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(54) **FLUOROGENIC SUBSTRATE FOR ADAMTS13**

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(57) **ABSTRACT**

Disclosed are fluorogenic substrates for measuring ADAMTS13 activity or ADAMTS13 inhibitor activity. Substrates can comprise an oligopeptide which can consist of up to 80 amino acids of sequence of von Willebrand Factor (VWF). The oligopeptide can include modifications of sequence of VWF, including an amino-terminal glycine, a scissile Y-M peptide, and a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction. A substrate can further comprise a fluorophore and a fluorescence quencher bound to the oligopeptide on opposite sides of the scissile Y-M peptide, wherein the fluorescence quencher is not identical to the fluorophore. An oligopeptide can be encoded by a nucleic acid sequence which can also encode a His tag. An oligopeptide can be expressed in a cell or microorganism. Also disclosed are methods of using a fluorogenic substrate to measure ADAMTS13 activity or ADAMTS13 inhibitor activity.

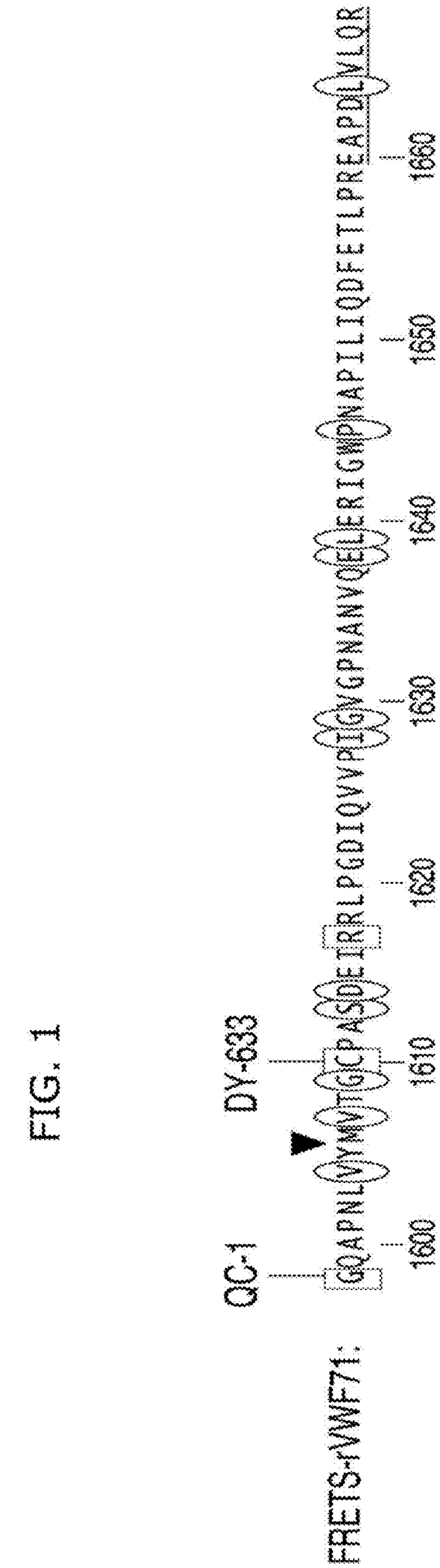

FRETS-rVWF71:
QC-1
DY-633
GQAPNLVYMVTGNPASD
1600
1610
ETRRLPGDIQVVPIGVGPNANVQELERIGWPNA
1620
1630
1640
PLIQEFLPREAPDLVLQR
1650
1660

