

Title

Monoclonal Antibody against Human Activated Protein C and Preparation and Use Thereof

Background of the Present Disclosure

Field of Disclosure

The present disclosure belongs to the technical field of monoclonal antibodies, and relates to a monoclonal antibody against human activated protein C (aPC) and use thereof.

Description of Related Arts

Protein C (PC) pathways play a crucial role in regulating coagulation and inflammation. Thrombin forms a complex with thrombomodulin (TM) on vascular endothelial cells, thereby activating protein C. Activated protein C (aPC) cleaves activated factor V and factor VIII, thereby negatively downregulating formation of thrombin and maintaining the balance between thrombosis and hemostasis in vivo. aPC protects animals against disoperation of endotoxin (LPS) or *Escherichia coli*. Recombinant human aPC products were approved by FDA as the first drug for treating severe sepsis. At present, the molecular mechanisms of aPC for anti-inflammatory and cytoprotective effects are not yet well understood. Studies on mutations showed that the anticoagulant activity of aPC is obviously not necessary for the antiapoptotic effect of aPC on endothelial cells. Vascular endothelial protein C receptors (EPCRs) are mainly expressed on the endothelium of large vessels, and promote activation of protein C through the thrombin-TM complex. Recently, it has been demonstrated that aPC bound to EPCR may cleave PAR-1 on endothelial cells. Moreover, this study shows that all anti-inflammatory and antiapoptotic effects of aPC signaling are mediated by PAR-1 in endothelial cells. However, under the attack of lethal dose of LPS, the phenotype of PAR-1-deficient mice was similar to that of wild-type control mice, which indicated that PAR-1 might not be the only participant in the regulation of inflammation and apoptosis by aPC signaling.

In view of the key role of aPC in regulating pathophysiological functions in vivo, a monoclonal antibody HAPC1573 has been produced. This antibody specifically recognizes the activated form of human protein C (aPC) rather than protein C (Xu, US8153766B2, US9127072B2). This antibody alters the amidolytic activity of aPC on chromogenic peptide substrates and blocks the anticoagulant activity of aPC in plasma. More importantly, this antibody does not affect the cleavage of extracellular histones H3 and H4 by aPC, nor the cytoprotective activity of aPC on thrombin-

induced permeability of endothelial cells. MAPC1591 is an anti-mouse aPC monoclonal antibody having similar functions to HAPC1573. MAPC1591 can block the anticoagulant activity of aPC in mice and maintain the cytoprotective activity of aPC, and thus protecting people with hemophilia A from traumatic hemorrhage. This indicates that the anti-aPC antibody can be applied to clinical settings such as hemophilia, trauma and sepsis.

Zhao et al. reported CDR sequences of HAPC1573 and humanized HAPC1573 against only anticoagulant activity rather than other activities of aPC. These CDR sequences showed an in vivo protective effect against bleeding in a monkey model of acute acquired hemophilia A (Zhao, US9657111B2, WO2017/087391A1, Nat. Communications 2020). The X-ray crystal structure of the aPC-humanized HAPC1573 Fab complex indicates that HAPC 1573 may not bind to the active site of aPC, but bind to an aPC autolysis loop containing the known aPC-FVa binding site. Therefore, humanized HAPC1573 may inhibit the anticoagulant function of aPC by interfering with the interaction between aPC and FVa. The inhibitory effect of humanized HAPC1573 is only limited to the anticoagulant activity of aPC, but does not affect its cytoprotective, anti-inflammatory and cell signaling functions. It is reported that the aPC concentration in human plasma is about 40 pm, and according to a surface plasmon resonance-based assay, the affinity of humanized HAPC 1573 to aPC is 10 nM. The concentration-dependent effect of HAPC 1573 in shortening prolonged APTT in human FVIII-deficient plasma and human FIX-deficient plasma was demonstrated in a Protac-dependent APTT coagulation assay. Even at the concentration of 50 µg/ml, HAPC 1573 still could not achieve the maximum inhibitory effect on the anticoagulant activity of aPC in this in vitro experiment. This observation was consistent with the in vivo observation of humanized HAPC 1573 in the monkey model of acute acquired hemophilia. In animal studies, it was found that only HAPC1573 at high doses of 10 mg/kg and 30 mg/kg could provide great protective effect. However, such high doses are not suitable for clinical use, so humanized HAPC 1573 with higher affinity is the prerequisite and guarantee for the potential use of aPC in treatment in the future.

Summary of the Present Disclosure

One aspect of the present disclosure provides an anti-aPC monoclonal antibody. The anti-aPC monoclonal antibody includes an original antibody and a mutant antibody obtained by mutating one or more of the following 6 mutation sites in the original antibody.

The original antibody includes:

(a) a heavy chain CDR represented by any one or more of SEQ ID NOs: 27, 28 and 29; and/or

(b) a light chain CDR represented by any one or more of SEQ ID NOs: 30, 31 and 32.

The 6 mutant sites are:

- 1) N57R/W located in VL CDR2;
- 2) E59G/P located in VL CDR2;
- 3) Q93V located in VL CDR3;
- 4) N31F located in VH CDR1;
- 5) S55K located in VH CDR2; and
- 6) Y104T located in VH CDR3.

In the present disclosure, a constant region of the anti-aPC monoclonal antibody includes one or more of human IgG1, IgG2, IgG3 and IgG4 sequences.

Preferably, in the present disclosure, the mutation sites of the anti-aPC monoclonal antibody where the original antibody is mutated include at least: N31F located in VH CDR1; S55K located in VH CDR2; N57R located in VL CDR2; and E59G located in VL CDR2.

Preferably, in the present disclosure, the mutation sites of the anti-aPC monoclonal antibody where the original antibody is mutated include at least: N57R located in VL CDR2; E59G located in VL CDR2; Q93V located in VL CDR3; N31F located in VH CDR1; and S55K located in VH CDR2.

Another aspect of the present disclosure provides a biological material related to the anti-aPC monoclonal antibody, which is any one or more of 1) to 10) as follows:

- 1) a nucleic acid molecule encoding the anti-aPC monoclonal antibody as described above;
- 2) an expression cassette containing the nucleic acid molecule as described in 1);
- 3) a construct containing the nucleic acid molecule as described in 1);
- 4) a construct containing the expression cassette as described in 2);
- 5) an expression system containing the nucleic acid molecule as described in 1);
- 6) an expression system containing the expression cassette as described in 2);
- 7) an expression system containing the construct as described in 3);
- 8) an expression system containing a recombinant vector as described in 4);
- 9) a pair of primers for amplifying the nucleic acid molecule encoding the amino acid sequence of the anti-aPC monoclonal antibody as described above; and

10) a fusion antibody containing the anti-aPC monoclonal antibody as described above.

Another aspect of the present disclosure provides a preparation product. The preparation product is a pharmaceutical composition or a detection kit. The preparation product includes the anti-aPC monoclonal antibody as described above or any one or more of the related biological materials thereof. When being the pharmaceutical composition, the preparation product further includes a pharmaceutically acceptable carrier.

Another aspect of the present disclosure provides a method for treating coagulation-related disorders, which includes: administering to an individual in need of treatment an effective amount of the anti-aPC monoclonal antibody or the related biological material thereof as described above. The individual suffers from coagulation deficiency or defect disorders, or the individual is in need of blood coagulation treatment, or the individual suffers from sepsis or hemophilia, or the individual is in need of regulation of hemostasis.

Another aspect of the present disclosure provides use of the anti-aPC monoclonal antibody or the related biological material thereof in preparation of a drug for prevention and/or treatment of coagulation deficiency or defect disorders. The coagulation deficiency or defect disorders include disorders causing coagulopathy due to deficiency of coagulation factors and thus causing bleeding, and coagulation disturbances caused by trauma or surgery. Further, the coagulation deficiency or defect disorders include hemophilia, sepsis, traumatic hemorrhage and the like.

Another aspect of the present disclosure provides use of the anti-aPC monoclonal antibody or the related biological material thereof in preparation of a promoter for promoting an ability of aPC to cleave histones H3 and H4, or in preparation of a drug for inhibiting anticoagulant activity of activated protein C in an individual, or in preparation of a drug for prevention and/or treatment in an individual in need of blood coagulation, or in preparation of a drug for prevention and/or treatment in an individual suffering from sepsis, or in preparation of a drug for prevention and/or treatment in an individual suffering from hemophilia, or in preparation of a drug for regulating hemostasis in an individual, or in immunoassays for non-disease diagnosis and treatment purposes or in preparation of a detection reagent or kit for aPC, or in combination with other reagents or drugs.

Another aspect of the present disclosure provides a preparation method of the anti-aPC monoclonal antibody as described above. The method includes: cultivating an expression system of the anti-aPC monoclonal antibody under conditions suitable for expressing the anti-aPC monoclonal antibody, so as to express the anti-aPC monoclonal antibody.

The present disclosure has the following beneficial effects: the anti-aPC monoclonal antibody

provided in the present disclosure is a humanized antibody obtained by humanizing HAPC 1573, or by performing screening of mutations on a single site and a combination of sites at the same time. The anti-aPC monoclonal antibody selectively binds to and inhibits activated protein C, but does not bind to or inhibit unactivated protein C. The anti-aPC monoclonal antibody selectively binds to human activated protein C, but does not bind to activated protein C of other species or unactivated protein C. The affinity of the antibody is significantly increased, and the affinity of some of the antibodies is 100 times higher than that of HAPC 1573. In a Protac-dependent APTT coagulation assay, a very small amount of the antibody, such as 1 µg/ml, is used in the present disclosure and can achieve the maximum inhibitory effect on the anticoagulant activity of aPC. The antibody can potentially block the anticoagulant activity of aPC, and has broad application prospects in the treatment of coagulation deficiency or defect disorders.

Brief Description of the Drawings

Fig. 1 shows binding abilities of humanized anti-aPC monoclonal antibodies (Humab001-1 to Humab001-28 or HAPC-1 to HAPC-28) to human aPC.

Fig. 2 shows binding abilities of humanized anti-aPC monoclonal antibodies (Humab001-1 to Humab001-28 or HAPC-1 to HAPC-28) to human PC.

Fig. 3 shows binding abilities of HAPC-14 mutant antibodies (HAPC14-1 to HAPC14-10 or HAPC-14-1 to HAPC-14-10) to hAPC/hPC; where abscissas in Figs. 3A-3D are respectively 10^{-6} nM, 10^{-4} nM, 10^{-2} nM, 10^0 nM, 10^2 nM and 10^4 nM.

Fig. 4 shows assays of affinities of HAPC-14 mutant antibodies (HAPC14-1 to HAPC14-10 or HAPC-14-1 to HAPC-14-10).

Fig. 5 shows effects of HAPC-14 mutant antibody HAPC-14-6 on the ability of aPC to cleave histones.

