTACACTGGTGACCGTGTCCAGC

A secretion signal peptide was added to the N terminus of the antibody 3005Hz6 mutant, and a linker GGGGS (SEQ ID NO: 37) and 6xHis were added to the C terminus of the antibody. The coding DNA sequence was obtained through gene synthesis (see Table 20), and subsequently inserted into the expression vector pCDNA3.1 (+) via the enzyme cutting sites NheI and XhoI. After the plasmid was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells was performed, and the cell culture supernatant was collected and subjected to Ni-column affinity purification. The SDS-PAGE results of the purified products are shown in FIG. 7(A), where the cell medium

supernatants from transient expression of 3005Hz6 (EG) and 3005Hz6 (DS) were labeled as Input, the flow-through fractions that failed to bind to the Ni column were labeled as FT, and the products that were eluted and purified were labeled as E. The cell supernatant from transient expression of the 3005Hz6 (DA) protein was purified via the Ni column and further purified by cation exchange chromatography. The SDS-PAGE results of the purified protein are shown in FIG. 7(B). The molecular weight of the three mutant proteins was 13 kDa.

2. Determination of activity of humanized antibody 3005Hz6 mutants

The ELISA assay for the binding activity to HSA was performed on the humanized antibody 3005Hz6 mutant proteins 3005Hz6 (DA), 3005Hz6 (DS), and 3005Hz6 (EG). In the ELISA experiment, the microplate was coated with 2 μg/mL HSA. Serially diluted 3005Hz6 or its mutants were added, and the level of the antibodies binding to HSA was determined using the HRP-labeled mouse anti-His tag monoclonal antibody (purchased from Proteintech, Cat. No. HRP-66005). The results of the ELISA experiment are shown in FIG. 8, and the EC50 of the ELISA experiment is shown in Table 21.

Table 21. Summary of EC50 for humanized antibody 3005Hz6 mutant proteins 3005Hz6 (DA), 3005Hz6 (DS), and 3005Hz6 (EG) binding to HSA

Name of antibody	EC50 (nM) for binding to HSA
3005Hz6	0.35
3005Hz6 (DA)	0.44
3005Hz6 (DS)	0.28
3005Hz6 (EG)	0.40

As can be seen from Table 21, the humanized antibody 3005Hz6 and its mutant proteins exhibited similar HSA binding activity.

Example 6. Fusion Constructs of Monovalent Anti-VEGFA Nanobody and Humanized Anti-Albumin Antibody

1. Design of fusion constructs of monovalent anti-VEGFA nanobody and humanized anti-albumin antibody 3005Hz6

A fusion construct of the anti-VEGFA antibody and the anti-albumin antibody 3005Hz6 was constructed using the anti-VEGFA nanobody as an effector molecule.

The technical solution for the screening of anti-VEGFA nanobodies in this example can be found in Patent Application No. PCT/CN2023/118600, which is briefly summarized as follows:

The anti-VEGFA nanobody sequence used in this example was screened from a human VEGFA-immunized alpaca library. The construction and screening processes of the phage library were as follows: Alpaca immunization was performed using human VEGFA165 (tag-free, purchased from Sino Biological, Cat. No. HPLC-10008-HNAH, hereinafter referred to as VEGFA) after emulsification, and a phage display library was constructed. After 4 rounds of enrichment and screening, 96 single clones were picked into a 96-well plate to determine the phage binding activity to VEGFA. Phages with VEGFA binding activity were subjected to plasmid sequencing and codon translation to obtain the amino acid sequences of candidate nanobodies. The amino acid sequences of the CDR regions of the preferred anti-VEGFA nanobody V1 are shown in Table 22, and the nucleic acid sequences are shown in Table 23. The amino acid sequences of the nanobody V1 and its 9 humanized antibodies are shown in Table 24, and the nucleic acid sequences are shown in Table 25.

Table 22. Amino acid sequences of CDR regions of anti-VEGFA nanobody V1

Name of anti-VEGFA nanobody	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
V1	16	SYTMG	17	AISKGGYKYDSVSLEA	18	TRAYGSSRLRLADTYEY

Table 23. Nucleic acid sequences encoding CDR regions of anti-VEGFA nanobody V1

Name of anti-VEGFA nanobody	SEQ ID NO	CDR-H1	SEQ ID NO	CDR-H2	SEQ ID NO	CDR-H3
V1	59	TCATATAC	60	GCAATCTCCAAAG	61	ACGAGAGCATACG

		CATGGGT		GAGGCTATAAGTAT GATTCCGTTTCCCT CGAGGCC		GCTCGTCTAGGCTG AGGCTCGCCGACA CCTATGAGTAT

Table 24. Amino acid sequences of anti-VEGFA nanobody V1 and its humanized antibodies

SEQ ID NO	Name of humanized anti-VEGFA nanobody	Amino acid sequence of anti-VEGFA nanobody
19	V1	QLQLVESGGGSVQPGGSLRLSCEVSGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISKDNTKNTVYLQMNSLKPE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTQVTVSS
20	V1-SA1	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRPE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
21	V1-DP	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNSKNTVYLQMNSLRPE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
22	V1-com-78	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNSKNTVYLQMNSLRAE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
23	V1-com-87	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNSKNTLYLQMNSLRPED TAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
24	V1-2m	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNSKNTVYLQMNSLRPE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
25	V1-3m	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRPE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS
26	V1-com-74-78-97	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRAE DTAVYYCASTRAYGSSRLRLADTYEYWGQGTLVTVSS
27	V1-4m	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRPE DTAVYYCASTRAYGSSRLRLADTYEYWGQGTLVTVSS
28	V1-SA3	EVQLVESGGGLVQPGGSLRLSCAASGRTFSSYTMGWFRQAPGKE REFVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRAE DTAVYYCAATRAYGSSRLRLADTYEYWGQGTLVTVSS

Table 25. Nucleic acid sequences encoding anti-VEGFA nanobody V1 and its humanized antibodies

SEQ ID NO	Name of humanized anti-VEGFA nanobody	Nucleic acid sequence of anti-VEGFA nanobody
62	V1	CAGTTGCAGCTCGTGGAGTCGGGTGGAGGCTCGGTGCAACCTGG GGGGTCTCTGAGACTCTCCTGTGAGGTCTCTGGAAGAACCTTCT CCTCATATACCATGGGTTGGTTCCGCCAGGCCCCAGGGAAGGAGC GTGAGTTCGTGGTGGCAATCTCCAAAGGAGGCTATAAGTATGATT CCGTTTCCCTCGAGGCCCGATTCACCATCTCCAAAGACAACACCA AGAACACGGTGTATCTGCAAATGAACAGCCTGAAACCTGAGGAC ACAGCCGTTTATTACTGTGCAGCAACGAGAGCATACGGCTCGTCT

		AGGCTGAGGCTCGCCGACACCTATGAGTATTGGGGCCAGGGGAC CCAGGTCACCGTCTCCTCA
63	V1-SA1	GAAGTGCAACTGGTCGAAAGCGGCGGAGGCCTGGTGCAGCCCG GAGGAAGCCTGAGACTGTCCTGCGCCGTCAGCGGCAGAACATTC AGCAGCTATACAATGGGCTGGTTTAGACAAGCCCCTGGCAAGGA GAGAGAGTTCGTGGTCGCCATTTCTAAGGGCGGCTACAAGTACG ACAGCGTGTCCCTGGAGGCTAGATTCACAATTAGCAGAGACAAC GCCAAAAACACAGTGTATCTGCAGATGAATAGCCTCAGACCTGA GGACACAGCCGTGTATTACTGCGCCGCCACAAGGGCCTACGGAA GCAGCAGACTCAGACTGGCCGACACATACGAGTACTGGGGACAA GGCACCCTCGTCACCGTGAGCAGT
64	V1-DP	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGTGTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATA GCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGCCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
65	V1-com-78	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATT CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGGCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
66	V1-com-87	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATA GCAAAAACACCCTGTACCTGCAGATGAACAGCCTGCGGCCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
67	V1-2m	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATT CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGCCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
68	V1-3m	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATG CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGCCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC

		AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
69	V1-com-74-78-97	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATG CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGGCTGAA GATACCGCCGTGTACTACTGCGCCTCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
70	V1-4m	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATG CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGCCTGAA GATACCGCCGTGTACTACTGCGCCTCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC
71	V1-SA3	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGCCTGGTGCAGCCCG GCGGATCTCTGCGGCTGTCTTGTGCCGCCTCTGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTTAGACAGGCCCCTGGCAAGGAA AGAGAGTTCGTGGTGGCCATCAGCAAGGGCGGATATAAGTACGA CAGCGTGTCCCTGGAGGCCAGATTCACCATCAGCCGGGACAATG CCAAAAACACCGTGTACCTGCAGATGAACAGCCTGCGGGCTGAA GATACCGCCGTGTACTACTGCGCCGCTACAAGAGCCTACGGCAGC AGCAGACTGCGCCTGGCCGACACATACGAGTACTGGGGCCAGGG CACACTGGTGACCGTGTCCTCC

Antibodies with high thermal stability are more favorable for antibody production and long-term storage. Therefore, the thermal stability *Tm* values (melting temperature) of different anti-VEGFA nanobodies were compared. For the anti-VEGFA nanobody V1 and its 9 humanized antibodies, the *Tm* values were determined using an UNCLE instrument (unchainedlabs), and the results are shown in Table 26.