FIG. 1

FIG. 2

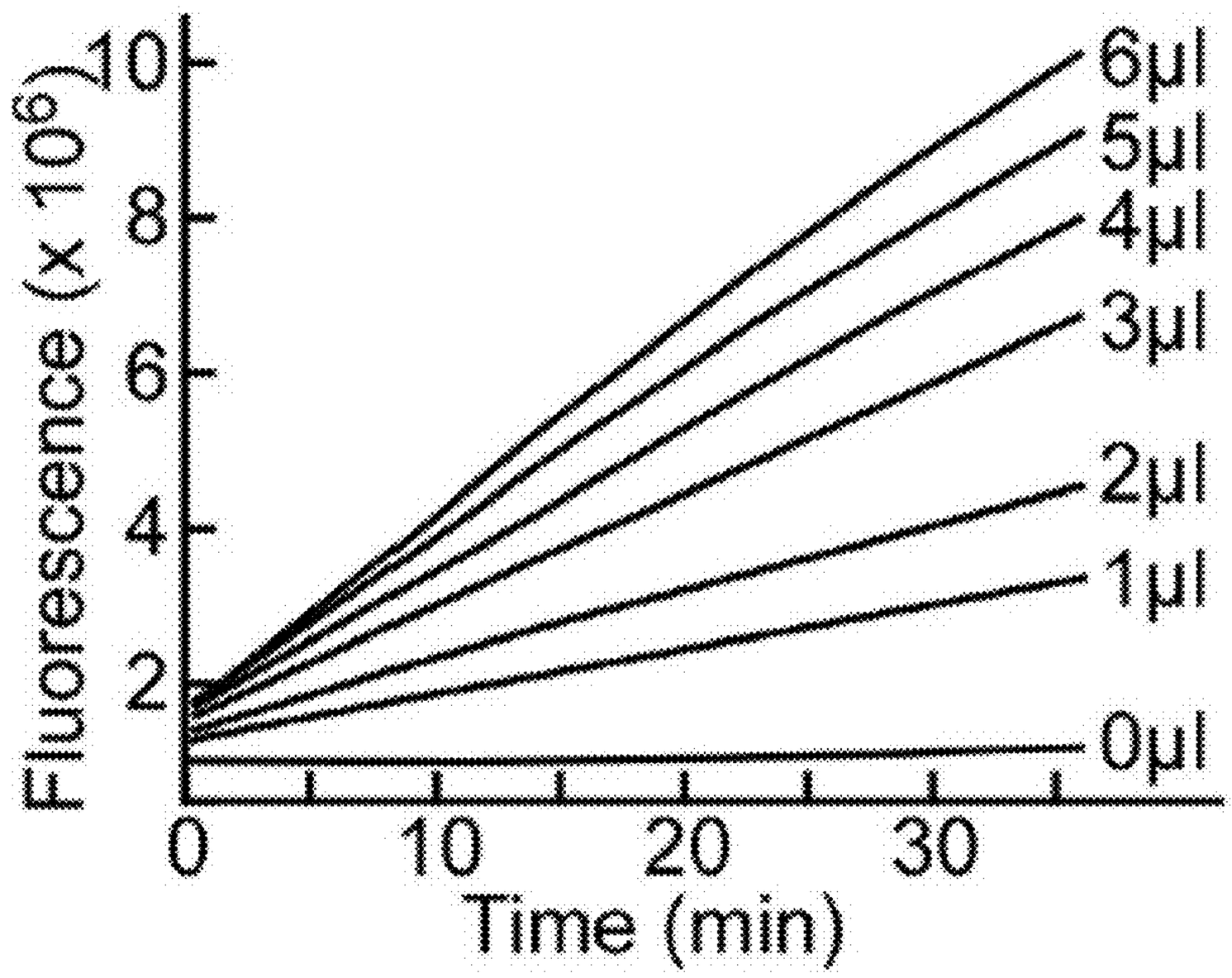

Fluorescence (x 10^6)
10
8
6
4
2
0
6μl
5μl
4μl
3μl
2μl
1μl
0μl
0
10
20
30
Time (min)