Fig. 6 shows in vitro pharmacodynamic experiments. Functions of HAPC-14 mutant antibodies HAPC-14-3 and HAPC-14-6 on inhibiting the anticoagulant activity of aPC in coagulation factor-deficient plasma: A: for coagulation factor FVIII-deficient plasma, B: for coagulation factor FIX-deficient plasma, C: for coagulation factor FXI-deficient plasma, D: for coagulation factor FVII-deficient plasma, E: for coagulation factor FXII-deficient plasma, F: for coagulation factor FV-deficient plasma, G: for coagulation factor FX-deficient plasma, H: for VWF-deficient plasma.

Fig. 7 shows in vivo pharmacodynamic experiments. A: effects of injecting coagulation

factor FVIII, HAPC1573 and HAPC-14-6 into coagulation factor FVIII-deficient mice on coagulation function of the deficient mice; B: effects of injecting different doses of HAPC-14-6 into coagulation factor FVIII-deficient mice on coagulation function of the deficient mice; C: effects of injecting HAPC1573 and different doses of HAPC-14-6 into coagulation factor FIX-deficient mice on coagulation function of the deficient mice.

Detailed Description of the Preferred Embodiments

In the present disclosure, HAPC 1573 is humanized and then optimized for affinity, and optimized mutation sites are screened in a Fab-based manner to finally obtain 6 optimal sites. Further, through the combination of mutations, the affinity of the obtained optimized mutants is 100 times higher than that of HAPC 1573, and in a Protac-dependent APTT coagulation assay, the mutant antibody at an amount of 1 µg/ml can achieve the maximum inhibitory effect on the anticoagulant activity of aPC, and thus has the possibility of further clinical application.

The present disclosure provides an anti-aPC antibody, namely an anti-aPC monoclonal antibody 14 (HAPC-14). The antibody includes:

(a) a heavy chain including a heavy chain CDR represented by any one or more of SEQ ID NOs: 27, 28 and 29; and/or

(b) a light chain including a light chain CDR represented by any one or more of SEQ ID NOs: 30, 31 and 32.

The anti-aPC monoclonal antibody 14 (HAPC-14) provided in the present disclosure has a heavy chain sequence as shown in SEQ ID NO: 5 and a light chain sequence as shown in SEQ ID NO: 6.

The present disclosure further provides a mutant antibody of a monoclonal antibody (anti-aPC monoclonal antibody 14) against human activated protein C (aPC). After design, screening and statistics of the mutant antibody on the basis of the sequence of the anti-aPC monoclonal antibody 14, it is found that mutations at one or more of the following six sites significantly affect the binding ability of a monoclonal antibody Fab fragment to a capture antibody, and the mutant antibody has high affinity, high functional activity and small differences from germline sequence. The six sites are:

- a) N57R/W located in VL CDR2 (N57R is preferred to N57W);
- b) E59G/P located in VL CDR2 (E59G is preferred to E59P);

- c) Q93V located in VL CDR3;
- d) N31F located in VH CDR1;
- e) S55K located in VH CDR2; and
- f) Y104T located in VH CDR3.

The most crucial mutant sites are N57R in the light chain and N31F in the heavy chain. In some embodiments, a concentration of the anti-aPC monoclonal antibody required to completely inhibit the anticoagulant activity of aPC and restore normal coagulation function in a humanized protein C (haPC) hemophilia mouse model is as low as 1 µg/ml.

For the convenience of description, the combinations of mutations will be described in short forms. N57R refers to N57R located in VL CDR2, N57W refers to N57W located in VL CDR2, E59G refers to E59G located in VL CDR2, E59P refers to E59P located in VL CDR2, Q93V refers to Q93V located in VL CDR3, N31F refers to N31F located in VH CDR1, S55K refers to S55K located in VH CDR2, and Y104T refers to Y104T located in VH CDR3.

In the present disclosure, the combination of mutations at the six sites includes, but not limited to, (1) only one mutation at any one of the following sites: N57R, N57W, E59G, E59P, Q93V, N31F or S55K; or (2) mutations at both of the following two sites: N57R and N57W; E59G and E59P; Q93V and N31F; N31F and S55K; N57R and E59G; N57R and E59P; N57R and Q93V; N57R and N31F; N57R and S55K; N57W and E59P; N57W and Q93V; N57W and N31F; N57W and S55K; E59G and Q93V; E59G and N31F; E59G and S55K; E59P and N31F; E59P and S55K; or Q93V and S55K; or (3) mutations at all of the following three sites: N57R, N57W and E59G; N57R, N57W and E59P; N57R, N57W and Q93V; N57R, N57W and N31F; N57R, N57W and S55K; N57R, E59G and E59P; N57R, E59G and Q93V; N57R, E59G and N31F; N57R, E59G and S55K; N57R, E59P and Q93V; N57R, E59P and N31F; N57R, E59P and S55K; N57R, Q93V and N31F; N57R, Q93V and S55K; N57R, N31F and S55K; N57W, E59G and E59P; N57W, E59G and Q93V; N57W, E59G and N31F; N57W, E59G and S55K; N57W, E59P and Q93V; N57W, E59P and N31F; N57W, E59P and S55K; N57W, Q93V and N31F; N57W, Q93V and S55K; N57W, N31F and S55K; E59G, E59P and Q93V; E59G, E59P and N31F; E59G, E59P and S55K; E59G, Q93V and N31F; E59G, Q93V and S55K; E59G, N31F and S55K; E59P, Q93V and N31F; E59P, Q93V and S55K; E59P, N31F and S55K; or Q93V, N31F and S55K; or (4) mutations at all of the following four sites: N57R, N57W, E59G and E59P; N57R, N57W, E59G and Q93V; N57R, N57W, E59G and N31F; N57R, N57W, E59G and S55K; N57R, E59G, E59P and Q93V; N57R, E59G, E59P and N31F; N57R, E59G, E59P and S55K; N57R, E59G, Q93V and N31F; N57R, E59G, Q93V and S55K; N57R, E59P, Q93V and

N31F; N57R, E59P, Q93V and S55K; N57R, E59P, N31F and S55K; N57R, Q93V, N31F and S55K; N57W, E59G, E59P and Q93V; N57W, E59G, E59P and N31F; N57W, E59G, E59P and S55K; N57W, E59P, Q93V and N31F; N57W, E59P, Q93V and S55K; N57W, E59P, N31F and S55K; N57W, Q93V, N31F and S55K; E59G, E59P, Q93V and N31F; E59G, E59P, Q93V and S55K; E59G, Q93V, N31F and S55K; or E59P, Q93V, N31F and S55K; or (5) mutations at all of the following five sites: N57R, N57W, E59G, E59P and Q93V; N57R, N57W, E59G, E59P and N31F; N57R, N57W, E59G, Q93V and N31F; N57R, N57W, E59P, Q93V and N31F; N57R, E59G, E59P, Q93V and N31F; N57W, E59G, E59P, Q93V and N31F; N57R, N57W, E59G, E59P and S55K; N57R, N57W, E59G, Q93V and S55K; N57R, N57W, E59P, Q93V and S55K; N57R, E59G, E59P, Q93V and S55K; N57W, E59G, E59P, Q93V and S55K; N57R, N57W, E59G, N31F and S55K; N57R, N57W, E59P, N31F and S55K; N57R, E59G, E59P, N31F and S55K; N57W, E59G, E59P, N31F and S55K; N57R, N57W, Q93V, N31F and S55K; N57R, E59G, Q93V, N31F and S55K; N57W, E59G, Q93V, N31F and S55K; N57R, E59P, Q93V, N31F and S55K; N57W, E59P, Q93V, N31F and S55K; or E59G, E59P, Q93V, N31F and S55K; or (6) mutations at all of the following six sites: N57R, N57W, E59G, E59P, Q93V and N31F; N57R, N57W, E59G, E59P, Q93V and S55K; N57R, N57W, E59G, E59P, N31F and S55K; N57R, N57W, E59G, Q93V, N31F and S55K; N57R, N57W, E59P, Q93V, N31F and S55K; or N57W, E59G, E59P, Q93V, N31F and S55K; or (7) mutations at all of the following seven sites: N57R, N57W, E59G, E59P, Q93V, N31F and S55K.

In a specific embodiment, the present disclosure provides an anti-aPC monoclonal antibody, which includes:

one or two or three of a heavy chain CDR1 represented by SEQ ID NO: 27 or 33, a heavy chain CDR2 represented by SEQ ID NO: 28 or 40, and a heavy chain CDR3 represented by SEQ ID NO: 29 or 77; and/or

one or two or three of a light chain CDR1 represented by SEQ ID NO: 30, a light chain CDR2 represented by any one of SEQ ID NOs: 31, 37, 43, 49 and 73, and a light chain CDR3 represented by SEQ ID NO: 32 or 44.

In a specific embodiment, the antibody is selected from the following:

an antibody including (a) heavy chain CDRs represented by SEQ ID NOs: 27, 28 and 29; and/or

(b) light chain CDRs represented by SEQ ID NOs: 30, 31 and 32;

an antibody including (c) heavy chain CDRs represented by SEQ ID NOs: 33, 34 and 35;

and/or

(d) light chain CDRs represented by SEQ ID NOs: 36, 37 and 38;

an antibody including (e) heavy chain CDRs represented by SEQ ID NOs: 39, 40 and 41;

and/or

(f) light chain CDRs represented by SEQ ID NOs: 42, 43 and 44;

an antibody including (g) heavy chain CDRs represented by SEQ ID NOs: 45, 46 and 47;

and/or

(h) light chain CDRs represented by SEQ ID NOs: 48, 49 and 50;

an antibody including (i) heavy chain CDRs represented by SEQ ID NOs: 51, 52 and 53;

and/or

(j) light chain CDRs represented by SEQ ID NOs: 54, 55 and 56;

an antibody including (k) heavy chain CDRs represented by SEQ ID NOs: 57, 58 and 59;

and/or

(l) light chain CDRs represented by SEQ ID NOs: 60, 61 and 62;

an antibody including (m) heavy chain CDRs represented by SEQ ID NOs: 63, 64 and 65;

and/or

(n) light chain CDRs represented by SEQ ID NOs: 66, 67 and 68;

an antibody including (o) heavy chain CDRs represented by SEQ ID NOs: 69, 70 and 71;

and/or

(p) light chain CDRs represented by SEQ ID NOs: 72, 73 and 74;

an antibody including (q) heavy chain CDRs represented by SEQ ID NOs: 75, 76 and 77;

and/or

(r) light chain CDRs represented by SEQ ID NOs: 78, 79 and 80;

an antibody including (s) heavy chain CDRs represented by SEQ ID NOs: 81, 82 and 83;

and/or

(t) light chain CDRs represented by SEQ ID NOs: 84, 85 and 86; and

an antibody including (u) heavy chain CDRs represented by SEQ ID NOs: 87, 88 and 89;

and/or

(v) light chain CDRs represented by SEQ ID NOs: 90, 91 and 92.