Table 26. Thermal stability *Tm* values of anti-VEGFA nanobody V1 and its 9 humanized antibodies

Name of anti-VEGFA nanobody	*Tm* (℃)
V1-DP	72.4
V1-SA1	71.0
V1	67.0
V1-SA3	66.7
V1-3m	66.4
V1-2m	65.8
V1-com-78	64.5
V1-com-87	63.5
V1-com-74-78-97	60.6
V1-4m	59.4

As can be seen from Table 26, V1-DP and V1-SA1 exhibited relatively high *Tm* values, indicating that the thermal stability of these two antibodies is likely superior to that of the other humanized antibodies.

The humanized anti-VEGFA nanobodies with relatively high melting temperatures (*Tm* values), namely V1-

SA1 (SEQ ID NO: 20) and V1-DP (SEQ ID NO: 21), were selected to design fusion constructs with the humanized anti-albumin antibody 3005Hz6. Based on different tandem arrangements, 3 types of fusion constructs using GGS as the linker were designed in total. The amino acid sequences are shown in Table 27, and the nucleic acid sequences are shown in Table 28.

Table 27. Amino acid sequences of fusion constructs of humanized antibody 3005Hz6 and monovalent anti-VEGFA nanobody

SEQ ID NO	Name of fusion construct	Amino acid sequence of fusion construct
29	V1SA-3005Hz6	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKEREF VVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRPEDTAVYY CAATRAYGSSRLRLADTYEYWGQGTLVTVSSGGSEVQLVESGGGLV QPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEWVSGISVDGSFL DYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARASGPQGL RLGAPRGQGTLVTVSS
30	3005Hz6-V1SA	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLE WVSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAV YYCARASGPQGLRLGAPRGQGTLVTVSSGGSEVQLVESGGGLVQPG GSLRLSCAVSGRTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVS LEARFTISRDNAKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLA DTYEYWGQGTLVTVSS
31	V1DP-3005Hz6	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKEREF VVAISKGGYKYDSVSLEARFTISRDNSKNTVYLQMNSLRPEDTAVYY CAATRAYGSSRLRLADTYEYWGQGTLVTVSSGGSEVQLVESGGGLV QPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEWVSGISVDGSFL DYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARASGPQGL RLGAPRGQGTLVTVSS

Table 28. Nucleic acid sequences encoding fusion constructs of humanized antibody 3005Hz6 and monovalent anti-VEGFA nanobody

SEQ ID NO	Name of fusion construct	Nucleic acid sequence of fusion construct
72	V1SA-3005Hz6	GAGGTGCAACTGGTGGAGTCCGGAGGTGGCCTGGTCCAGCCTGG CGGCAGCCTGAGACTCTCCTGCGCCGTGAGCGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTCAGACAAGCTCCAGGCAAAGAA AGGGAGTTCGTGGTGGCTATTAGCAAAGGCGGCTATAAGTACGAT AGCGTGAGCCTGGAAGCTCGGTTCACCATTAGCAGAGATAACGC CAAGAACACCGTGTACCTGCAAATGAATAGCCTGAGACCTGAGG ATACCGCCGTGTACTACTGTGCCGCCACAAGAGCCTATGGCAGCT CCAGGCTCAGACTGGCTGATACCTACGAATATTGGGGCCAAGGCA CCCTGGTGACAGTCAGCAGCGGCGGATCCGAAGTGCAGCTGGTC GAGAGCGGCGGAGGACTGGTGCAACCTGGCGGCAGCCTCAGAC TGAGCTGTGCTGCTTCTGGCTTCACCTTCAGCAACTACTATATGAG CTGGGCCAGACAGGCCCCAGGCAAAGGCCTGGAATGGGTTTCCG GCATCAGCGTGGACGGCTCTTTTCTGGACTACGCCGATGCCGTGA AGGGCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGCCTGT ACCTGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTAC TACTGCGCCCGGGCCAGCGGCCCCCAGGGCCTGCGGCTGGGAGC CCCTAGAGGCCAGGGTACACTGGTGACCGTGTCCAGC
73	3005Hz6-V1SA	GAAGTGCAGCTGGTCGAGAGCGGCGGAGGACTGGTGCAACCTG GCGGCAGCCTCAGACTGAGCTGTGCTGCTTCTGGCTTCACCTTCA GCAACTACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGC CTGGAATGGGTTTCCGGCATCAGCGTGGACGGCTCTTTTCTGGAC TACGCCGATGCCGTGAAGGGCAGATTCACAATCTCTCGGGATAAT GCCAAGAACAGCCTGTACCTGCAGATGAACAGCCTGAGACCTGA

		GGACACCGCCGTGTACTACTGCGCCCGGGCCAGCGGCCCCCAGG GCCTGCGGCTGGGAGCCCCTAGAGGCCAGGGTACACTGGTGACC GTGTCCAGCGGCGGATCCGAGGTGCAACTGGTGGAGTCCGGAGG TGGCCTGGTCCAGCCTGGCGGCAGCCTGAGACTCTCCTGCGCCG TGAGCGGCAGAACCTTCAGCAGCTACACCATGGGCTGGTTCAGA CAAGCTCCAGGCAAAGAAAGGGAGTTCGTGGTGGCTATTAGCAA AGGCGGCTATAAGTACGATAGCGTGAGCCTGGAAGCTCGGTTCAC CATTAGCAGAGATAACGCCAAGAACACCGTGTACCTGCAAATGA ATAGCCTGAGACCTGAGGATACCGCCGTGTACTACTGTGCCGCCA CAAGAGCCTATGGCAGCTCCAGGCTCAGACTGGCTGATACCTACG AATATTGGGGCCAAGGCACCCTGGTGACAGTCAGCAGC
74	V1DP-3005Hz6	GAAGTTCAGTTGGTCGAATCAGGAGGAGGATTGGTTCAACCTGG TGGTTCTCTCAGATTGTCTTGCGCTGTGAGTGGCAGGACATTCAG CAGCTACACTATGGGATGGTTTCGCCAGGCTCCAGGCAAGGAGA GAGAGTTTGTAGTCGCCATTTCTAAGGGGGGATACAAGTACGACT CTGTGAGCCTGGAGGCTAGGTTCACAATCAGCAGGGATAACTCTA AGAATACCGTCTACCTGCAGATGAATAGCCTCAGACCTGAGGACA CTGCCGTCTACTACTGCGCTGCCACTCGGGCTTACGGTTCCAGCA GGCTTAGGCTGGCCGATACATATGAATACTGGGGCCAGGGCACCC TTGTTACAGTGAGCAGTGGCGGATCCGAAGTGCAGCTGGTCGAG AGCGGCGGAGGACTGGTGCAACCTGGCGGCAGCCTCAGACTGA GCTGTGCTGCTTCTGGCTTCACCTTCAGCAACTACTATATGAGCTG GGCCAGACAGGCCCCAGGCAAAGGCCTGGAATGGGTTTCCGGCA TCAGCGTGGACGGCTCTTTTCTGGACTACGCCGATGCCGTGAAGG GCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGCCTGTACC TGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTACTAC TGCGCCCGGGCCAGCGGCCCCCAGGGCCTGCGGCTGGGAGCCCC TAGAGGCCAGGGTACACTGGTGACCGTGTCCAGC

A secretion signal peptide was added to the N terminus of the fusion construct, and a linker GGGGS (SEQ ID NO: 37) and 6xHis were added to the C terminus of the antibody. The coding DNA sequence was obtained through gene synthesis (see Table 28), and subsequently inserted into the expression vector pCDNA3.1 (+) via the enzyme cutting sites NheI and XhoI. After the plasmid was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells was performed, and the cell culture supernatant was collected and subjected to Ni-column affinity purification. The SDS-PAGE results of the purified products are shown in FIG. 9, where the cell medium supernatant from transient expression of each protein was labeled as Input, the flow-through fractions that failed to bind to the Ni column were labeled as FT, and the products that were eluted and purified were labeled as E. The molecular weight of the target protein was about 28 kDa.