FIG. 3

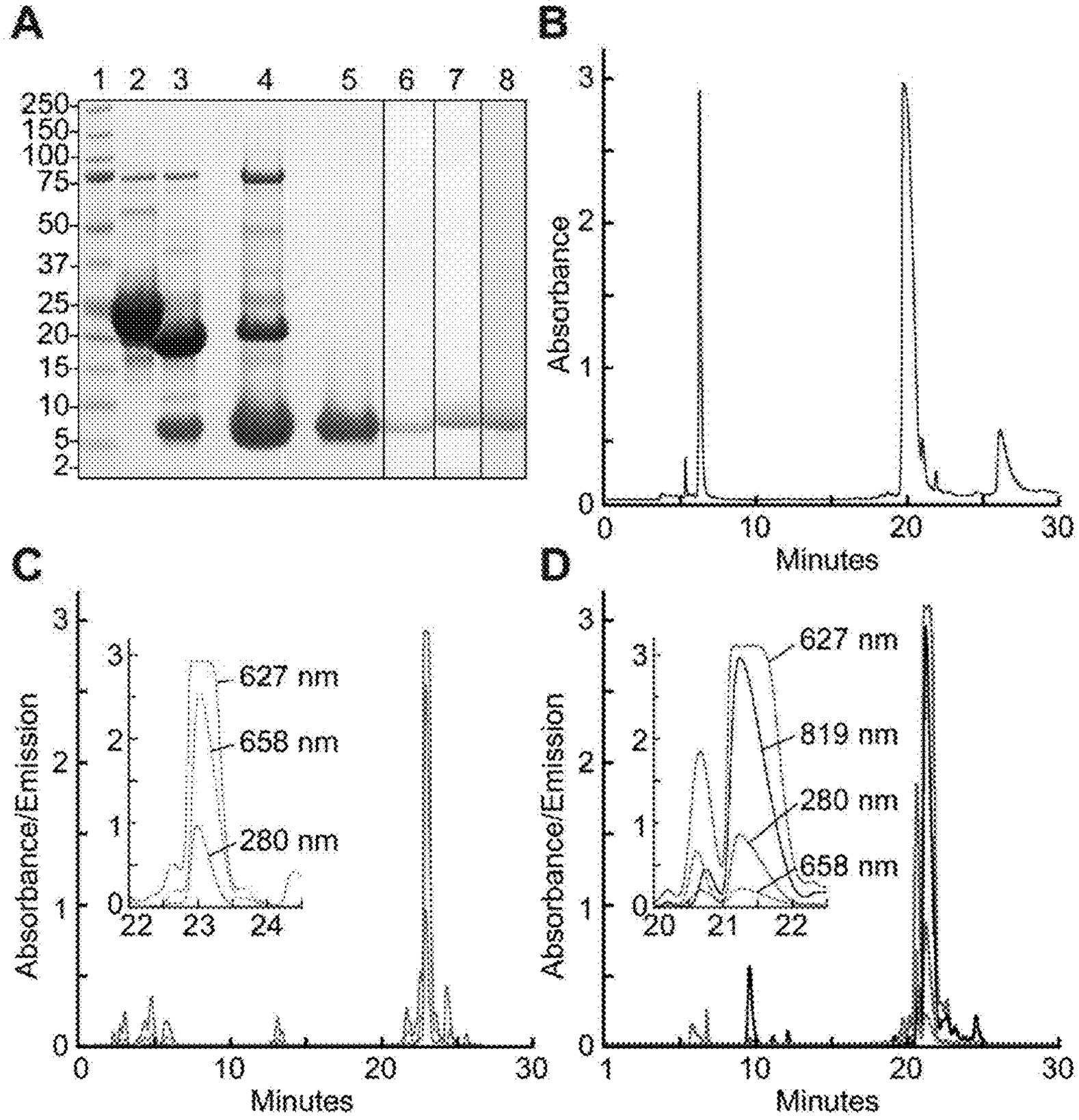

A
1 2 3 4 5 6 7 8
250
150
100
75
50
37
25
20
15
10
5
2
B
Absorbance
Minutes
C
Absorbance/Emission
627 nm
658 nm
280 nm
Minutes
D
Absorbance/Emission
627 nm
819 nm
280 nm
658 nm
Minutes