In the present disclosure, a heavy chain framework region of the anti-aPC monoclonal antibody may be any existing framework region suitable for exerting functions of the anti-aPC monoclonal antibody of the present disclosure, and may be human-originated or mouse-originated.

In the present disclosure, a light chain framework region of the anti-aPC monoclonal antibody may be any existing framework region suitable for exerting functions of the anti-aPC monoclonal antibody of the present disclosure, and may be human-originated or mouse-originated.

In a specific embodiment, the anti-aPC monoclonal antibody includes:

a heavy chain sequence as shown in any of SEQ ID NOs: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25; and/or

a light chain sequence as shown in any of SEQ ID NOs: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26.

In a specific embodiment, the anti-aPC monoclonal antibody is selected from the following:

an antibody HaPC14 including a heavy chain sequence as shown in SEQ ID NO: 5 and a light chain sequence as shown in SEQ ID NO: 6;

an antibody HaPC14-1 including a heavy chain sequence as shown in SEQ ID NO: 7 and a light chain sequence as shown in SEQ ID NO: 8;

an antibody HaPC14-2 including a heavy chain sequence as shown in SEQ ID NO: 9 and a light chain sequence as shown in SEQ ID NO: 10;

an antibody HaPC14-3 including a heavy chain sequence as shown in SEQ ID NO: 11 and a light chain sequence as shown in SEQ ID NO: 12;

an antibody HaPC14-4 including a heavy chain sequence as shown in SEQ ID NO: 13 and a light chain sequence as shown in SEQ ID NO: 14;

an antibody HaPC14-5 including a heavy chain sequence as shown in SEQ ID NO: 15 and a light chain sequence as shown in SEQ ID NO: 16;

an antibody HaPC14-6 including a heavy chain sequence as shown in SEQ ID NO: 17 and a light chain sequence as shown in SEQ ID NO: 18;

an antibody HaPC14-7 including a heavy chain sequence as shown in SEQ ID NO: 19 and a light chain sequence as shown in SEQ ID NO: 20;

an antibody HaPC14-8 including a heavy chain sequence as shown in SEQ ID NO: 21 and a light chain sequence as shown in SEQ ID NO: 22;

an antibody HaPC14-9 including a heavy chain sequence as shown in SEQ ID NO: 23 and a light chain sequence as shown in SEQ ID NO: 24; and

an antibody HaPC14-10 including a heavy chain sequence as shown in SEQ ID NO: 25 and a light chain sequence as shown in SEQ ID NO: 26.

In the present disclosure, some sequences have the same composition, but different numbers (for details, referring to specific amino acid sequences in the sequence listing).

For example, among the sequences representing the heavy chain CDR1 of the antibody, sequences 27 and 87 are the same, and sequences 33, 39, 45, 51, 57, 63, 69, 75 and 81 are the same.

Among the sequences representing the heavy chain CDR2 of the antibody, sequences 28, 34, 52 and 70 are the same, and sequences 40, 46, 58, 64, 76, 82 and 88 are the same.

Among the sequences representing the heavy chain CDR3 of the antibody, sequences 29, 35, 41, 47, 53, 59, 65 and 71 are the same, and sequences 77, 83 and 89 are the same.

Among the sequences representing the light chain CDR1 of the antibody, sequences 30, 36, 42, 48, 54, 60, 66, 72, 78, 84 and 90 are the same.

Among the sequences representing the light chain CDR2 of the antibody, sequences 37 and 61 are the same, and sequences 49, 55, 67, 79, 85 and 91 are the same.

Among the sequences representing the light chain CDR3 of the antibody, sequences 32, 38, 68, 74 and 86 are the same, and sequences 44, 50, 56, 62, 80 and 92 are the same.

In a specific embodiment, the present disclosure further provides a biological material related to the anti-aPC monoclonal antibody, which is any one or more of 1) to 10) as follows:

- 1) a nucleic acid molecule encoding the anti-aPC monoclonal antibody as described above;
- 2) an expression cassette containing the nucleic acid molecule as described in 1);
- 3) a construct containing the nucleic acid molecule as described in 1);
- 4) a construct containing the expression cassette as described in 2);
- 5) an expression system containing the nucleic acid molecule as described in 1);
- 6) an expression system containing the expression cassette as described in 2);
- 7) an expression system containing the construct as described in 3);

8) an expression system containing a recombinant vector as described in 4);

9) a pair of primers for amplifying the nucleic acid molecule encoding the amino acid sequence of the anti-aPC monoclonal antibody as described above; and

10) a fusion antibody containing the anti-aPC monoclonal antibody as described above. The expression system may be a cell or a cell line, and the cell or the cell line encodes the anti-aPC monoclonal antibody.

In a specific embodiment, the present disclosure provides a preparation product. The preparation product is a pharmaceutical composition or a detection reagent or a detection kit.

In a specific embodiment, the pharmaceutical composition provided in the present disclosure includes the anti-aPC monoclonal antibody as described above, or any one or more of the biological materials as described above, and a pharmaceutically acceptable carrier. The carrier may include various excipients and diluents, which are not essential active ingredients as such and have no undue toxicity after administration. Suitable carriers are well known to those skilled in the art. For example, relevant contents of pharmaceutically acceptable carriers are described in Remington's Pharmaceutical Sciences (Mack Pub.Co., N.J., 1991).

In a specific embodiment, the detection reagent or detection kit provided in the present disclosure includes the anti-aPC monoclonal antibody as described above, or any one or more of the biological materials as described above. The antibody is labeled.

Labeling may be carried out in any existing manner, such as chemiluminescence labeling, fluorophore, radioactive labeling, chromophore or the like.

The detection reagent or kit may further include buffers, diluents, operating instructions and the like.

The antibody may be present in any form suitable for its functioning or activity, for example, in the form of an aqueous suspension.

In some embodiments, the anti-aPC monoclonal antibody of the present disclosure promotes an ability of aPC to cleave histones H3 and H4, which indicates that the anti-aPC monoclonal antibody can provide a cytoprotective effect against cytotoxicity of the histones H3 and H4.

In a specific embodiment, the present disclosure provides a method for treating coagulation deficiency or defect disorders as defined above, and specifically provides a method for treating hereditary and acquired coagulation deficiency or defect such as hemophilia A and hemophilia B or sepsis. The present disclosure also provides a method for shortening bleeding time by administering

an anti-aPC monoclonal antibody to a patient in need (for example, a trauma patient or a patient in need of blood coagulation). The present disclosure further provides a method for inhibiting anticoagulant activity of activated protein C in a subject. The present disclosure further provides a method for regulating thrombosis in a subject. According to the method of the present disclosure, an effective amount of the anti-aPC monoclonal antibody described above in the present disclosure is administered to an individual in need of treatment. The present disclosure also provides a method for producing a monoclonal antibody against human aPC.

In a specific embodiment, the present disclosure provides a method for treating coagulation deficiency or defect disorders, which includes administering an effective amount of the anti-aPC monoclonal antibody or the related biological material thereof as described above to an individual in need of treatment.

In a specific embodiment, the present disclosure provides a method for treating coagulation related disorders, which includes administering an effective amount of the anti-aPC monoclonal antibody as described above or any one or more of the biological materials as described above to an individual in need of treatment. The individual suffers from coagulation deficiency or defect disorders, or the individual is in need of blood coagulation treatment, or the individual suffers from sepsis or hemophilia, or the individual is in need of regulation of hemostasis.

The coagulation deficiency or defect disorders may be hereditary or acquired, including disorders causing coagulopathy due to deficiency of one or more of coagulation factors in plasma and thus causing bleeding, and specifically including: hemophilia A, hemophilia B and hemophilia C, which are caused by the deficiency of coagulation factors VIII, IX and XI, respectively; and rare coagulation factor deficiencies, which are hemorrhagic diseases caused by absence or abnormal working of one or more of other coagulation factors (i.e., coagulation factors I, II, V, V+VIII, VII, X or XIII), for example, coagulation disturbances caused by trauma, and excessive bleeding caused by treatment of sepsis, transplantation, cardiac surgery and cosmetic surgery.

In a specific embodiment, according to the method for promoting the ability of aPC to cleave histones H3 and H4 in the present disclosure, after the administration of the anti-aPC monoclonal antibody or the related biological material or pharmaceutical composition thereof, the ability of aPC to cleave histones H3 and H4 can be promoted, so that the cytoprotective effect against cytotoxicity of histones H3 and H4 can be provided.

In a specific embodiment, according to the method for inhibiting the anticoagulant activity of activated protein C in an individual in the present disclosure, the administration of the anti-aPC

monoclonal antibody or the related biological material or pharmaceutical composition thereof can inhibit the anticoagulant activity of the activated protein C in the individual, so that the coagulation function of the individual can be regulated.

In a specific embodiment, the present disclosure provides use of the anti-aPC monoclonal antibody or the related biological material thereof

i) in preparation of a drug for prevention and/or treatment of coagulation deficiency or defect disorders;

ii) in preparation of a promoter for promoting the ability of aPC to cleave histones H3 and H4;

iii) in preparation of a drug for inhibiting the anticoagulant activity of activated protein C in an individual;

iv) in preparation of a drug for treatment in an individual in need of blood coagulation;

vi) in preparation of a drug for regulating hemostasis in an individual;

vii) in immunoassays for disease or non-disease diagnosis and treatment purposes or in preparation of a detection reagent or kit for aPC; and

viii) in combination with other reagents or drugs.

In a specific embodiment, the present disclosure further provides a preparation method of the anti-aPC monoclonal antibody as described above. The method includes: cultivating an expression system of the anti-aPC monoclonal antibody under conditions suitable for expressing the anti-aPC monoclonal antibody, so as to express the anti-aPC monoclonal antibody.

In the present disclosure, a polypeptide having a certain identity with the amino acid sequence of the anti-aPC monoclonal antibody is also within the protection scope of the present disclosure, for example, an antibody or antibody fragment (having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity) having 70%, 75%, 80%, 85%, 90%, 95%, 99% or more sequence identity with the anti-aPC monoclonal antibody and having the functions of the anti-aPC monoclonal antibody, and specifically, a polypeptide fragment obtained by substituting, deleting or adding one or more (specifically 1-50, 1-30, 1-20, 1-10, 1-5, 1-3, 1, 2 or 3) amino acids in the anti-aPC monoclonal antibody, or by adding one or more (specifically 1-50, 1-30, 1-20, 1-10, 1-5, 1-3, 1, 2 or 3) amino acids to the N-terminal and/or C-terminal of the anti-aPC monoclonal antibody, and having the functions of the anti-aPC monoclonal antibody.