2. Determination of activity of fusion constructs of humanized antibody 3005Hz6 and monovalent anti-VEGFA nanobody

In this example, whether the two nanobody elements constituting the fusion construct maintained the original activity was determined.

The HSA binding activity of the anti-albumin nanobody 3005Hz6 in the fusion constructs was determined. The determination method was identical to that described in "2. Determination of activity of humanized antibodies" of Example 4. The ELISA results are shown in FIG. 10, and the EC50 values are shown in Table 29.

Table 29. Summary of EC50 for fusion constructs or 3005Hz6 binding to HSA

Name of fusion construct	EC50 (nM) for binding to HSA
V1SA-3005Hz6	0.32
3005Hz6-V1SA	0.33
V1DP-3005Hz6	0.39
3005Hz6	0.25

As can be seen from Table 29, the binding activity of the fusion constructs to HSA was similar to that of 3005Hz6 alone, indicating that the fusion of 3005Hz6 with other antibody fragments did not affect the binding activity of 3005Hz6 to HSA. Moreover, the anti-VEGFA antibody can be fused at the N or C terminus of the anti-albumin antibody, and the different positions did not affect the binding activity of the fusion protein to albumin.

The activity of the anti-VEGFA nanobody in the fusion constructs was determined by a VEGFR2 competition ELISA method. The activity of the fusion constructs was compared with that of the VEGFA antibody alone.

Specifically, the microplate was coated with a goat anti-human IgG Fc protein (purchased from Solarbio, Cat. No. SPA105) at a concentration of 5 μg/mL at 4 °C overnight. After the plate was washed with PBST and blocked with the milk powder, the VEGFR2 extracellular domain-human IgG1 Fc fusion protein (VEGFR2-Fc, prepared in-house) was added. Amino acid sequence of VEGFR2-Fc:

ASVGLPSVSLDLPRLSIQKDILTIKANTTLQITCRGQRDLDWLWPNNQSGSEQRVEVTECSDGLFCK
TLTIPKVIGNDTGAYKCFYRETDLASVIYVYVQDYRSPFIASVSDQHGVVYITENKNKTVVIPCLGS
ISNLNVSLCARYPEKRFVPDGNRISWDSKKGFTIPSYMISYAGMVFCEAKINDESYQSIMYIVVVVG
YRIYDVVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEM
KKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEKPFVAFGSGMESLVEATVGERVRIPA
KYLGYPPPEIKWYKNGIPLESNHTIKAGHVLTIMEVSERDTGNYTVILTNPISKEKQSHVVSLVVYV
PPQIGEKSLISPVDSYQYGTTQTLTCTVYAIPPPHHIHWYWQLEEECANEPSQAVSVTNPYPCEEWR
SVEDFQGGNKIEVNKNQFALIEGKNKTVSTLVIQAANVSALYKCEAVNKVGRGERVISFHVTRGPE
ITLQPDMQPTEQESVSLWCTADRSTFENLTWYKLGPQPLPIHVGELPTPVCKNLDTLWKLNATMFS
NSTNDILIMELKNASLQDQGDYVCLAQDRKTKKRHCVVRQLTVLERVAPTITGNLENQTTSIGESI
EVSCTASGNPPPQIMWFKDNETLVEDSGIVLKDGNRNLTIRRVRKEDEGLYTCQACSVLGCAKVE
AFFIIEGAQEKTNLEPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 39)

The serially diluted fusion construct was incubated with biotin-modified VEGFA165-Avi-His at a constant final concentration of 0.4 nM for 1 h. VEGFA165-Avi-His was prepared in-house by adding a linker, an Avi tag, and a 6xHis tag to the C terminus of human VEGFA165. The VEGFA165-Avi-His expression plasmid and a BirA enzyme expression plasmid were co-transfected into 293F cells. During the expression of the protein, the BirA enzyme catalyzed the modification with biotin on the Avi tag. Amino acid sequence of VEGFA165-Avi-His:

APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEG
LECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQENPCGPCSERRKHLFVQ
DPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRRGSGSGLNDIFEAQKIEWHEGGGGSHHHHH
H (SEQ ID NO: 40)

The mixture of the fusion construct and VEGFA165-Avi-His was added to the microplate with VEGFR2-Fc captured, and the microplate was incubated at 37 °C for 1 h. The biotin signal of VEGFA165-Avi-His was determined using the HRP-labeled streptavidin antibody Streptavidin-HRP (purchased from Sangon Biotech, Cat. No. D111054-0001), and the plate was incubated at room temperature for 45 min. After the plate was washed, the TMB color development solution was added to the microplate at 100 μL/well, and color development was performed for 15 min; the stopping solution was added at 50 μL/well, and the microplate was placed in a microplate reader to read the light absorption value at 450 nm (OD450).

The VEGFR2 competition activity of the fusion constructs and the monovalent nanobody V1-SA1 is shown in FIG. 11, and the summary of IC50 from the VEGFR2 competition experiment is shown in Table 30.

Table 30. Summary of IC50 of fusion constructs and monovalent nanobody V1-SA1 competing for VEGFR2

Name of fusion construct	VEGFR2 competition ELISA IC50 (nM)	Ratio of fusion construct IC50 to V1-SA1 IC50
V1SA-3005Hz6	0.84	1.08
3005Hz6-V1SA	0.75	0.96
V1DP-3005Hz6	0.63	0.81
V1-SA1	0.78	1.00

3005Hz6	N/A	N/A

As can be seen from Table 30, the three fusion constructs generated by fusing 3005Hz6 with V1-SA1 or V1-DP in tandem exhibited VEGFR2 competition activity comparable to that of the monovalent nanobody V1-SA1 (IC50 ratio: 0.81-1.08), indicating that fusion of the anti-VEGFA antibody with 3005Hz6 did not affect the anti-VEGFA activity. Moreover, the anti-VEGFA antibody can be fused at the N or the C terminus of the anti-albumin antibody without affecting the activity of the anti-VEGFA antibody in the fusion construct.

The data in Tables 29 and 30 collectively demonstrated that the fusion constructs of the monovalent anti-VEGFA nanobody (V1-SA1 or V1-DP) and the anti-albumin nanobody (3005Hz6) had strong VEGFR2 competition activity and HSA binding activity. This indicates that in the fusion construct, the antibody elements binding to different antigens can function independently, and the mutual interference between the antibody elements is relatively small. Therefore, antibodies binding to other antigens can be fused and expressed with the anti-albumin humanized antibody 3005Hz6 to construct multifunctional fusion constructs.

Example 7. Fusion Constructs of Bivalent Anti-VEGFA Nanobodies and Humanized Anti-Albumin Antibody

1. Design of fusion constructs of bivalent anti-VEGFA nanobody and anti-albumin nanobody

Since VEGFA exists as a homodimer *in vivo*, in order to improve the efficiency of the nanobody binding to VEGFA and to improve the efficiency of the nanobody blocking VEGFA/VEGFR interaction, the anti-VEGFA nanobody adopted a bivalent form, which was tandem arrangement of two nanobodies with the same sequence. In order to increase the molecular weight of the bivalent anti-VEGFA nanobody *in vivo* and to prolong its half-life by binding to albumin, the bivalent anti-VEGFA nanobody was fused and expressed with the humanized anti-albumin antibody 3005Hz6. The amino acid sequences of the fusion constructs of the humanized antibody 3005Hz6 and the bivalent anti-VEGFA nanobody V1-SA1 or V1-DP are shown in Table 31, and the nucleic acid sequences are shown in Table 32.