FIG. 4

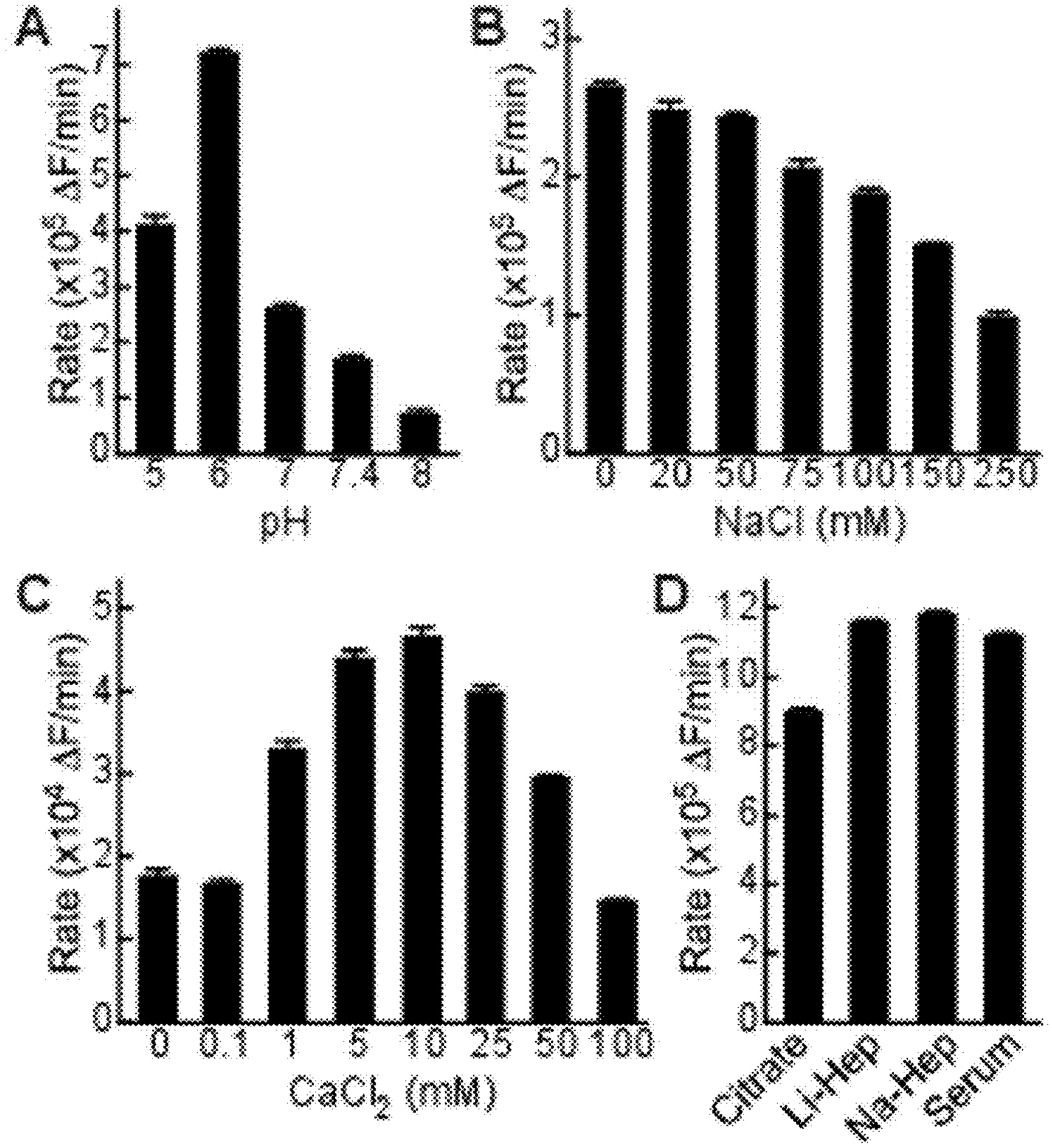

A
Rate (x10³ ΔF/min)
0
1
2
3
4
5
6
7
5
6
7
7.4
8
pH
B
Rate (x10³ ΔF/min)
0
1
2
3
0
20
50
75
100
150
250
NaCl (mM)
C
Rate (x10⁴ ΔF/min)
0
1
2
3
4
5
0
0.1
1
5
10
25
50
100
CaCl2 (mM)
D
Rate (x10³ ΔF/min)
0
2
4
6
8
10
12
Citrate
Li-Hep
Na-Hep
Serum