The term "protein C" or "PC" refers to any variant, isoform and/or species homolog of Protein C in its zymogen form, which is naturally expressed by cells and is present in plasma, and is different from the activated form of protein C.

The term "activated protein C" or "aPC" refers to the activated form of protein C.

The term "anti-aPC monoclonal antibody" refers to an antibody that specifically binds to an epitope of aPC, and is a monoclonal antibody against human aPC. The anti-aPC monoclonal antibody may be in a variety of antibody forms, including but not limited to a complete antibody, an antibody fragment, a human antibody, a humanized antibody and a genetically engineered antibody, such as a monoclonal antibody, a chimeric antibody or a recombinant antibody as well as fragments of these antibodies, provided that they retain their properties according to the present disclosure.

The antibody fragment is further defined as Fab', Fab, F(ab')₂, a single-domain antibody, Fv or scFv. The anti-aPC monoclonal antibody used herein refers to an antibody molecule preparation with a single molecular component. The monoclonal antibody component exhibits single binding specificity and affinity to a particular epitope, and may have variable and constant regions derived from human germline immunoglobulin sequences. For example, a Fab fragment refers to a monovalent fragment composed of VL, VH, CL and CH1 domains; a F(ab')₂ fragment refers to a divalent fragment containing two Fab fragments linked by disulfide bridges in a hinge region; and a Fv fragment refers to a Fv fragment composed of VL and VH domains of a single arm of an antibody.

The term "identity" may be evaluated with naked eyes or with computer software. Using the computer software, the identity between two or more sequences may be expressed in percentage (%), which can be used to evaluate the identity between related sequences.

The term "individual" generally includes humans and non-human primates, such as mammals, dogs, cats, horses, goats, pigs and cattle, which can benefit from the treatment using the antibody, drug, composition, related preparation, kit or combined preparation described above.

The term "therapeutically effective amount" generally refers to an amount that can achieve the effect of treating the diseases listed above after an appropriate period of administration.

The term "monoclonal antibody" refers to an antibody molecule preparation of a single amino acid composition, which may have variable and constant regions derived from human germline immunoglobulin sequences. In an embodiment, the monoclonal antibody is prepared from a hybridoma including B cells, which is obtained from a transgenic non-human animal, such as a transgenic mouse, having a genome including human heavy chain transgenes and human light chain transgenes fused with immortalized cells.

The term "fusion antibody" refers to a product obtained by fusing an antibody fragment with other bioactive proteins using a genetic engineering technology. It is a recombinant protein with the above two domains, in which a target gene is linked with genes of a fragment of Ig at the gene level and expressed in eukaryotic or prokaryotic cells. The fusion antibodies can be divided into two categories according to the different fragments of Ig linked to the target protein: Fab(Fv) fusion proteins; and Fc fusion proteins. Two different proteins are linked together into one macromolecule. The linkage may be realized by a chemical method or by fusion of genes. The method for preparing a fusion antibody involves conventional recombinant DNA and gene transfection techniques well known in the art. Because of the different fusion proteins, this antibody fusion protein has many biological functions, and the expressed recombinant protein affects neither the antigen binding ability of the single chain antibody nor the biological characteristics of the protein fused with it.

The term "humanized antibody" refers to an antibody in which a framework region or "complementary-determining region" (CDR) has been modified into a CDR of an immunoglobulin having different specificities compared to the parental immunoglobulin.

When referring to inhibiting and/or blocking the binding of an aPC substrate to aPC, the term inhibiting and blocking also includes a measurable decrease in the binding affinity of aPC to a physiological substrate when aPC is in contact with an anti-aPC antibody compared with the situation that aPC is not in contact with the anti-aPC antibody, for example, blocking the interaction of aPC with its substrates including Factor Va or Factor VIIIa by at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100%.

"Complementary-determining region" or "CDR" refers to one of three hypervariable regions in the heavy chain variable region or light chain variable region of an antibody molecule, which forms an N-terminal antigen-binding surface complementary to a three-dimensional structure of a bound antigen. Starting from the N-terminal of the heavy chain or light chain, these complementarity-determining regions are denoted as "CDR1", "CDR2" and "CDR3" respectively. The CDRs involve antigen-antibody binding, and CDR3 includes a unique region specific for antigen-antibody binding. Therefore, an antigen-binding site may include six CDRs, which include CDRs from each of the heavy chain and light chain variable regions.

The term "epitope" means a range or region of an antigen to/with which an antibody specifically binds or interacts, and in some embodiments, "epitope" indicates where the antigen physically contacts the antibody.

The term "conservative substitution" means a polypeptide modification, which involves the replacement of one or more amino acids with amino acids that have similar biochemical characteristics but do not cause loss of biological or biochemical functions of the polypeptide. "Conservative amino acid substitution" means the replacement of an amino acid residue with an amino acid residue having similar side chains. The families of amino acid residues having similar side chains have been defined in the art. Common conservative substitutions are, for example, mutual substitutions between aromatic amino acids Phe, Trp and Tyr, mutual substitutions between hydrophobic amino acids Leu, Ile and Val, mutual substitutions between polar amino acids Gln and Asn, mutual substitutions between basic amino acids Lys, Arg and His, mutual substitutions between acidic amino acids Asp and Glu, and mutual substitutions between hydroxyl amino acids Ser and Thr.

The term "identity" refers to the proportion of nucleotides being identical when two nucleic acids or two polypeptides are compared, for example, at least about 80% of the nucleotides or amino acid, usually at least about 85%, in some embodiments about 90%, 91%, 92%, 93%, 94% or 95%, in at least one embodiment at least about 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4% or 99.5% of the nucleotides or amino acids are identical.

Nucleic acids may be present in whole cells, in cell lysates or in a partially purified or substantially pure form. Nucleic acids are "isolated" or "made substantially pure" when being purified from other cellular components that are normally associated with nucleic acids in a natural environment. Nucleic acids can be isolated using techniques well known in the art, such as alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and the like.

Relevant terms used in the present disclosure are explained as follows:

CDRs	Complementary-determining regions
CV	Column volume
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment
hr	Hour
HRP	Horseradis peroxydase
KDa	Kilodaltons
koff	Dissociation Rate

OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Periplasmic extract
SA	Streptavidin
scFv	Single chain variable fragment
SPR	Surface plasmon resonance
TMB	Tetra-methyl-benzidine
VH	Heavy chain variable domain
VL	Light chain variable domain

Further descriptions of the present disclosure are provided below with reference to embodiments. The following embodiments are used to describe the present disclosure, but are not intended to limit the scope of the present disclosure. Except for the content specifically mentioned below, the process, conditions, experimental methods, and the like for implementing the present disclosure are all general knowledge and well-known knowledge in the field, and the present disclosure is not particularly limited.

Example 1 Humanization of mouse anti-aPC monoclonal antibody

Variable region sequences of mouse hybridoma antibodies were humanized. VH/VL amino acid sequences were numbered using a Kabat system. Then, the VH/VL sequences were analyzed for the presence of hot spot sites, including unpaired cysteine residues, N-linked glycosylation sites and deamination sites inside CDRs. N-linked glycosylation sites close to CDRs would also be considered as high risk sites. If such sites are recognized, they need to be removed. Based on the VH/VL sequence alignment, the best human germline gene frame sequences were selected, and J region sequences were selected and spliced based on the best homology. Homology modeling was carried out on murine VH/VL sequences to form 3D structures. FR residues within a distance of 5 Å from CDRs, residues on a VH/VL binding surface that affect antigen binding after being altered and the like will be considered as potential sites in need of back mutations. The importance of back mutations was determined by analysis using a computer, and different combinations containing a single or several back mutations were designed.

Target segment DNA was amplified through PCR, and plasmids encoding humanized

antibody sequences were constructed. The antibodies were subjected to transient transfection and expression and purified by a protein A affinity chromatography column. The humanized antibodies were assayed by SEC-HPLC and SDS-PAGE, and 28 humanized anti-aPC monoclonal antibody clones were obtained.

1.1 ELISA assays of affinities of anti-aPC monoclonal antibodies to human aPC (haPC)

Affinities of chimeric antibodies and the above 28 humanized antibodies to haPC were assayed by ELISA and FACS assays.

The assay results are shown in Figs. 1. NOs. 1-28 (Humab001-1 to Humab001-28 or HAPC-1 to HAPC28) humanized antibodies, 3657 positive control antibody (3657 PC mAb) and chimeric antibody (30120-mAb001×hIgG) had good binding to haPC. NOs. 13-20 antibodies had low EC50 values, which indicated that these antibodies had higher binding abilities to haPC.

1.2 ELISA assays of binding abilities of anti-aPC monoclonal antibodies to human PC (hPC)

NOs. 13-20 humanized anti-aPC monoclonal antibodies (Humab001-13 to Humab001-20 or HAPC-13 to HAPC-20) were selected and assayed for their binding abilities to hPC.

The results are shown in Fig. 2. The results showed that the NOs. 13-20 humanized anti-aPC monoclonal antibodies did not bind to hPC at all.

NO. 13 antibody (HAPC-13), NO. 14 antibody (HAPC-14) and NO. 16 antibody (HAPC-16) were three parental antibodies selected for optimization of affinity. These three antibodies had the same heavy chain framework regions and different light chain framework regions. HAPC-13 was the original, HAPC-14 had 1 back mutation based on HAPC-13, and HAPC-16 had 5 back mutations based on HAPC-13. In the following embodiments, the antibody HAPC-14 was selected for subsequent optimization of affinity.

Example 2 Optimization of affinity of anti-aPC monoclonal antibody HAPC-14

On the basis of NO. 14 antibody (Humab001-14 or HAPC14 or HAPC-14) described above, the affinity of this antibody was optimized, and mutants was constructed.

Materials	Vendor/Generator	Catalog No.
Blocker Casein in PBS	Pierce	37528
SA-HRP	Sigma	S5512
Goat Anti-Human IgG Fd	Southern Biotech	2046-01

Goat anti-Human IgG F(ab') ₂ -HRP	Jackson	109-035-006
Goat anti-c-myc HRP conjugated	BETHYL	A190-104P
Blocker Casein in PBS	Pierce	37528
SA-HRP	Sigma	S5512
Expi293F™ Cells	Thermo Fisher	A14527
ExpiFectamine293 transfection kit	Thermo Fisher	A14524
Expi293F™ expression medium	Thermo Fisher	A1435101
Lipofectamine™ 2000 Transfection Reagent	Thermo Fisher	11668019
Fetal bovine serum (FBS)	Corning	35-076-CV
Opti-MEM	Thermo Fisher	31985070
MabSelect SuRe column	GE healthcare	11-0034-93
NuPAGE4%-12% Bis-Tris Gel	Thermo Fisher	NP0322BOX
PageRuler™ Unstained Protein Ladder	Thermo Fisher	26614
HPLC-SEC column	TOSOH	08541
Series S Sensor Chip CM5	GE healthcare	29-1496-03
Amine Coupling Kit	GE healthcare	BR100633
10×HBS-EP+ buffer	GE healthcare	BR100669
anti-human Fc IgG	Jackson	109-005-098
10 mM Glycine, pH 1.5	Cool pharm	KH-59224

2.1 Site-directed mutation

Each amino acid of six complementary-determining regions (CDRs) of a parental HAPC-14 Fab clone was respectively mutated into 19 other amino acids by using site-directed mutation. DNA primers containing NNS codons encoding 20 amino acids were used to introduce mutations into each target CDR position. Phosphorylated degenerate primers were used in site-directed mutation reactions.