Table 31. Amino acid sequences of fusion constructs of bivalent anti-VEGFA nanobody and humanized anti-albumin antibody 3005Hz6

SEQ ID NO	Name of fusion construct	Amino acid sequence of fusion construct
32	V1SA-3005Hz6-V1SA	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKERE FVVAISKGGYKYDSVSLEARFTISRDNAKNTVYLQMNSLRPEDTAVY YCAATRAYGSSRLRLADTYEYWGQGTLVTVSSGGSEVQLVESGGGL VQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEWVSGISVDGSF LDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARASGPQ GLRLGAPRGQGTLVTVSSGGSEVQLVESGGGLVQPGGSLRLSCAVSG RTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVSLEARFTISRDN AKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLADTYEYWGQG TLVTVSS
33	3005Hz6-2V1SA	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLE WVSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAV YYCARASGPQGLRLGAPRGQGTLVTVSSGGSEVQLVESGGGLVQPG GSLRLSCAVSGRTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVS LEARFTISRDNAKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRL ADTYEYWGQGTLVTVSSGGSEVQLVESGGGLVQPGGSLRLSCAVSG RTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVSLEARFTISRDN AKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLADTYEYWGQG TLVTVSS
34	V1DP-3005Hz6-V1DP	EVQLVESGGGLVQPGGSLRLSCAVSGRTFSSYTMGWFRQAPGKERE FVVAISKGGYKYDSVSLEARFTISRDNSKNTVYLQMNSLRPEDTAVY YCAATRAYGSSRLRLADTYEYWGQGTLVTVSSGGSEVQLVESGGGL VQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLEWVSGISVDGSF LDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAVYYCARASGPQ GLRLGAPRGQGTLVTVSSGGSEVQLVESGGGLVQPGGSLRLSCAVSG RTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVSLEARFTISRDN

		SKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLADTYEYWGQG TLVTVSS
35	3005Hz6-2V1DP	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWARQAPGKGLE WVSGISVDGSFLDYADAVKGRFTISRDNAKNSLYLQMNSLRPEDTAV YYCARASGPQGLRLGAPRGQGTLVTVSSGGSEVQLVESGGGLVQPG GSLRLSCAVSGRTFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVS LEARFTISRDNSKNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLA DTYEYWGQGTLVTVSSGGSEVQLVESGGGLVQPGGSLRLSCAVSGR TFSSYTMGWFRQAPGKEREFVVAISKGGYKYDSVSLEARFTISRDNS KNTVYLQMNSLRPEDTAVYYCAATRAYGSSRLRLADTYEYWGQGT LVTVSS

Table 32. Nucleic acid sequences encoding fusion constructs of bivalent anti-VEGFA nanobody and humanized anti-albumin antibody 3005Hz6

SEQ ID NO	Name of fusion construct	Nucleic acid sequence of fusion construct
75	V1SA-3005Hz6-V1SA	GAAGTGCAACTGGTCGAAAGCGGCGGAGGCCTGGTGCAGCCCGG AGGAAGCCTGAGACTGTCCTGCGCCGTCAGCGGCAGAACATTCA GCAGCTATACAATGGGCTGGTTTAGACAAGCCCCTGGCAAGGAGA GAGAGTTCGTGGTCGCCATTTCTAAGGGCGGCTACAAGTACGACA GCGTGTCCCTGGAGGCTAGATTCACAATTAGCAGAGACAACGCCA AAAACACAGTGTATCTGCAGATGAATAGCCTCAGACCTGAGGACA CAGCCGTGTATTACTGCGCCGCCACAAGGGCCTACGGAAGCAGCA GACTCAGACTGGCCGACACATACGAGTACTGGGGACAAGGCACC CTCGTCACCGTGAGCAGTGGCGGATCCGAAGTGCAGCTGGTCGA GAGCGGTGGAGGACTGGTGCAACCTGGCGGAAGCCTCAGACTGA GCTGTGCTGCCTCTGGCTTCACCTTCAGCAACTACTATATGAGCTG GGCCAGACAGGCCCCAGGCAAAGGCCTGGAATGGGTTTCCGGCA TCAGCGTGGACGGCAGCTTTCTGGACTACGCCGATGCCGTGAAGG GCAGATTCACAATCTCTCGGGATAATGCCAAGAACAGCCTGTACC TGCAGATGAACAGCCTGAGACCTGAGGACACCGCCGTGTACTACT GCGCAAGGGCTTCCGGTCCTCAGGGCCTGCGGCTGGGAGCTCCC AGAGGTCAGGGTACACTGGTGACCGTGTCCAGCGGCGGAAGCGA GGTGCAACTGGTGGAGTCCGGAGGTGGCCTGGTCCAGCCTGGCG GCAGCCTGAGACTCTCCTGCGCCGTGAGCGGCAGAACCTTCAGC AGCTACACCATGGGCTGGTTCAGACAAGCTCCAGGCAAAGAAAG GGAGTTCGTGGTGGCTATTAGCAAAGGCGGCTATAAGTACGATAG CGTGAGCCTGGAAGCTCGGTTCACCATTAGCAGAGATAACGCCAA GAACACCGTGTACCTGCAAATGAATAGCCTGAGACCTGAGGATAC CGCCGTGTACTACTGTGCCGCTACAAGAGCCTATGGCAGCTCCAG GCTCAGACTGGCTGATACCTACGAATATTGGGGCCAAGGCACCCT GGTGACAGTCTCTAGC
76	3005Hz6-2V1SA	GAAGTGCAGCTGGTCGAGAGCGGTGGAGGACTGGTGCAACCTGG CGGAAGCCTCAGACTGAGCTGTGCTGCCTCTGGCTTCACCTTCAG CAACTACTATATGAGCTGGGCCAGACAGGCCCCAGGCAAAGGCCT GGAATGGGTTTCCGGCATCAGCGTGGACGGCAGCTTTCTGGACTA CGCCGATGCCGTGAAGGGCAGATTCACAATCTCTCGGGATAATGC CAAGAACAGCCTGTACCTGCAGATGAACAGCCTGAGACCTGAGG ACACCGCCGTGTACTACTGCGCAAGGGCTTCCGGTCCTCAGGGCC TGCGGCTGGGAGCTCCCAGAGGTCAGGGTACACTGGTGACCGTG TCCAGCGGCGGATCCGAAGTGCAACTGGTCGAAAGCGGCGGAGG CCTGGTGCAGCCCGGAGGAAGCCTGAGACTGTCCTGCGCCGTCA GCGGCAGAACATTCAGCAGCTATACAATGGGCTGGTTTAGACAAG CCCCTGGCAAGGAGAGAGAGTTCGTGGTCGCCATTTCTAAGGGC GGCTACAAGTACGACAGCGTGTCCCTGGAGGCTAGATTCACAATT