FIG. 5

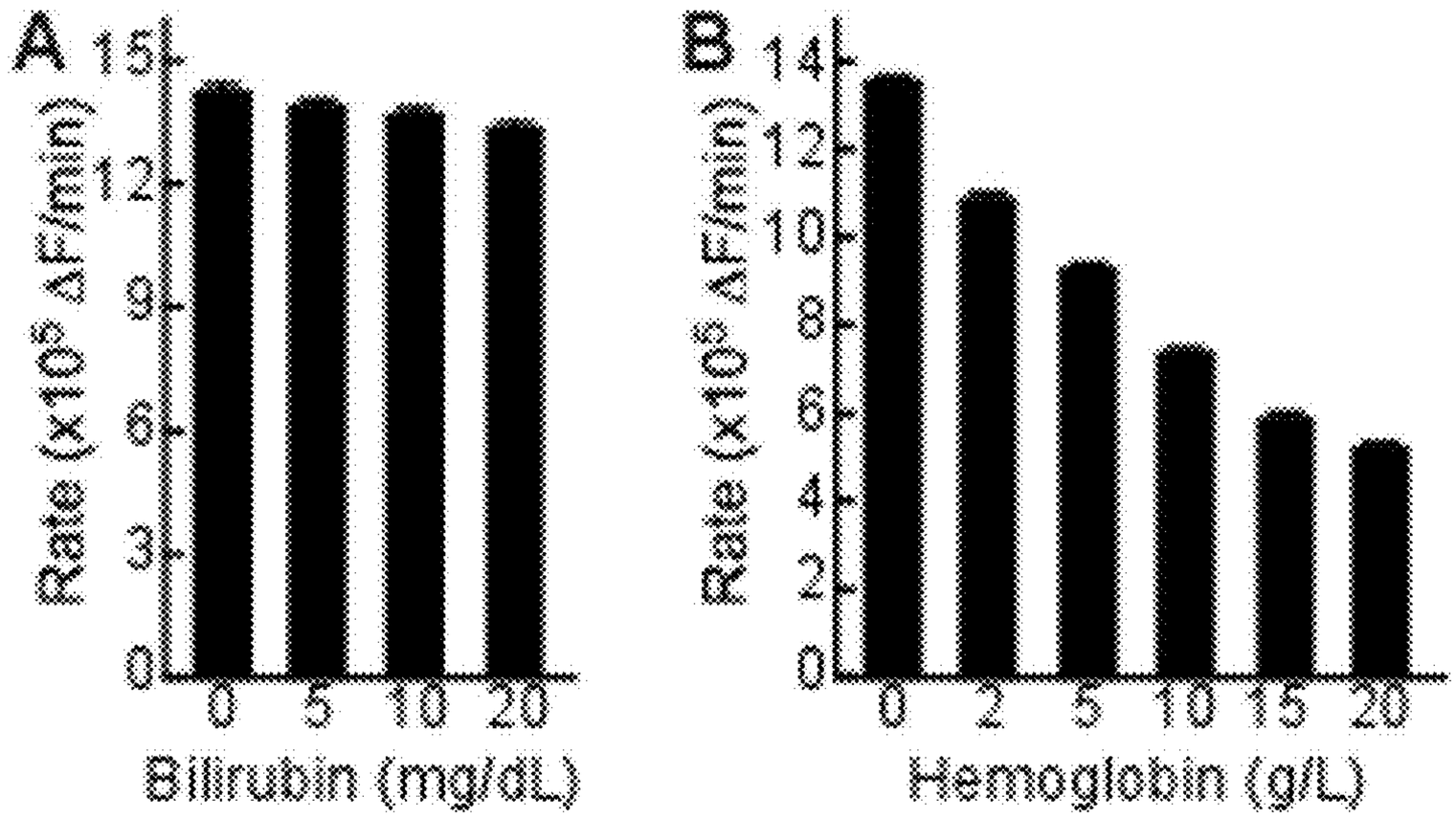

A
Rate (x10⁵ ΔF/min)
0
3
6
9
12
15
0
5
10
20
Bilirubin (mg/dL)
B
Rate (x10⁵ ΔF/min)
0
2
4
6
8
10
12
14
0
2
5
10
15
20
Hemoglobin (g/L)