2.2 PCR

PCR was carried out at 94°C for 2 minutes (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 5 minutes) for 16 cycles, and then at 72°C for 10 minutes.

A PCR product was purified, and then introduced into *Escherichia coli* TG1 by electroporation for colony formation and Fab fragment production.

2.3 Preliminary screening of Fab mutant library

The preliminary screening included single-point ELISA (SPE) assay carried out by capture ELISA.

Each well of 96-well plates was coated with an anti-Fab antibody in a coating buffer PBS with pH 7.4 at 4°C. The next day, the plates were blocked with casein in PBS with pH 7.4 at 25°C for 1 hour. Then, Fab PE was added to the plates, and incubated at 25°C for 1 hour. After the plates were washed, biotinylated antigen was added to the wells and incubated at 25°C for 1 hour. Then, the biotinylated antigen was incubated with an SA-horseradish peroxidase (HRP) conjugate at 25°C for 1 hour. HRP activity was detected with a tetramethylbenzidine (TMB) substrate, and the reaction was quenched with 2M HCl. The plates were read at 450 nm. Clones exhibiting an optical density (OD) signal at 450 nm that was 3 times greater than that of the parental clone were selected and sequenced. Binding-improved mutants with unique sequences of HAPC-14 were demonstrated by capture ELISA at pH 7.4. The binding-improved mutants with unique sequences of HAPC-14 were ranked by direct antigen coating ELISA. The results are shown in Table 1 below.

Table 1

Antibody	Mutation	Capture Elisa OD450	Bmax	EC50
HAPC-14-Fab	WT	about 0.6196	0.221	221.3
HAPC-14-R1-1H1-F2	N to F	2.5193	0.2776	20.33
HAPC-14-R1-2H6-C2	S to K	1.8358	0.2621	19.42
HAPC-14-R1-3H4-C5	Y to S	1.77415	0.3275	102.80
HAPC-14-R1-3H4-C6	Y to T	1.4995	0.3245	55.82
HAPC-14-R1-3H7-F2	Y to P	1.42405	0.1933	25.13
HAPC-14-R1-1L7-B9	D to A	1.34885	0.1509	28.41
HAPC-14-R1-2L4-A1	N to L	2.1465	0.2441	13.38
HAPC-14-R1-2L4-B2	N to W	2.99075	0.6618	4.82
HAPC-14-R1-2L4-D4	N to R	3.0343	0.5669	10.45
HAPC-14-R1-2L4-G9	N to K	2.3219	0.2868	13.19
HAPC-14-R1-2L6-G1	E to G	2.00495	0.4919	35.65
HAPC-14-R1-2L6-F2	E to K	1.37175	0.2995	28.24
HAPC-14-R1-2L6-G11	E to P	1.56065	0.2199	18.04
HAPC-14-R1-3L1-D11	Q to W	2.06855	0.2612	13.64
HAPC-14-R1-3L1-G8	Q to V	1.4895	0.2405	30.79

As shown in Table 1, the affinity-improved mutations in the nine individuals (HAPC-14-R1-1H1-F2, HAPC-14-R1-2H6-C2, HAPC-14-R1-3H4-C6, HAPC-14-R1-2L4-B2, HAPC-14-R1-2L4-D4, HAPC-14-R1-2L6-G1, HAPC-14-R1-2L6-G11, HAPC-14-R1-3L1-D11 and HAPC-14-R1-3L1-

G8) identified in the preliminary screening of HAPC-14 were further used for designing and constructing a combinatorial mutant library.

2.4 Verification of main Fab mutant library by direct KD ELISA ranking

KD ranking of the binding-improved Fab of each mutant was determined by KD ELISA of direct antigen coating ELISA.

Each well of 96-well plates was coated with hPro1. biotin in 1xPBS buffer with pH 7.4, and incubation was carried out overnight at 4°C. The next day, the plates were blocked with casein in 1xPBS buffer with pH 7.4 at 25°C for 1 hour. Fab PE of each mutant diluted according to one concentration was added to the plates and incubated at 25°C for 1 hour. Then the Fab PE was incubated with goat anti-c-myc HRP conjugated at 25°C for 1 hour. HRP activity was detected with a tetramethylbenzidine (TMB) substrate, and the reaction was quenched with 2M HCl. OD values of each plate were read at 450 nm.

2.5 Combinatorial screening of HAPC-14 Fab library

Point mutations in VH and VL determined to be beneficial for antigen binding were further combined for additional binding synergy. In short, each degenerate primer was phosphorylated and then used with uracilated ssDNA at a ratio of 10:1. The mixture was heated to 85°C for 5 minutes and then cooled to 55°C for more than 1 hour. Then, T4 ligase and T4 DNA polymerase were added, and the mixture was incubated at 37°C for 1.5 hours. 100 ng of combinatorial library DNA was introduced into TG1 by electroporation to form colonies and produce Fab fragments.

The combinatorial mutants were expressed as Fab, and screened using capture ELISA. Clones exhibiting an optical density (OD) signal at 450 nm that was 3 times greater than that of the first-round precursor clone were selected and sequenced. The binding-improved mutants with unique sequences were further demonstrated by capture ELISA and sequenced by direct antigen coating ELISA.

A plurality of combinatorial mutants of HAPC-14 were screened, and the top 16 combinatorial mutants with significantly improved affinity were confirmed by KD ELISA (as shown in Nos.1-16 in Table 2).

Table 2

		Capture Elisa	KD Elisa ranking		VHCDR1	VHCDR2	VHCDR3	VLCDR2	VLCDR3
No.	Name	OD450	Bmax	EC50	<u>NY</u> YLN	DIRLK <u>S</u> NNYEKHVAESVKG	EGD <u>Y</u> FDY	LAS <u>N</u> LES	<u>Q</u> QNNEDPYT
	HAPC-14	0.0758	/	/					

1	R2-24F3	2.7928	1.366	1.373	F	 K		. . . W . G .	V
2	R2-23H2	2.6867	1.26	1.934	F	 K		. . . W . G .	
3	R2-25G5	2.6576	1.045	5.659	F	 K		. . . W . P .	V
4	R2-26E4	2.5923	1.211	1.838	F	 K		. . . R . G .	V
5	R2-25B7	2.5891	1.067	2.724	F			. . . W . P .	V
6	R2-27G7	2.58	0.7152	1.004	F	 K		. . . R . G .	
7	R2-29D9	2.5494	0.9603	7.854	F	 K	. . . T . . .	. . . R . P .	V
8	R2-24H1	2.4255	1.251	2.517	F			. . . R . G .	V
9	R2-22A10	2.3922	1.015	2.679	F	 K		. . . R . . .	V
10	R2-24D4	2.373	0.7169	2.104	F	 K	. . . T . . .	. . . W . P .	
11	R2-30F7	2.3418	0.6171	7.274	F			. . . R . P .	V
12	R2-23E10	2.3192	1.235	1.299	F			. . . W . G .	
13	R2-22B1	2.3056	1.225	1.194	F	 K	. . . T . . .	. . . W . P .	V
14	R2-32E6	2.1754	1.41	3.879		 K		. . . W . G .	V
15	R2-22B6	2.1719	1.214	3.971	F			. . . R . G .	
16	R2-9C1	1.9471	1.089	1.8	F	 K		. . . W . . .	V

After screening and statistics, it was found that the mutations at the following 6 sites would significantly affect the binding ability of the Fab fragment to the capture antibody. These six sites were:

- a) N57R/W located in VL CDR2 (N57R is preferred to N57W);
- b) E59G/P located in VL CDR2 (E59G is preferred to E59P);
- c) Q93V located in VL CDR3;
- d) N31F located in VH CDR1;
- e) S55K located in VH CDR2; and
- f) Y104T located in VH CDR3.

The most crucial mutant sites were N57R located in the light chain and N31F located in the heavy chain.

Example 3 Anti-aPC monoclonal antibodies based on HAPC-14 combinatorial mutation sites (constructing whole IgG1)

3.1 Construction of anti-aPC monoclonal antibodies (HAPC14-1 to HAPC14-10) based on HAPC14 combinatorial mutation sites

Based on the 6 mutation sites in Example 2, a combinatorial construction was carried out to obtain 10 mutants (14-1 to 14-10 in Table 3 below) (HAPC14-1 to HAPC14-10), and then the

sequences were constructed into a whole IgG form for expression and purification. The expression level, affinity and other bioactivities of the antibodies were investigated.

Amino acid sequences of HAPC-14 and the 10 mutants are shown in SEQ ID NOs: 5-26.

Humanized antibody 13 (HAPC-13)

Heavy chain (SEQ ID NO: 1)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGQGT^TTVTVSS

Light chain (SEQ ID NO: 2)

DIQMTQSPSSLSASVGDRVTITCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASNLESGV
PSRFGSGSGTDFTLTISLQPEDFATYYCQQNNEDPYTFGQGTKLEIK

Humanized antibody 16 (HAPC-16)

Heavy chain (SEQ ID NO: 3)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGQGT^TTVTVSS

Light chain (SEQ ID NO: 4)

NIVLTQSPSSLSASVGDRVTITCRASESVDSFGATFMHWYQQKPGKPPKLLIYLASNLESGV
PSRFGSGSGRTDFTLTISLQPEDFATYYCQQNNEDPYTFGQGTKLEIK

Humanized antibody 14 (HAPC-14)

Heavy chain (SEQ ID NO: 5)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGQGT^TTVTVSS

Light chain (SEQ ID NO: 6)

DIQLTQSPSSLSASVGDRVTITCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASNLESGV
PSRFGSGSGTDFTLTISLQPEDFATYYCQQNNEDPYTFGQGTKLEIK

HAPC-14-1

Heavy chain (SEQ ID NO: 7)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 8)

DIQLTQSPSSLSASVGDRVITTCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASWLGSV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQQNNEDPYTFGGGTKLEIK

HAPC-14-2

Heavy chain (SEQ ID NO: 9)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 10)

DIQLTQSPSSLSASVGDRVITTCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASWLESGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGGGTKLEIK

HAPC-14-3

Heavy chain (SEQ ID NO: 11)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 12)

DIQLTQSPSSLSASVGDRVITTCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGGGTKLEIK

HAPC-14-4

Heavy chain (SEQ ID NO: 13)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 14)

DIQLTQSPSSLSASVGDRVITTCRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGGGTKLEIK

HAPC-14-5

Heavy chain (SEQ ID NO: 15)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 16)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASWLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGQGGTKLEIK

HAPC-14-6

Heavy chain (SEQ ID NO: 17)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 18)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQNNEDPYTFGQGGTKLEIK

HAPC-14-7

Heavy chain (SEQ ID NO: 19)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKSNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 20)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQNNEDPYTFGQGGTKLEIK

HAPC-14-8

Heavy chain (SEQ ID NO: 21)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 22)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGQGGTKLEIK

HAPC-14-9

Heavy chain (SEQ ID NO: 23)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNWVRQAPGKGLEWVGDIRLKKNNYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYWGGQTTVTVSS

Light chain (SEQ ID NO: 24)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQQNNEDPYTFGQGGTKLEIK

HAPC-14-10

Heavy chain (SEQ ID NO: 25)

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNWVRQAPGKGLEWVGDIRLKKNYYEK
HYAESVKGRFTISRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYWGQGTTVTVSS

Light chain (SEQ ID NO: 26)

DIQLTQSPSSLSASVGDRVITICRASESVDSFGATFMHWYQQKPGKAPKLLIYLASRLGSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCVQNNEDPYTFGQGGTKLEIK

HAPC-14

Heavy chain CDR1 (SEQ ID NO: 27)

NYYLN

HAPC-14

Heavy chain CDR2 (SEQ ID NO: 28)

DIRLKSNNYEKHYAESVKG

HAPC-14

Heavy chain CDR3 (SEQ ID NO: 29)

EGDYFDY

HAPC-14

Light chain CDR1 (SEQ ID NO: 30)

RASESVDSFGATFMH

HAPC-14

Light chain CDR2 (SEQ ID NO: 31)

LASNLES

HAPC-14

Light chain CDR3 (SEQ ID NO: 32)

QQNNEDPYT

HAPC-14-1

Heavy chain CDR1 (SEQ ID NO: 33)

FYYLN

HAPC-14-1

Heavy chain CDR2 (SEQ ID NO: 34)

DIRLKSNNYEKHYAESVKG

HAPC-14-1

Heavy chain CDR3 (SEQ ID NO: 35)

EGDYFDY

HAPC-14-1

Light chain CDR1 (SEQ ID NO: 36)

RASESVDSFGATFMH

HAPC-14-1

Light chain CDR2 (SEQ ID NO: 37)

LASWLGS

HAPC-14-1

Light chain CDR3 (SEQ ID NO: 38)

QQNNEDPYT

HAPC-14-2

Heavy chain CDR1 (SEQ ID NO: 39)

FYYLN

HAPC-14-2(SEQ ID NO: 40)

Heavy chain CDR2

DIRLKKNYEKHYAESVKG

HAPC-14-2

Heavy chain CDR3 (SEQ ID NO: 41)

EGDYFDY

HAPC-14-2

Light chain CDR1 (SEQ ID NO: 42)

RASESVDSFGATFMH

HAPC-14-2

Light chain CDR2 (SEQ ID NO: 43)

LASWLES

HAPC-14-2

Light chain CDR3 (SEQ ID NO: 44)

VQNNEDPYT

HAPC-14-3

Heavy chain CDR1 (SEQ ID NO: 45)

FYYLN

HAPC-14-3

Heavy chain CDR2 (SEQ ID NO: 46)

DIRLKKNNYEKHYAESVKG

HAPC-14-3

Heavy chain CDR3 (SEQ ID NO: 47)

EGDYFDY

HAPC-14-3

Light chain CDR1 (SEQ ID NO: 48)

RASESVDSFGATFMH

HAPC-14-3

Light chain CDR2 (SEQ ID NO: 49)

LASRLGS

HAPC-14-3

Light chain CDR3 (SEQ ID NO: 50)

VQNNEDPYT

HAPC-14-4

Heavy chain CDR1 (SEQ ID NO: 51)

FYYLN

HAPC-14-4

Heavy chain CDR2 (SEQ ID NO: 52)

DIRLKSNNYEKHYAESVKG

HAPC-14-4

Heavy chain CDR3 (SEQ ID NO: 53)

EGDYFDY

HAPC-14-4

Light chain CDR1 (SEQ ID NO: 54)

RASESVDSFGATFMH

HAPC-14-4

Light chain CDR2 (SEQ ID NO: 55)

LASRLGS

HAPC-14-4

Light chain CDR3 (SEQ ID NO: 56)

VQNNEDPYT

HAPC-14-5

Heavy chain CDR1 (SEQ ID NO: 57)

FYYLN

HAPC-14-5

Heavy chain CDR2 (SEQ ID NO: 58)

DIRLKKNNYEKHYAESVKG

HAPC-14-5

Heavy chain CDR3 (SEQ ID NO: 59)

EGDYFDY

HAPC-14-5

Light chain CDR1 (SEQ ID NO: 60)

RASESVDSFGATFMH

HAPC-14-5

Light chain CDR2 (SEQ ID NO: 61)

LASWLGS

HAPC-14-5

Light chain CDR3 (SEQ ID NO: 62)

VQNNEDPYT

HAPC-14-6

Heavy chain CDR1 (SEQ ID NO: 63)

FYYLN

HAPC-14-6

Heavy chain CDR2 (SEQ ID NO: 64)

DIRLKKNNYEKHYAESVKG

HAPC-14-6

Heavy chain CDR3 (SEQ ID NO: 65)

EGDYFDY

HAPC-14-6

Light chain CDR1 (SEQ ID NO: 66)

RASESVDSFGATFMH

HAPC-14-6

Light chain CDR2 (SEQ ID NO: 67)

LASRLGS

HAPC-14-6

Light chain CDR3 (SEQ ID NO: 68)

QQNNEDPYT

HAPC-14-7

Heavy chain CDR1 (SEQ ID NO: 69)

FYYLN

HAPC-14-7

Heavy chain CDR2 (SEQ ID NO: 70)

DIRLKSNNYEKHYAESVKG

HAPC-14-7

Heavy chain CDR3 (SEQ ID NO: 71)

EGDYFDY

HAPC-14-7

Light chain CDR1 (SEQ ID NO: 72)

RASESVDSFGATFMH

HAPC-14-7

Light chain CDR2 (SEQ ID NO: 73)

LASRLES

HAPC-14-7

Light chain CDR3 (SEQ ID NO: 74)

QQNNEDPYT

HAPC-14-8

Heavy chain CDR1 (SEQ ID NO: 75)

FYYLN

HAPC-14-8

Heavy chain CDR2 (SEQ ID NO: 76)

DIRLKKNNYEKHYAESVKG

HAPC-14-8

Heavy chain CDR3 (SEQ ID NO: 77)

EGDTFDY

HAPC-14-8

Light chain CDR1 (SEQ ID NO: 78)

RASESVDSFGATFMH

HAPC-14-8

Light chain CDR2 (SEQ ID NO: 79)

LASRLGS

HAPC-14-8

Light chain CDR3 (SEQ ID NO: 80)

VQNNEDPYT

HAPC-14-9

Heavy chain CDR1 (SEQ ID NO: 81)

FYYLN

HAPC-14-9

Heavy chain CDR2 (SEQ ID NO: 82)

DIRLKKNNYEKHYAESVKG

HAPC-14-9

Heavy chain CDR3 (SEQ ID NO: 83)

EGDTFDY

HAPC-14-9

Light chain CDR1 (SEQ ID NO: 84)

RASESVDSFGATFMH

HAPC-14-9

Light chain CDR2 (SEQ ID NO: 85)

LASRLGS

HAPC-14-9

Light chain CDR3 (SEQ ID NO: 86)

QQNNEDPYT

HAPC-14-10

Heavy chain CDR1 (SEQ ID NO: 87)

NYYLN

HAPC-14-10

Heavy chain CDR2 (SEQ ID NO: 88)

DIRLKKNNYEKHYAESVKG

HAPC-14-10

Heavy chain CDR3 (SEQ ID NO: 89)

EGDTFDY

HAPC-14-10

Light chain CDR1 (SEQ ID NO: 90)

RASESVDSFGATFMH

HAPC-14-10

Light chain CDR2 (SEQ ID NO: 91)

LASRLGS

HAPC-14-10

Light chain CDR3 (SEQ ID NO: 92)

VQNNEDPYT

Detailed information is shown in Table 3.

Table 3

Backbone	hIgG1		
Entry	Mutations	H chain	L chain
1	L57W,L59G,H31F	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASWLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCQQ NNEDPYTFGQGTKLEIK
2	L57W,L93V,H31F,H55K	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASWLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK
3	L57R,L59G,L93V,H31F,H55K	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK
4	L57R,L59G,L93V,H31F	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK
5	L57W,L59G,L93V,H31F,H55K	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASWLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK
6	L57R,L59G,H31F,H55K	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCQQ NNEDPYTFGQGTKLEIK
7	L57R,H31F	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCQQ NNEDPYTFGQGTKLEIK
8	L57R,L59G,L93V,H31F,H55K,H104T	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK
9	L57R,L59G,H31F,H55K,H104T	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCQQ NNEDPYTFGQGTKLEIK
10	L57R,L59G,L93V,H55K,H104T	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFLNW VRQAPGKLEWVGDIRLKNNYEKHYAESVKGRFTI SRDDSKSITYLQMNSLRAEDTAVYYCAREGDTFDYW GQGTTIVVSS	DIQLTQSPSSLSASVGDRTITCRASESVDSFGATFMHWYQQKPGKA PKLLIYLASRLGSVPSRFRSGSGSGTDFTLTISLQPEDFATYYCVQ NNEDPYTFGQGTKLEIK

The underlined parts are CDRs, and the other parts are framework regions.

Humanized antibody 14 (anti-aPC monoclonal antibody 14)

Heavy chain

```
EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNW
VRQAPGKGLEWVGDIRLKSNNYEKHYAESVKGRFTI
SRDDSKSITYLQMNSLRAEDTAVYYCAREGDYFDYW
GQGTITVTVSS
```

Light chain

```
DIQLTQSPSSLSASVGRVTITCRASESVDSFGATFMHWYQQKPGKA
PKLLIYLASNLESGVPSRFSGSGSDTFTLTISLQPEDFATYYCQQ
NNEDPYTFGQGTKLEIK
```

The underlined parts are CDRs, the other parts are framework regions, and white fonts indicate humanized sites.