		AGCAGAGACAACGCCAAAAACACAGTGTATCTGCAGATGAATAG CCTCAGACCTGAGGACACAGCCGTGTATTACTGCGCCGCCACAAG GGCCTACGGAAGCAGCAGACTCAGACTGGCCGACACATACGAGT ACTGGGGACAAGGCACCCTCGTCACCGTGAGCAGTGGCGGAAGC GAGGTGCAACTGGTGGAGTCCGGAGGTGGCCTGGTCCAGCCTGG CGGCAGCCTGAGACTCTCCTGCGCCGTGAGCGGCAGAACCTTCA GCAGCTACACCATGGGCTGGTTCAGACAAGCTCCAGGCAAAGAA AGGGAGTTCGTGGTGGCTATTAGCAAAGGCGGCTATAAGTACGAT AGCGTGAGCCTGGAAGCTCGGTTCACCATTAGCAGAGATAACGCC AAGAACACCGTGTACCTGCAAATGAATAGCCTGAGACCTGAGGAT ACCGCCGTGTACTACTGTGCCGCTACAAGAGCCTATGGCAGCTCC AGGCTCAGACTGGCTGATACCTACGAATATTGGGGCCAAGGCACC CTGGTGACAGTCTCTAGC
77	V1DP-3005Hz6-V1DP	GAGGTCCAGCTGGTGGAGTCTGGTGGAGGTCTGGTGCAGCCTGG TGGCTCTCTGAGACTGAGCTGTGCTGTGAGTGGCAGAACCTTCTC CAGCTACACCATGGGTTGGTTCAGGCAGGCTCCAGGTAAAGAGA GAGAGTTCGTGGTGGCCATCAGCAAGGGCGGTTACAAGTACGATA GCGTTTCTCTGGAAGCCAGATTCACCATTTCTCGCGACAACAGTA AGAACACTGTGTACCTGCAAATGAACAGTCTGAGGCCAGAAGAT ACTGCCGTGTACTACTGTGCAGCTACTCGGGCCTATGGCTCTAGCA GACTGAGACTGGCCGATACCTATGAGTACTGGGGCCAGGGTACAC TCGTCACAGTGTCTTCTGGCGGTTCTGAAGTCCAGTTGGTCGAGA GCGGAGGTGGTCTCGTGCAACCAGGCGGTAGCTTGAGACTGTCTT GTGCTGCAAGCGGTTTCACCTTTAGCAACTACTACATGTCTTGGGC AAGGCAAGCTCCAGGCAAGGGCTTGGAGTGGGTCAGCGGTATCT CCGTGGATGGAAGCTTTCTGGACTACGCTGATGCCGTGAAGGGCC GGTTCACCATCTCTCGCGATAACGCAAAGAATTCTCTCTACCTGCA GATGAACAGCTTGCGACCTGAAGACACAGCTGTCTATTATTGCGC TCGGGCTTCTGGACCTCAGGGCTTGAGACTCGGAGCACCTAGAG GTCAGGGTACTCTGGTGACAGTGAGCAGTGGCGGTAGCGAGGTG CAGTTGGTGGAAAGCGGCGGTGGCCTGGTGCAACCTGGTGGAAG CCTGAGGCTGTCATGCGCCGTGTCAGGCCGGACATTTAGCTCTTA CACAATGGGCTGGTTCCGACAGGCACCAGGCAAAGAACGGGAAT TCGTCGTGGCTATCTCTAAGGGCGGCTATAAGTATGATTCCGTCTC ACTGGAAGCAAGGTTTACCATTAGCAGAGACAACAGCAAGAACA CAGTCTACCTCCAGATGAATAGCCTGCGGCCTGAAGATACAGCCG TCTACTACTGCGCCGCTACACGGGCATACGGCTCCAGCAGACTCA GGCTCGCAGACACCTACGAATACTGGGGTCAGGGAACCTTGGTCA CAGTCAGCTCA
78	3005Hz6-2V1DP	GAAGTCCAGTTGGTGGAGTCCGGTGGAGGTCTGGTGCAACCTGG CGGATCACTGCGGTTGTCTTGCGCTGCCAGCGGATTTACCTTCAG CAACTACTATATGTCTTGGGCTAGGCAAGCACCAGGCAAAGGCCT GGAGTGGGTCTCTGGCATCTCTGTCGACGGTAGCTTTCTGGATTAC GCCGACGCTGTCAAGGGTCGCTTCACCATCAGCAGGGACAACGC TAAGAACTCTCTGTATCTGCAAATGAACAGTCTCAGACCAGAAGA TACAGCCGTCTACTATTGTGCCAGAGCCAGTGGACCACAGGGTTT GCGCTTGGGTGCACCAAGAGGCCAGGGAACACTCGTTACCGTCT CCAGCGGCGGAAGCGAAGTCCAACTGGTGGAATCTGGAGGTGGC CTGGTCCAGCCAGGTGGCTCTCTGAGACTGAGCTGCGCAGTGTCC GGAAGAACCTTCTCCAGCTATACCATGGGCTGGTTTAGACAAGCA CCTGGTAAGGAGAGGGAGTTCGTGGTCGCTATCTCCAAGGGCGG ATACAAGTACGACAGCGTCTCTCTGGAAGCTAGGTTTACCATTAGC CGGGACAACTCCAAGAATACCGTCTATCTGCAGATGAACAGCCTT AGGCCTGAAGACACAGCCGTGTATTACTGCGCTGCTACAAGGGCC TACGGCAGCTCTAGGCTGAGACTGGCAGACACCTATGAGTACTGG GGTCAGGGTACTCTGGTCACCGTGAGCTCAGGCGGATCTGAAGTC

		CAGCTGGTCGAATCCGGTGGTGGACTGGTTCAGCCTGGTGGTAGC CTGCGCCTTTCTTGTGCTGTGAGCGGCCGGACCTTCTCTAGCTACA CCATGGGTTGGTTCAGACAGGCACCTGGCAAAGAGAGAGAATTC GTCGTGGCCATCTCTAAGGGTGGCTACAAATACGACTCCGTGAGC CTTGAAGCACGCTTTACCATCTCCAGAGATAACAGCAAGAACACC GTCTACCTTCAGATGAACTCTCTGAGGCCAGAAGACACTGCCGTG TACTACTGTGCAGCTACCAGAGCATACGGTAGCAGCAGGCTTAGA CTGGCCGATACCTACGAGTATTGGGGACAGGGTACACTGGTGACA GTGAGCTCT

A secretion signal peptide MDAMKRGLCCVLLLCGAVFVSPS (SEQ ID NO: 36) was added to the N terminus of the fusion construct, and a linker GGGGS (SEQ ID NO: 37) and 6xHis were added to the C terminus of the antibody. The coding DNA sequence was obtained through gene synthesis (see Table 32), and subsequently inserted into the expression vector pCDNA3.1 (+) via the enzyme cutting sites NheI and XhoI. After the plasmid was verified by sequencing, endotoxin-free large-scale extraction was performed. Transient expression in suspension 293F cells was performed, and the cell culture supernatant was collected and subjected to Ni-column affinity purification. For the fusion construct 3005Hz6-2V1SA (SEQ ID NO: 33), a tag-free protein was simultaneously constructed. After a secretion signal peptide was added to the N terminus of the protein, the sequence was ligated to an expression vector, and transient expression was performed in 293F cells.

V1SA-3005Hz6-V1SA (SEQ ID NO: 32) and 3005Hz6-2V1SA (SEQ ID NO: 33) purified via the Ni column were purified by cation exchange chromatography. Each component of the purified products was subjected to SDS-PAGE, and the results are shown in FIG. 12(A), where the flow-through fractions were labeled as FT, the eluted products of V1SA-3005Hz6-V1SA were labeled as components A1 and A2, and the eluted products of 3005Hz6-2V1SA were labeled as components A5 and A6. Cation exchange chromatography was performed on 3005Hz6-2V1SA without any tag, and the Coomassie-stained image of the purified product is shown in FIG. 12(B). Ni column purification was performed on V1DP-3005Hz6-V1DP and 3005Hz6-2V1DP, and the Coomassie-stained image of the purified product is shown in FIG. 12(C). The molecular weight of the fusion constructs was about 41 kDa.

2. ELISA assay and binding and dissociation rate determination (SPR) of fusion constructs

The HSA binding activity of the fusion constructs V1SA-3005Hz6-V1SA (SEQ ID NO: 32), 3005Hz6-2V1SA (SEQ ID NO: 33), and 3005Hz6-2V1DP (SEQ ID NO: 35) was determined by the ELISA method. In the experiment, human serum albumin HSA (Baxter AG) was diluted to 2 μg/mL and added to a microplate at 100 μL/well for coating at 4 °C overnight. After blocking with the 5% skim milk powder and washing with PBST (PBS containing 0.1% Tween 20, pH = 7.4), serial dilutions of the fusion construct containing the His tag were added to a microplate at 100 μL/well, and the microplate was incubated at 37 °C for 1 h. After the plate was washed with PBST, an HRP-labeled mouse anti-His tag monoclonal antibody (purchased from Proteintech, Cat. No. HRP-66005) was added, and the mixture was incubated at room temperature for 45 min. After the plate was washed, 100 μL of TMB (purchased from Tiangen Biotech, Cat. No. PA107-01) was added to each well for color development at 37 °C for 15 min, followed by the addition of 50 μL of the stopping solution. The absorbance at 450 nm (OD450) was measured using a microplate reader (purchased from Thermo Scientific, model: Multiskan SkyHigh, Cat. No. A51119700C).

The ELISA results are shown in FIG. 13, and the EC50 values are shown in Table 33.

Table 33. Summary of EC50 for fusion constructs V1SA-3005Hz6-V1SA, 3005Hz6-2V1SA, and 3005Hz6-2V1DP binding to HSA

Name of fusion construct	EC50 (nM) for binding to HSA
V1SA-3005Hz6-V1SA	0.35
3005Hz6-2V1SA	0.26
3005Hz6-2V1DP	0.30

As can be seen from Table 33, the fusion constructs V1SA-3005Hz6-V1SA, 3005Hz6-2V1SA, and 3005Hz6-2V1DP all had relatively strong HSA binding activity, indicating that the antibody 3005Hz6 in the fusion construct can exert complete biological activity.