FIG. 6

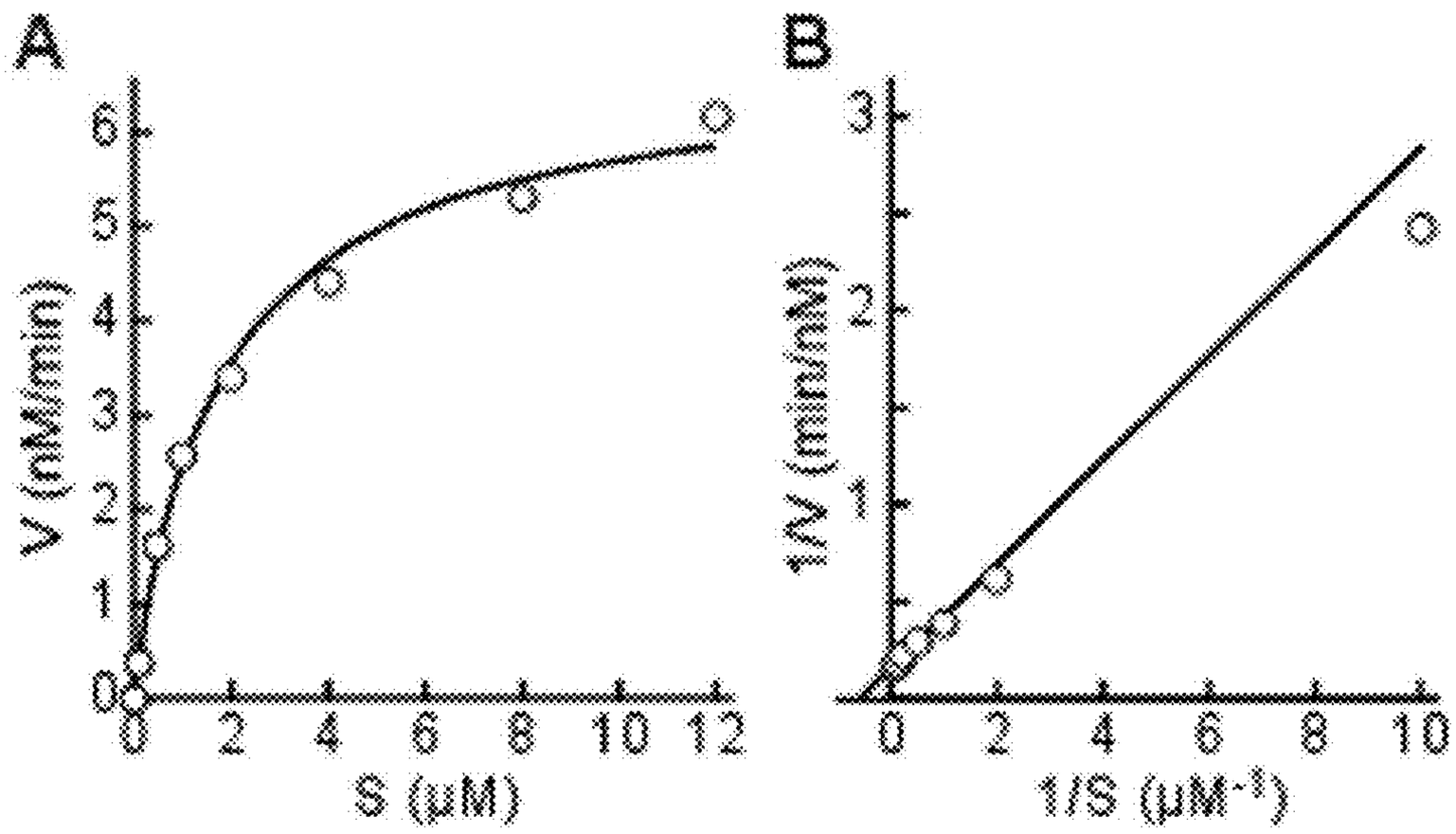

A
V (nM/min)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
S (μM)
B
1/V (min/nM)
0
1
2
3
0
2
4
6
8
10
1/S (μM-1)