Protein number	mutation sites
HPAC14-1	L57W,L59G,H31F
HPAC 14-2	L57W,L93V,H31F,H55K
HPAC 14-3	L57R,L59G,L93V,H31F,H55K
HPAC 14-4	L57R,L59G,L93V,H31F
HPAC 14-5	L57W,L59G,L93V,H31F,H55K
HPAC 14-6	L57R,L59G,H31F,H55K
HPAC 14-7	L57R,H31F
HPAC 14-8	L57R,L59G,L93V,H31F,H55K,H104T
HPAC 14-9	L57R,L59G,H31F,H55K,H104T
HPAC 14-10	L57R,L59G,L93V,H55K,H104T
HAPC14	

3.2 Assays of binding abilities of anti-aPC monoclonal antibodies constructed based on HAPC14 combinatorial mutation sites to haPC/hPC (HAPC14-1 to HAPC14-10)

Assay method:

The binding abilities of the anti-aPC monoclonal antibodies to haPC/hPC were assayed by Elisa.

1. ELISA plates were coated with haPC/hPC (1 $\mu\text{g/mL}$ in PBS), 100 $\mu\text{L/well}$, at 4°C overnight.
2. The plates were washed 3 times with PBST (0.1% Tween 20), 200 $\mu\text{L/well}$.

3. The plates were blocked with 2% BSA (PBS solution), 200 μ L/well, at room temperature for 1 hour.
4. The plates were washed 3 times with PBST (0.1% Tween 20), 200 μ L/well.
5. The plates were incubated with anti-aPC monoclonal antibody as primary antibody (diluted from 10 μ g/mL in 2% BSA), 100 μ L/well, at room temperature for 1 hour.
6. The plates were washed 3 times with PBST (0.1% Tween 20), 200 μ L/well.
7. The plates were incubated with anti-hIgG-Fc antibody-HRP (diluted 5000-fold in 2% BSA buffer) at room temperature for 1 hour.
8. The plates were washed 6 times with PBST (0.1% Tween 20), 200 μ L/well.
9. The plates were incubated with a TMB substrate (prepared from solutions A and B at 1:1 (v/v)), 100 μ L/well, away from light for 10 minutes.
10. The reaction was quenched with HCl (1 M), 100 μ L/well, and OD450 was read.

The assay results are shown in Fig. 3, Table 4 and Table 5. As can be seen, the anti-aPC monoclonal antibodies bind to hAPC and do not bind to hPC.

Table 4

Antigen	Antibody	EC50 (nM)	Span
hAPC	HAPC14-1	0.1141	2.106
	HAPC14-2	0.111	1.775
	HAPC14-3	0.0618	1.615
	HAPC14-4	0.06052	1.52
	HAPC14-5	0.09239	1.631
	HAPC14-6	0.08212	1.854
	HAPC14-7	0.1304	1.578
	HAPC14-8	0.1382	1.046
	HAPC14-9	0.07608	1.07
	HAPC14-10	0.2799	0.7453
	HAPC14	0.2203	0.2909

Table 5

Antigen	Antibody	Span
---------	----------	------

hPC	HAPC14-1	0.023
	HAPC14-2	0.01042
	HAPC14-3	about 0.2673
	HAPC14-4	0.1571
	HAPC14-5	about 0.04753
	HAPC14-6	about 0.01332
	HAPC14-7	about 0.006612
	HAPC14-8	-0.004647
	HAPC14-9	0.012
	HAPC14-10	about 0.001450
	HAPC14	about 0.03871

3.3 Assays of affinities of anti-aPC monoclonal antibodies constructed based on HAPC-14 combinatorial mutation sites using Octet system for biomolecular interactions (HAPC-14-1 to HAPC-14-10)

Assay method: Octet analysis was carried out at 25°C using a Protein A Octet biosensor in 1x Kinetics buffer. During the kinetics assay, the baseline step assay was run in 1x Kinetics buffer for 120 seconds, and then run for 240 seconds after the antibody was loaded to the biosensor. Next, the baseline 2 step assay was run in 1x Kinetics buffer for 120 seconds. Then, the biosensor with the capture antibody was immersed in wells containing antigen (huAPC) of 5 diluted concentrations (3.13 nM, 6.25 nM, 12.5 nM, 25 nM and 50 nM) for 180 seconds, and then dissociation was carried out in 1x Kinetics buffer for 400 seconds. The 1x Kinetics buffer was used as a blank control to show the natural dissociation of the antibody in the buffer. 10 mM glycine at pH 1.5 was used for the regeneration step. Octet data analysis software was used to calculate binding kinetics, and a 1:1 binding model was used for curve fitting.

The affinities of the mutant antibodies were assayed at pH 7.4. The results are shown in Fig. 4 and Table 6:

Table 6

Sample	KD (M)	kon (1/Ms)	kdis (1/s)	Full R ²
HAPC14-1	3.83E-09	3.06E+05	1.17E-03	0.9932
HAPC14-2	3.78E-09	3.07E+05	1.16E-03	0.9877
HAPC14-3	2.67E-09	3.20E+05	8.54E-04	0.9933
HAPC14-4	4.06E-09	3.10E+05	1.26E-03	0.9911

HAPC14-5	2.83E-09	3.22E+05	9.11E-04	0.9924
HAPC14-6	3.64E-09	2.97E+05	1.08E-03	0.9917
HAPC14-7	7.72E-09	2.56E+05	1.97E-03	0.9929
HAPC14-8	1.05E-08	2.50E+05	2.62E-03	0.9901
HAPC14-9	9.69E-09	2.45E+05	2.38E-03	0.9917
HAPC14-10	1.90E-08	2.25E+05	4.28E-03	0.9906
HAPC14	2.54E-07	6.40E+04	1.62E-02	0.9866

As can be seen from the results in Fig. 4 and Table 6, the mutated antibodies could still bind to haPC, but could not bind to hPC, and their affinities were increased as compared with the unmutated No. 14 antibody HAPC-14. Especially No. 6 (HAPC14-6) and No. 3 (HAPC14-3) showed a nearly 100-times increase.

Example 4 Cleavage of histones by anti-aPC monoclonal antibody HAPC14-6 and aPC

Assay method:

Reagent	Content (ug/ μ L)	Incubation	
Calf thymus histone in each tube	0.2	37°C	4h
aPC present or absent in each tube	When aPC was present, its content was 0.012		
HAPC present or absent in each tube	When HAPC was present, its content was 0.06		
PBS Buffer at pH 7.3 was added until the reaction volume reached 100 μ l			

After SDS-PAGE, Coomassie Blue staining was carried out, and whether the HAPC monoclonal antibody promoted or inhibited the cleavage of histones by aPC was observed.

The assay results are shown in Fig. 5 (from left to right in Fig. 5: containing histones only; containing histones and APC; containing histones, APC and HAPC1573; containing histones, APC and HAPC14-6). As can be seen, HAPC1573 and HAPC14-6 promoted the ability of aPC to cleave histones, which indicated that the HAPC14-6 monoclonal antibody of the present disclosure could provide the cytoprotective effect against cytotoxicity of the histones H3 and H4.

Consistent with HAPC1573, HAPC-14-6 did not inhibit, but actually promoted the cleavage of histones H3 and H4 by aPC. Different from HAPC1573, HAPC-14-6 slightly inhibited the cleavage of histone H1 by aPC. This indicated that HAPC-14-6 could provide the cytoprotective effect against cytotoxicity of the histones H3 and H4.

Example 5 In vitro pharmacodynamic studies of anti-aPC monoclonal antibodies

Assay method: STAGO Compact Max APTT-procedure.

Coagulation factor FIX-deficient plasma + antibodies at various dilutions in Table 7 (final concentration) + 0.01 U/ml Protac (final concentration) + OWREN-KOLLER buffer, at room temperature for 15 min.

150 µl total 150 ul

Control: coagulation factor FIX-deficient plasma 150 μ l + OWREN-KOLLER buffer 150 μ l.

Assay method: STAGO Compact Max APTT-procedure.

Assay result: As shown in Table 7, in the coagulation factor VIII and VIX-deficient plasma, the anti-aPC monoclonal antibodies HAPC14-1 to HAPC14-10 could inhibit the anticoagulant activity of aPC to different degrees. At the same antibody concentration, the anti-aPC monoclonal antibodies HAPC14-1 to HAPC14-10 all had better effects in inhibiting the anticoagulant activity of aPC than the murine antibodies 1573, HAPC13 and HAPC14.

Table 7

Antibody	APC-antibody concentration ($\mu\text{g/ml}$)	Mean clotting time (s)	
		FVIII-deficient plasma	FIX-deficient plasma
1573	5	388.9	403.5
	1	629	566.8
	0.5	600	584.2
HAPC-13	5	300.7	325.7
	1	546.3	402.3
	0.5	628.8	502.1
HAPC-14	5	324.4	310.2
	1	457.3	415.3
	0.5	574.5	457
HAPC14-1	5	165.8	171.3
	1	222.5	194.6
	0.5	327.1	219.3
HAPC14-2	5	158.9	142.9
	1	195.6	210.8
	0.5	358.4	233.2
HAPC14-3	5	176.4	185.1
	1	188	199.6

	0.5	205.2	208.4
HAPC14-4	5	158.3	176.2
	1	184.3	193.7
	0.5	220.6	219.6
HAPC14-5	5	171.2	166.1
	1	200.2	187.5
	0.5	223.9	197.4
HAPC14-6	5	161.7	179.5
	1	186.9	198.5
	0.5	182.3	181.3
HAPC14-7	5	175.5	146.5
	1	197.9	145.4
	0.5	296.7	240.9
HAPC14-8	0.5	189.7	276.4
	0.1	251.9	392
	0.05	315.9	732
HAPC14-9	0.5	181.6	170
	0.25	-	497.8
	0.1	217.3	610.8
	0.05	261.6	670.1
HAPC14-10	1	-	158.2
	0.5	254.5	330.7
	0.1	309.1	595.5
	0.05	414.6	733.3

(2) Experimental result of effects of different antibodies at different concentrations on coagulation factor FVIII-deficient plasma

Experimental method: The antibodies were diluted to corresponding concentrations with OWREN-KOLLER buffer.

Coagulation factor FVIII-deficient plasma + antibodies at various dilutions in Fig. 6A (final concentration) + 0.05 U/ml Protac (final concentration) + OWREN-KOLLER buffer, at room temperature for 15 min.

150 µl

total 150 µl

Control: coagulation factor FVIII-deficient plasma 150 µl + OWREN-KOLLER buffer 150

Coagulation factor FV-deficient plasma (containing 5% standard plasma) + antibodies at various dilutions in Fig. 6F (final concentration) + 0.05 U/ml Protac (final concentration) + OWREN-KOLLER buffer, at room temperature for 15 min.

150 μl total 150 ul

Control: coagulation factor FV-deficient plasma (containing 5% standard plasma) 150 µl + OWREN-KOLLER buffer 150 µl.

Assay method: STAGO Compact Max APTT-procedure.