The affinity of the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA for HSA was determined using the surface plasmon resonance (SPR) method.

In the experiment, a Biacore 8K instrument (Cytiva) was used, and Series S Sensor Chip NTA was utilized to capture the His-tagged anti-albumin antibody 3005Hz6 or fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA. The analyte was serially diluted HSA and was diluted with a running buffer (composition: 10 mM HEPES, 150 mM NaCl, 0.05% v/v Tween-20, pH = 7.4). The binding time between the ligand and the analyte was 150 s, and the dissociation time was 900 s. The binding and dissociation curves of the ligand with the analyte are shown in FIG. 14.

The "1:1 binding" model was used for fitting to obtain the binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of 3005Hz6, V1SA-3005Hz6-V1SA, and 3005Hz6-2V1SA with HSA. The results are shown in Table 34.

Table 34. Binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of anti-albumin antibody 3005Hz6 and fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA with HSA

Ligand	Analyte	ka (1/Ms)	kd (1/s)	K_D (M)
3005Hz6	HSA	1.09E+05	1.38E-03	1.27E-08
V1SA-3005Hz6-V1SA	HSA	2.48E+04	1.72E-03	6.94E-08
3005Hz6-2V1SA	HSA	4.11E+04	1.47E-03	3.58E-08

As can be seen from Table 34, the fusion construct 3005Hz6-2V1SA had relatively high affinity for HSA, and the K_D was similar to the K_D of the antibody 3005Hz6 with HSA (the K_D was 12.7-35.8 nM).

3. Determination of binding and dissociation rates of fusion constructs with VEGFA (SPR)

The affinity of the fusion constructs V1SA-3005Hz6-V1SA (His-tag) and 3005Hz6-2V1SA (tag-free) and the positive control aflibercept for VEGFA was determined by the SPR method.The SPR experiment was performed utilizing Biacore 8K (Cytiva). A Series S Sensor Chip CAP was used to capture biotin-modified VEGFA165-Avi-His (SEQ ID NO: 40) with a capture level of about 40 RU. Each protein was diluted with a running buffer HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Tween 20) to set a 2-fold protein concentration gradient. In the kinetic analysis, the binding time between the ligand and the analyte was 150 s, the dissociation time was 900 s, and the flow rate was 30 μL/min.

The binding and dissociation curves of V1SA-3005Hz6-V1SA, 3005Hz6-2V1SA, and the positive control aflibercept with VEGFA are shown in FIGs. 15(A), 15(B), and 15(C), respectively. The binding and dissociation curves were fitted using the "1:1 binding" model to obtain the binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D). The results are shown in Table 35.

Table 35. Binding rates (ka), dissociation rates (kd), and equilibrium dissociation constants (K_D) of fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA and positive control aflibercept with VEGFA

Analyte	Ligand	ka (1/Ms)	kd (1/s)	K_D (M)
V1SA-3005Hz6-V1SA	VEGFA165-Avi-His	7.35E+06	1.79E-05	2.43E-12
3005Hz6-2V1SA	VEGFA165-Avi-His	7.86E+06	4.24E-05	5.39E-12
Aflibercept	VEGFA165-Avi-His	1.19E+06	1.31E-04	1.10E-10

As can be seen from Table 35, under the SPR experimental conditions for capturing the biotin-modified VEGFA165-Avi-His, the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA had extremely high binding affinity (K_D = 2.4-5.4 pM) for VEGFA; under the same experimental conditions, the affinity of the positive control drug aflibercept for VEGFA was one order of magnitude lower than those of V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA, with K_D of about 110 pM.

Example 8. Inhibition of VEGFA-Stimulated HUVEC Proliferation Activity by Fusion Constructs

In this example, primary human umbilical vein endothelial cells (HUVECs) were used to determine whether the fusion constructs can block VEGFA-stimulated HUVEC proliferation at the cellular level.

Since the fusion constructs bind to HSA in humans, in this experiment, the differences in the activity of the fusion constructs in inhibiting HUVEC proliferation with or without pre-incubation of HSA were compared. In the experiment, a 210 ng/mL solution of human VEGFA165 (purchased from GenScript, Cat. No. Z03073) was prepared using an endothelial cell medium ECM (purchased from Zhong Qiao Xin Zhou, Cat. No. ZQ-1304) containing 0.5% FBS. Serial dilutions of the fusion construct 3005Hz6-2V1SA with or without the same molar concentration of HSA (labeled as 3005Hz6-2V1SA + HSA and 3005Hz6-2V1SA, respectively) and serial dilutions of the fusion construct V1SA-3005Hz6-V1SA with or without the same molar concentration of HSA (labeled as V1SA-3005Hz6-V1SA + HSA and V1SA-3005Hz6-V1SA, respectively)

were prepared, and the positive control was the marketed drug aflibercept (Bayer) with the same molar concentration gradient. In a 96-well plate, 50 μL of each serially diluted protein to be tested (3005Hz6-2V1SA + HSA, 3005Hz6-2V1SA, V1SA-3005Hz6-V1SA + HSA, V1SA-3005Hz6-V1SA, and aflibercept) was mixed with 50 μL of the VEGFA165 solution. A control without VEGFA165 (expected to exhibit the slowest proliferation) and a control with VEGFA165 only (expected to exhibit the fastest proliferation) were set. The mixed solution of the protein to be tested and VEGFA165 was placed in an incubator at 37 °C with 5% CO_2 for 1.5-2 h. Primary HUVEC cells (purchased from the National Stem Cell Translational Resource Center, Cat. No. DFSC-EC-01) were digested, centrifuged, and resuspended in ECM containing 0.5% FBS. The cell suspension was added to a 96-well plate pre-mixed with the protein to be tested and VEGFA165 at 50 μL/well (1.2×10^4 cells/well). At this point, the final concentration of VEGFA165 was 70 ng/mL. The cells were cultured in an incubator at 37 °C with 5% CO_2 for 68-72 h. After the culture was completed, 16 μL of the CCK-8 staining solution (purchased from Solarbio, Cat. No. CA-1210) was added to each well, and the plate was incubated in an incubator at 37 °C for 2.5-4 h. The proliferation activity of HUVEC cells was determined by reading the absorbance value (wavelength: 450 nm) using a microplate reader. The IC50 values were calculated using GraphPad Prism 8 software through four-parameter nonlinear regression analysis.

The inhibition rates of cell proliferation by the proteins to be tested at different concentrations were calculated according to the following formula:

$$\frac{[\text{OD450 (with VEGFA only)} - \text{OD450 (containing mixture of VEGFA and serially diluted protein to be tested)}]}{[\text{OD450 (with VEGFA only)} - \text{OD450 (without VEGFA)}]} * 100\%$$

The experimental results of the inhibition of VEGFA-stimulated HUVEC proliferation by fusion constructs V1SA-3005Hz6-V1SA (with or without pre-incubation of HSA) and 3005Hz6-2V1SA (with or without pre-incubation of HSA) are shown in FIGs. 16(A) and 16(B), respectively.

The IC50 and maximum inhibition rates of the inhibition of HUVEC proliferation by the fusion construct V1SA-3005Hz6-V1SA are shown in Table 36. The IC50 and maximum inhibition rates of the inhibition of HUVEC proliferation by the fusion construct 3005Hz6-2V1SA are shown in Table 37.

Table 36. IC50 and maximum inhibition rates of fusion construct V1SA-3005Hz6-V1SA in HUVEC proliferation inhibition experiment

Protein	HUVEC proliferation inhibition	
	IC50 (nM)	Maximum inhibition rate (%)
Aflibercept	2.40	92.6
V1SA-3005Hz6-V1SA	2.67	80.4
V1SA-3005Hz6-V1SA + HSA	2.59	86.3

Table 37. IC50 and maximum inhibition rates of fusion construct 3005Hz6-2V1SA in HUVEC proliferation inhibition experiment

Protein	HUVEC proliferation inhibition	
	IC50 (nM)	Maximum inhibition rate (%)
Aflibercept	4.26	92.2
3005Hz6-2V1SA	2.84	89.0
3005Hz6-2V1SA + HSA	2.59	90.2

The results in Tables 36 and 37 showed that both fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA can efficiently block the downstream signaling pathway activated by human VEGFA165 at the cellular level and inhibit human VEGFA165-stimulated HUVEC proliferation, and their activity was similar to that of the positive control aflibercept (receptor drug). Moreover, the results showed that whether HSA was pre-incubated with the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA had no significant effect on their HUVEC proliferation inhibition activity.