FIG. 7

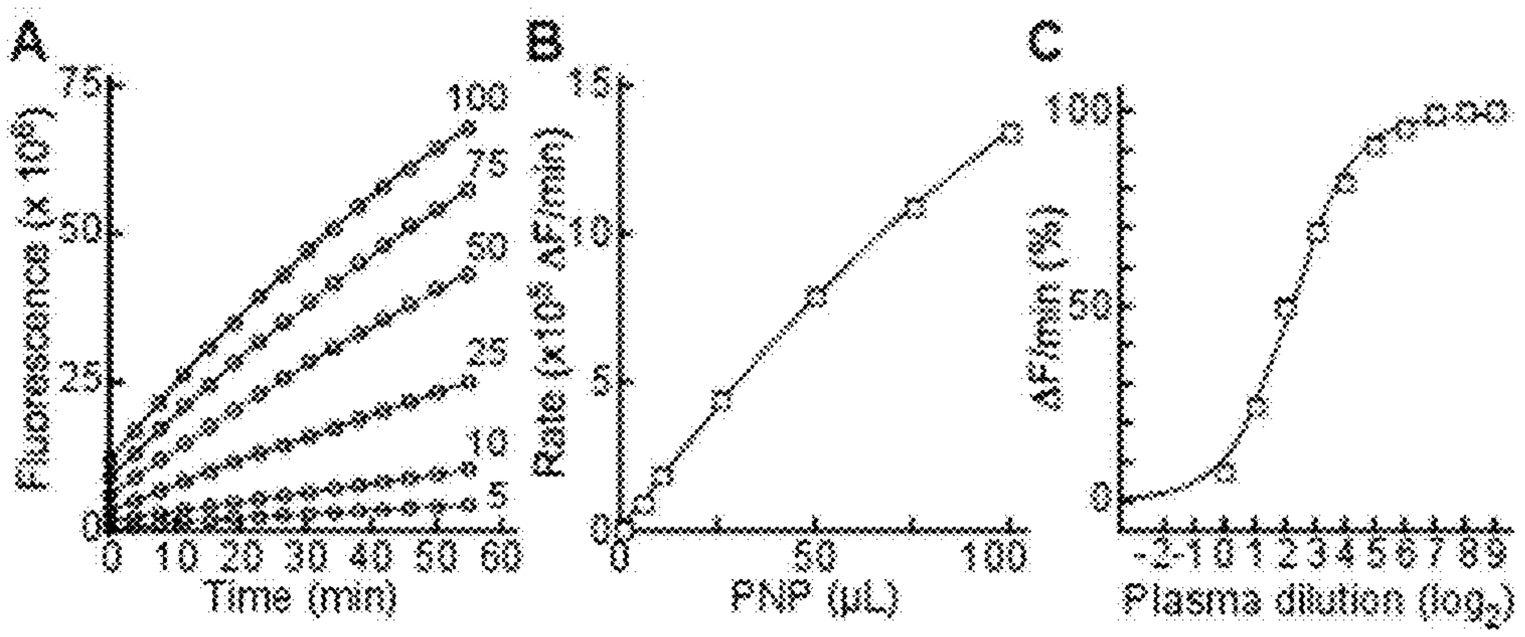
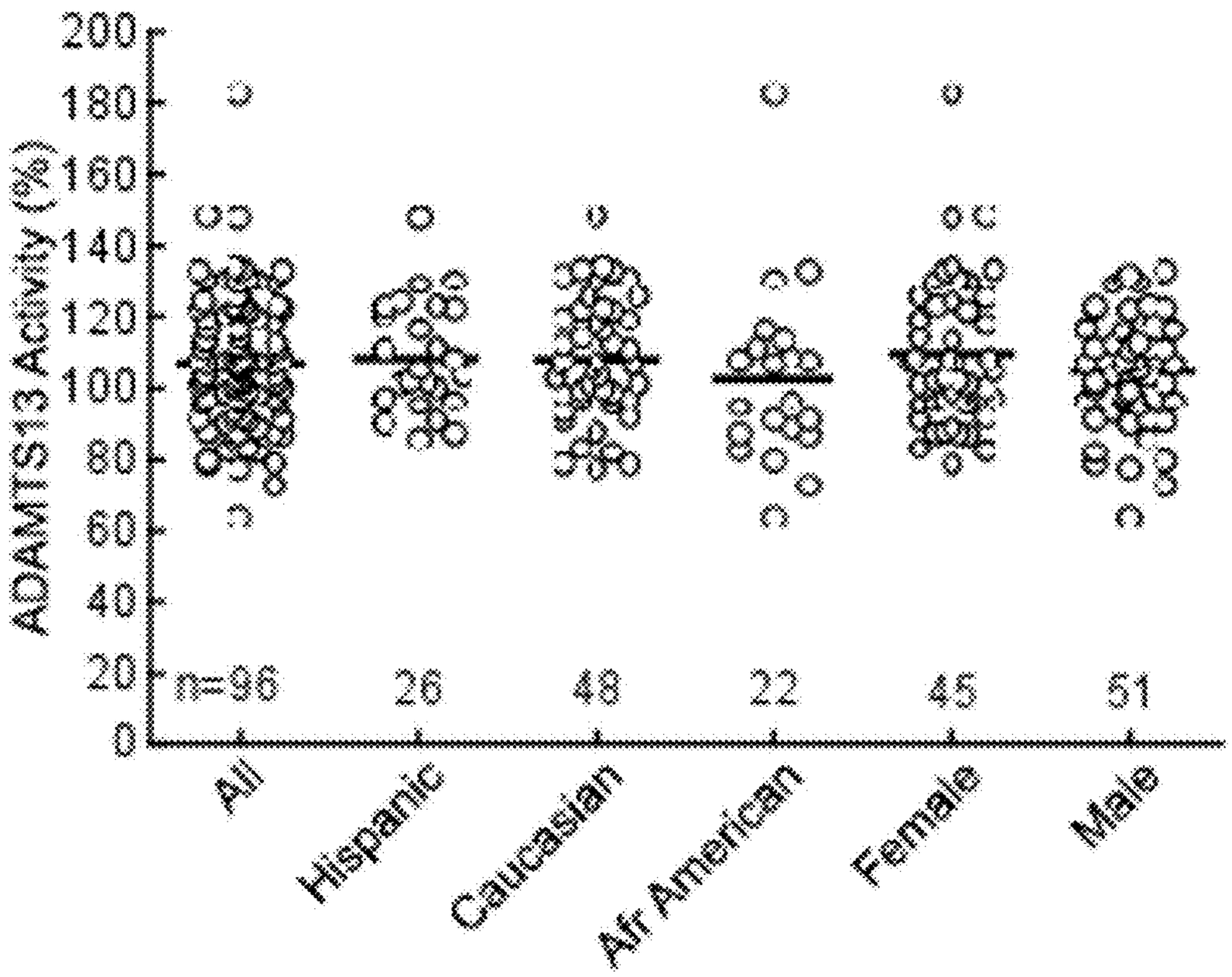