Assay result: As shown in Fig. 6F, in the coagulation factor FV-deficient plasma, the anti-aPC monoclonal antibodies completely inhibited the anticoagulant activity of aPC.

(8) Experimental result of effects of different antibodies at different concentrations on coagulation factor FX-deficient plasma

Experimental method: The antibodies were diluted to corresponding concentrations with OWREN-KOLLER buffer.

Coagulation factor FX-deficient plasma (containing 1% standard plasma) + antibodies at various dilutions in Fig. 6G (final concentration) + 0.05 U/ml Protac (final concentration) + OWREN-KOLLER buffer, at room temperature for 15 min.

150 µl total 150 ul

Control: coagulation factor FX-deficient plasma (containing 1% standard plasma) 150 µl + OWREN-KOLLER buffer 150 µl.

Assay method: STAGO Compact Max APTT-procedure.

Assay result: As shown in Fig. 6G, in the coagulation factor FX-deficient plasma, the anti-aPC monoclonal antibodies completely inhibited the anticoagulant activity of aPC.

(9) Experimental result of effects of different antibodies at different concentrations on coagulation factor VWF-deficient plasma

Experimental method: The antibodies were diluted to corresponding concentrations with OWREN-KOLLER buffer.

Coagulation factor VWF-deficient plasma + antibodies at various dilutions in Fig. 6H (final concentration) + 0.01 U/ml Protac (final concentration) + OWREN-KOLLER buffer, at room temperature for 15 min.

restored to the normal level by at most 1 μ g of HAPC-14-6.

As shown in Fig. 7C, in the humanized protein C hemophilia B mice, after different doses of HAPC-14-6 were respectively injected, the coagulation function of the deficient mice could be restored to the normal level by at most 1 μ g of HAPC-14-6.

The result indicated that in the present disclosure, after the protein C activating enzyme (protac)-induced APTT experiments were used to test the minimum dose of anti-aPC monoclonal antibodies to inhibit the anticoagulant activity of aPC in human plasma deficient in multiple coagulation factors, it was found that a concentration of at most 1 μ g/mg of the anti-aPC monoclonal antibodies could completely inhibit the anticoagulant activity of aPC, while a concentration of 100 μ g/ml or above was required for the murine antibody HAPC1573.

The foregoing embodiments are merely intended for describing the technical solutions of the present disclosure, but not for limiting the present disclosure. Although the present disclosure is described in detail with reference to the foregoing embodiments, persons of ordinary skill in the art should understand that they may still make modifications to the specific implementations described in the foregoing embodiments or make equivalent replacements to some technical features thereof. Changes and advantages that can be conceived by persons skilled in the art without departing from the spirit and scope of the concept of the disclosure, should fall in the scope of the technical solutions claimed in the present disclosure.

What is claimed is:

1. An anti-aPC monoclonal antibody, wherein a heavy chain CDR and/or a light chain CDR of the anti-aPC monoclonal antibody comprises one or more of the following mutations:

- 1) N57R and/or N57W mutation relative to a light chain CDR2 as shown in SEQ ID NO: 31;
- 2) E59G and/or E59P mutation relative to the light chain CDR2 as shown in SEQ ID NO: 31;
- 3) Q93V mutation relative to a light chain CDR3 as shown in SEQ ID NO: 32;
- 4) N31F mutation relative to a heavy chain CDR1 as shown in SEQ ID NO: 27;
- 5) S55K mutation relative to a heavy chain CDR2 as shown in SEQ ID NO: 28; and
- 6) Y104T mutation relative to a heavy chain CDR3 as shown in SEQ ID NO: 29.

2. The anti-aPC monoclonal antibody according to claim 1, wherein an original sequence of the anti-aPC monoclonal antibody before mutations comprises:

i) a heavy chain comprising the heavy chain CDR1 as shown in SEQ ID NO: 27, the heavy chain CDR2 as shown in SEQ ID NO: 28 and the heavy chain CDR3 as shown in SEQ ID NO: 29; and

ii) a light chain comprising a light chain CDR1 as shown in SEQ ID NO: 30, the light chain CDR2 as shown in SEQ ID NO: 31 and the light chain CDR3 as shown in SEQ ID NO: 32.

3. The anti-aPC monoclonal antibody according to claim 1, wherein

a light chain CDR2 of the anti-aPC monoclonal antibody comprises the N57R and E59G mutations relative to the light chain CDR2 as shown in SEQ ID NO: 31.

4. The anti-aPC monoclonal antibody according to claim 1, wherein the anti-aPC monoclonal antibody is selected from one of the following:

an antibody comprising (a) heavy chain CDRs represented by SEQ ID NOs: 27, 28 and 29; and/or (b) light chain CDRs represented by SEQ ID NOs: 30, 31 and 32;

an antibody comprising (c) heavy chain CDRs represented by SEQ ID NOs: 33, 34 and 35; and/or (d) light chain CDRs represented by SEQ ID NOs: 36, 37 and 38;

an antibody comprising (e) heavy chain CDRs represented by SEQ ID NOs: 39, 40 and 41; and/or (f) light chain CDRs represented by SEQ ID NOs: 42, 43 and 44;

an antibody comprising (g) heavy chain CDRs represented by SEQ ID NOs: 45, 46 and 47; and/or (h) light chain CDRs represented by SEQ ID NOs: 48, 49 and 50;

an antibody comprising (i) heavy chain CDRs represented by SEQ ID NOs: 51, 52 and 53;

and/or (j) light chain CDRs represented by SEQ ID NOs: 54, 55 and 56;

an antibody comprising (k) heavy chain CDRs represented by SEQ ID NOs: 57, 58 and 59;

and/or (l) light chain CDRs represented by SEQ ID NOs: 60, 61 and 62;

an antibody comprising (m) heavy chain CDRs represented by SEQ ID NOs: 63, 64 and 65;

and/or (n) light chain CDRs represented by SEQ ID NOs: 66, 67 and 68;

an antibody comprising (o) heavy chain CDRs represented by SEQ ID NOs: 69, 70 and 71;

and/or (p) light chain CDRs represented by SEQ ID NOs: 72, 73 and 74;

an antibody comprising (q) heavy chain CDRs represented by SEQ ID NOs: 75, 76 and 77;

and/or (r) light chain CDRs represented by SEQ ID NOs: 78, 79 and 80;

an antibody comprising (s) heavy chain CDRs represented by SEQ ID NOs: 81, 82 and 83;

and/or (t) light chain CDRs represented by SEQ ID NOs: 84, 85 and 86; and

an antibody comprising (u) heavy chain CDRs represented by SEQ ID NOs: 87, 88 and 89;

and/or (v) light chain CDRs represented by SEQ ID NOs: 90, 91 and 92.

5. The anti-aPC monoclonal antibody according to claim 4, comprising:

a heavy chain as shown in any of SEQ ID NOs: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25;

and/or

a light chain as shown in any of SEQ ID NOs: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26.

6. The anti-aPC monoclonal antibody according to claim 4, wherein the anti-aPC monoclonal antibody is selected from the following:

an antibody comprising a heavy chain as shown in SEQ ID NO: 5 and a light chain as shown in SEQ ID NO: 6;

an antibody comprising a heavy chain as shown in SEQ ID NO: 7 and a light chain as shown in SEQ ID NO: 8;

an antibody comprising a heavy chain as shown in SEQ ID NO: 9 and a light chain as shown in SEQ ID NO: 10;

an antibody comprising a heavy chain as shown in SEQ ID NO: 11 and a light chain as shown in SEQ ID NO: 12;

an antibody comprising a heavy chain as shown in SEQ ID NO: 13 and a light chain as shown in SEQ ID NO: 14;

an antibody comprising a heavy chain as shown in SEQ ID NO: 15 and a light chain as shown in SEQ ID NO: 16;

an antibody comprising a heavy chain as shown in SEQ ID NO: 17 and a light chain as shown

in SEQ ID NO: 18;

an antibody comprising a heavy chain as shown in SEQ ID NO: 19 and a light chain as shown in SEQ ID NO: 20;

an antibody comprising a heavy chain as shown in SEQ ID NO: 21 and a light chain as shown in SEQ ID NO: 22;

an antibody comprising a heavy chain as shown in SEQ ID NO: 23 and a light chain as shown in SEQ ID NO: 24; and

an antibody comprising a heavy chain as shown in SEQ ID NO: 25 and a light chain as shown in SEQ ID NO: 26.

7. The anti-aPC monoclonal antibody according to claim 1, wherein the anti-aPC monoclonal antibody is a complete antibody or an antibody fragment thereof; wherein the antibody fragment is Fab', Fab, F(ab')₂, a single-domain antibody, Fv or scFv.

8. A biological material related to the anti-aPC monoclonal antibody according to any one of claims 1 to 7, wherein the biological material is any one of 1) to 3) as follows:

- 1) a nucleic acid molecule encoding the anti-aPC monoclonal antibody according to claim 1;
- 2) a construct containing the nucleic acid molecule as described in 1); and
- 3) an expression system containing the nucleic acid molecule as described in 1).

9. A preparation product, wherein the preparation product is a pharmaceutical composition or a detection kit;

the preparation product comprises the anti-aPC monoclonal antibody according to claim 1 or any one or more of the biological materials according to claim 8; and

when being the pharmaceutical composition, the preparation product further comprises a pharmaceutically acceptable carrier.

10. A method for treating coagulation related disorders, comprising administering to an individual in need of treatment an effective amount of the anti-aPC monoclonal antibody according to any one of claims 1 to 7 or any one or more of the biological materials according to claim 8; wherein the individual suffers from coagulation deficiency or defect disorders, or the individual is in need of blood coagulation treatment, or the individual suffers from sepsis or hemophilia, or the individual is in need of regulation of hemostasis.

11. Use of the anti-aPC monoclonal antibody according to any one of claims 1 to 7 or the biological material according to claim 9 in any one or more of the following:

- i) in preparation of a drug for prevention and/or treatment of coagulation deficiency or defect disorders;
- ii) in preparation of a promoter for promoting an ability of aPC to cleave histones H3 and H4;
- iii) in preparation of a drug for inhibiting anticoagulant activity of activated protein C in an individual;
- iv) in preparation of a drug for treatment in an individual in need of blood coagulation;
- vi) in preparation of a drug for regulating hemostasis in an individual;
- vii) in immunoassays for disease or non-disease diagnosis and treatment purposes or preparation of a detection reagent or kit for aPC; and
- viii) in combination with other reagents or drugs.

12. The use according to claim 12, wherein the coagulation deficiency or defect disorders comprise disorders causing coagulopathy due to deficiency of coagulation factors and thus causing bleeding, and coagulation disturbances caused by trauma or surgery.

13. The use according to claim 12, wherein the coagulation deficiency or defect disorders comprise hemophilia, sepsis and traumatic hemorrhage.