Example 9. Study on Activity of Fusion Constructs in Inhibiting VEGFA-Induced VEGFR2 Phosphorylation

In this example, primary HUVEC cells were used to verify the inhibition effect of the fusion constructs on VEGFA-induced VGFR2 phosphorylation.

In the experiment, primary HUVEC cells were digested, centrifuged, resuspended in an ECM medium (containing 5% FBS, 1% epidermal growth factor, and 1% penicillin-streptomycin) (purchased from Zhong Qiao Xin Zhou, Cat. No. ZQ-1304), and plated in a 96-well plate at a density of 3×10^5 cells/mL, with 100 μL of the cell suspension per well. The cells were cultured overnight in an incubator at 37 °C with 5% CO_2. VEGFA165 and the fusion constructs were prepared using the ECM medium, and tag-free human VEGFA165 (purchased from GenScript, Cat. No. Z03073) at a working concentration of 50 ng/mL (molar concentration: 1.3 nM) was separately pre-mixed with the fusion constructs V1SA-3005Hz6-V1SA (pre-incubated with HSA at the same molar concentration) and 3005Hz6-2V1SA (pre-incubated with HSA at the same molar concentration) at different concentrations. Meanwhile, a control without VEGFA165 and a control with VEGFA165 only were set. The mixed solution was left to stand at 37 °C for 30 min and added to HUVEC cells plated overnight in advance, and the mixture was treated for 5 min. The cells were washed once with PBS, and 65 μL of a strong RIPA lysis solution (purchased from Beyotime, Cat. No. P0013B) containing PMSF was added to each well for lysis on ice for 20 min. The lysate was transferred to a 1.5 mL centrifuge tube and centrifuged at 13000 rpm for 15 min (4 °C), and the supernatant was collected. 5× loading buffer was added, and the mixture was heated at 95 °C for 6 min. The supernatant was taken and subjected to the western blot assay for VEGFR2 phosphorylation. In the western blot experiment, the anti-GAPDH antibody for determining the endogenous reference GAPDH was purchased from Proteintech (Cat. No. 60004-1-Ig), and the antibody for determining phosphorylation of tyrosine at position 1175 of VGFR2 (p-VEGFR2) was purchased from Cell Signaling Technology (Cat. No. 2478S).

The signal intensities of the phosphorylated VEGFR2 (p-VEGFR2) band and the GAPDH band in the results of the western blot experiment were quantified, and the signal intensity ratio of the two bands (p-VEGFR2/GADPH) was calculated to obtain the inhibition rate of the protein to be tested on VEGFR2 phosphorylation.

The calculation formula for the inhibition rate of the protein to be tested on VEGFR2 phosphorylation is as follows:

$$[1 - \frac{\text{[p-VEGFR2/GADPH with co-treatment of protein to be tested and VEGFA]}}{\text{[p-VEGFR2/GADPH with VEGFA treatment alone]}}]*100\%$$

The inhibition rates of the fusion construct V1SA-3005Hz6-V1SA on VEGFR2 phosphorylation are shown in Table 38.

Table 38. Inhibition rate of fusion construct V1SA-3005Hz6-V1SA on VEGFR2 phosphorylation

HUVEC treatment mode	Fusion construct/VEGFA (molar ratio)	VEGFR2 phosphorylation inhibition rate
Co-treatment with V1SA-3005Hz6-V1SA and VEGFA	0.5	22.1%
	1	40.0%
	3	74.7%
	5	90.5%

Table 38 showed that V1SA-3005Hz6-V1SA can effectively inhibit human VEGFA-induced VEGFR2 phosphorylation in HUVEC cells. Under the condition of incubation with HSA, V1SA-3005Hz6-V1SA could achieve a VEGFR2 phosphorylation inhibition rate of 90% or higher when the molar concentration was 5 times that of VEGFA.

The inhibition rates of the fusion construct 3005Hz6-2V1SA on VEGFR2 phosphorylation are shown in Table 39.

Table 39. Inhibition rate of fusion construct 3005Hz6-2V1SA on VEGFR2 phosphorylation

HUVEC treatment mode	Fusion construct/VEGFA (molar ratio)	VEGFR2 phosphorylation inhibition rate
Co-treatment with 3005Hz6-2V1SA and VEGFA	0.5	-5.5%
	1	40.4%
	2	63.0%
	3	90.4%

Table 39 showed that 3005Hz6-2V1SA can effectively inhibit human VEGFA-induced VEGFR2

phosphorylation in HUVEC cells. Under the condition of incubation with HSA, 3005Hz6-2V1SA could achieve a VEGFR2 phosphorylation inhibition rate of 90% or higher when the molar concentration was 3 times that of VEGFA.

Example 10. Study on Activity of Fusion Constructs in VEGF Reporter Cells

In this example, the VEGF reporter gene system was used to assess the activity of the fusion constructs in blocking the VEGF downstream signaling pathway.

HEK-293/VEGF/NFAT stably transfected reporter gene cell strain (purchased from the National Institutes for Food and Drug Control) was generated by transfecting HEK-293 cells to co-express VEGFR2 (KDR) and the NFAT-RE-luc 2p gene. When VEGFA recognized and activated the KDR receptor, an intracellular downstream signaling pathway was initiated, and the transcription factor NFAT was activated to initiate the expression of the Luciferase reporter gene. At this time, chemiluminescence could be generated after a luciferase substrate was added.

HEK-293/VEGF/NFAT reporter gene cells were digested, centrifuged, and resuspended in DMEM containing 1% FBS at a cell density of 5×10^4 cells/mL. The cells were plated on a white-bottom 96-well plate (purchased from Costar, Cat. No. 3917) at 80 μL/well and incubated in an incubator at 37 °C with 5% CO_2 overnight. The next day, VEGFA165 (purchased from GenScript, Cat. No. Z03073) at 80 ng/mL was prepared in DMEM containing 1% FBS and mixed with V1SA-3005Hz6-V1SA (with or without pre-incubation of an equimolar concentration of HSA) or 3005Hz6-2V1SA (with or without pre-incubation of an equimolar concentration of HSA) at different concentrations, and the mixture was incubated at 37 °C for 30 min. The negative control contained no VEGFA165, and the positive control contained VEGFA165 alone. 20 μL of the mixed solution (the protein to be tested and VEGFA165) was added to 80 μL of cells, and the mixture was incubated in an incubator at 37 °C for 6 h. Luciferase reporter gene substrate (purchased from Yeasen, Cat. No. 11404ES60) was added at 100 μL/well, and the relative light units (RLU) of luciferin were measured using a multi-mode microplate reader (purchased from Agilent BioTek, model: Synergy H1).

The determination results of the activity of the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA in inhibiting the luminescence of the luciferin substrate under the experimental conditions described above are shown in FIGs. 17(A) and 17(B), respectively, and the fitted IC50 values are shown in Tables 40 and 41, respectively.

Table 40. Summary of IC50 for inhibition of luciferin luminescence by fusion construct V1SA-3005Hz6-V1SA with or without pre-incubation of HSA in VEGF reporter gene system

Protein	IC50 (nM) of VEGF reporter gene experiment
V1SA-3005Hz6-V1SA	0.24
V1SA-3005Hz6-V1SA + HSA	0.22

Table 41. Summary of IC50 for inhibition of luciferin luminescence by fusion construct 3005Hz6-2V1SA with or without pre-incubation of HSA in VEGF reporter gene system

Protein	IC50 (nM) of VEGF reporter gene experiment
3005Hz6-2V1SA	0.24
3005Hz6-2V1SA + HSA	0.25

The results of FIG. 17 and Tables 40 and 41 showed that the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA were both able to block the activation of the VEGFA165-stimulated downstream signaling pathway at the cellular level, thereby reducing the expression of the Luciferase reporter gene activated by the transcription promoter NFAT into the nucleus, and their IC50 values were both 0.24 nM. In this example, whether the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA bind to HSA in advance had substantially no effect on the blocking activity against VEGFA downstream signals.

Based on the activity determination experiments at the protein level and the cellular level described above, the fusion constructs V1SA-3005Hz6-V1SA (SEQ ID NO: 32) and 3005Hz6-2V1SA (SEQ ID NO: 33) composed of the bivalent anti-VEGFA nanobodies (V1-SA1, SEQ ID NO: 20) and the humanized anti-albumin nanobody (3005Hz6, SEQ ID NO: 9) both have strong HSA binding activity and affinity for VEGFA, and the fusion constructs can efficiently inhibit VEGFA165-induced HUVEC proliferation and VEGFR2 phosphorylation, and inhibit the luciferin luminescence of VEGF reporter gene cells at the cellular level. These data showed that the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA have strong VEGFA neutralizing activity, can efficiently block the VEGFA downstream signaling pathway, inhibit

VEGFA-induced cell proliferation, and can be potentially used for treating diseases associated with VEGFA-induced abnormal vascular proliferation, such as wet AMD, diabetic macular edema, and tumors.

Meanwhile, the data also showed that the antibody elements in the fusion construct binding to different antigens can function independently, the mutual interference between the antibody elements is relatively small, and the binding of HSA does not affect the biological functions of the effector molecules. Therefore, it is expected that the fusion constructs V1SA-3005Hz6-V1SA and 3005Hz6-2V1SA can normally exert the activity of blocking the VEGFA signaling pathway and inhibiting the proliferation of endothelial cells after binding to albumin *in vivo*.

The preferred embodiments of the present disclosure are described in detail above. However, the present disclosure is not limited to the specific details in the embodiments described above. Within the scope of the technical concept of the present disclosure, various simple modifications can be made to the technical solutions of the present disclosure, and all such simple modifications will fall within the protection scope of the present disclosure.

In addition, it should be noted that the various specific technical features described in the specific embodiments described above can be combined in any suitable manner provided that no contradiction arises. In order to avoid unnecessary repetition, various possible combinations will not be illustrated separately in the present disclosure.

Claims

1. An anti-VEGFA fusion construct, comprising an anti-albumin antibody or an antigen-binding fragment thereof and an anti-VEGFA antibody or an antigen-binding fragment thereof, wherein the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the anti-albumin antibody or the antigen-binding fragment thereof.
2. The fusion construct according to claim 1, wherein the anti-albumin antibody or the antigen-binding fragment thereof comprises CDR-H1, CDR-H2, and CDR-H3 of a variable domain of a heavy chain, wherein
   an amino acid sequence of the CDR-H1 comprises SEQ ID NO: 1 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 1;
   an amino acid sequence of the CDR-H2 comprises SEQ ID NO: 41 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 41;
   an amino acid sequence of the CDR-H3 comprises SEQ ID NO: 3 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 3.
3. The fusion construct according to claim 1 or 2, wherein X_1X_2 in SEQ ID NO: 41 (GISVX$_1$X$_2$SFLDYADAVKG) represents DS, DA, EG, or DG.
4. The fusion construct according to any one of claims 1-3, wherein the amino acid sequences of the CDR-H1, the CDR-H2, and the CDR-H3 comprise any one of the following groups:
   A) SEQ ID NOs: 1, 2, and 3;
   B) SEQ ID NOs: 1, 10, and 3;
   C) SEQ ID NOs: 1, 11, and 3;
   D) SEQ ID NOs: 1, 12, and 3.
5. The fusion construct according to claim 1, wherein the anti-VEGFA antibody or the antigen-binding fragment thereof comprises CDR-H1, CDR-H2, and CDR-H3 of a variable domain of a heavy chain, wherein
   an amino acid sequence of the CDR-H1 comprises SEQ ID NO: 16 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 16;
   an amino acid sequence of the CDR-H2 comprises SEQ ID NO: 17 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 17;
   an amino acid sequence of the CDR-H3 comprises SEQ ID NO: 18 or an amino acid sequence having at least 80% identity to the amino acid sequence set forth in SEQ ID NO: 18.
6. The fusion construct according to any one of claims 1-5, wherein the anti-albumin antibody or the antigen-binding fragment thereof or the anti-VEGFA antibody or the antigen-binding fragment thereof comprises a humanized sequence, wherein an engineered site of the humanized sequence is located in a non-CDR region, and preferably, the engineered site of the humanized sequence is located in a framework region and/or a constant region of the antibody.
7. The fusion construct according to any one of claims 1-6, wherein a structure of the anti-albumin antibody or the antigen-binding fragment thereof or the anti-VEGFA antibody or the antigen-binding fragment thereof comprises a nanobody, a chimeric antibody, a Fab fragment, a Fab' fragment, an Fd fragment, an Fv fragment, a dAb fragment, a F(ab')2 fragment, a single chain antibody fragment, or a linear antibody.
8. The fusion construct according to any one of claims 1-7, wherein an amino acid sequence of the anti-albumin antibody or the antigen-binding fragment thereof comprises any one of the amino acid sequences of SEQ ID NOs: 4, 6-9, and 13-15, or has at least 80% identity to any one of the amino acid sequences of SEQ ID NOs: 4, 6-9, and 13-15.
9. The fusion construct according to any one of claims 1-7, wherein an amino acid sequence of the anti-VEGFA antibody or the antigen-binding fragment thereof comprises any one of the amino acid sequences of SEQ ID NOs: 19-28, or has at least 80% identity to any one of the amino acid sequences of SEQ ID NOs: 19-28.
10. The fusion construct according to any one of claims 1-9, wherein the anti-albumin antibody or the antigen-binding fragment thereof is linked to the anti-VEGFA antibody or the antigen-binding fragment thereof directly or indirectly, and preferably, the indirect linkage may be a linkage via a linker, a functional domain, and/or a linker for conjugation,
    wherein the linker is selected from a linker peptide, an oligopeptide, an oligopeptide polymer, a polypeptide, a polypeptide polymer, PEG, a nucleic acid, a polysaccharide, a fatty chain, biotin, streptavidin, and avidin;
    the functional domain is one or a combination of two or more of an Fc fragment, serum albumin, a

cytokine, transferrin, or a scaffold protein;
the linker for conjugation comprises a functional group linker, and preferably, the functional group linker comprises sulfhydryl, amino, hydroxy, and/or carboxyl reactive groups;
wherein more preferably, the fusion construct may comprise one or more anti-VEGFA antibodies or antigen-binding fragments thereof, wherein the anti-VEGFA antibody or the antigen-binding fragment thereof is linked to the N terminus, the C terminus, and/or an internal residue of the anti-albumin antibody or the antigen-binding fragment thereof directly or indirectly;
further preferably, the fusion construct comprises any one of the sequences of SEQ ID NOs: 29-35, or has at least 80% identity to the amino acid sequences set forth in SEQ ID NOs: 29-35.

11. A nucleic acid, wherein the nucleic acid encodes the fusion construct according to any one of claims 1-10.
12. The nucleic acid according to claim 11, comprising any one of the nucleotide sequences of SEQ ID NOs: 62-71 or a degenerate sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 62-71 and having a function of encoding an anti-VEGFA antibody or an antigen-binding fragment thereof.
13. The nucleic acid according to claim 11 or 12, comprising any one of the nucleotide sequences of SEQ ID NOs: 45, 47-50, and 56-58 or a degenerate sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 45, 47-50, and 56-58 and having a function of encoding an anti-albumin antibody or an antigen-binding fragment thereof.
14. The nucleic acid according to any one of claims 11-13, comprising any one of the nucleotide sequences of SEQ ID NOs: 72-78 or a degenerate sequence thereof, or a nucleotide sequence having at least 80% identity to any one of the nucleotide sequences of SEQ ID NOs: 72-78 and having a function of encoding the fusion construct.
15. A vector, comprising the nucleic acid according to any one of claims 11-14.
16. A host cell, comprising the nucleic acid according to any one of claims 11-14 or the vector according to claim 15.
17. A preparation method for the fusion construct according to any one of claims 1-10, comprising culturing the host cell according to claim 16 and expressing the fusion construct.
18. Use of the fusion construct according to any one of claims 1-10, the nucleic acid according to any one of claims 11-14, the vector according to claim 15, or the host cell according to claim 16 in preparing a product for treating and/or diagnosing a disease.
19. A product for treating and/or diagnosing a disease, comprising any one of the following:
A) the fusion construct according to any one of claims 1-10;
B) the nucleic acid according to any one of claims 11-14;
C) the vector according to claim 15; or
D) the host cell according to claim 16.
20. A method for detecting VEGFA, comprising contacting a sample to be tested with the fusion construct according to any one of claims 1-10, and subsequently, detecting a content of a complex formed by VEGFA and the fusion construct, wherein preferably, the VEGFA is from a mammal, and more preferably, the mammal is a human, a murine, or a monkey.