A
B
C
Fluorescence (x 10⁶)
75
50
25
0
100
75
50
25
10
5
0 10 20 30 40 50 60
Time (min)
Rate (x10⁵ AF/min)
15
10
5
0
0
50
100
PNP (µL)
AF/min (%)
100
50
0
-2-1 0 1 2 3 4 5 6 7 8 9
Plasma dilution (log2)

FIG. 8


ADAMTS13 Activity (%)
200
180
160
140
120
100
80
60
40
20
0
n=96
26
48
22
45
51
All
Hispanic
Caucasian
Afr American
Female
Male

FLUOROGENIC SUBSTRATE FOR ADAMTS13

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Ser. No. 61/508,782 filed on Jul. 18, 2011, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grants R01 HL072917-08 and R01 HL089746-04 awarded by the National Institutes of Health. The Government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN COMPUTER READABLE FORM

[0003] The Sequence Listing, which is a part of the present disclosure, includes a computer readable form. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety. The information recorded in computer readable form is identical to the written sequence listing.

INTRODUCTION

[0004] ADAMTS13 is a metalloprotease that cleaves von Willebrand Factor (VWF) and inhibits the growth of platelet thrombi. ADAMTS13 is a normal component of blood. In some disease states, such as, for example idiopathic thrombotic thrombocytopenic purpura (idiopathic TTP), levels of ADAMTS13 levels can be below normal.

[0005] Several studies suggest that ADAMTS13 activity and inhibitor assays can provide useful prognostic information. Measurement of ADAMTS13 activity in blood, plasma or serum can be useful, for example to identify patients with severe ADAMTS13 deficiency (Tsai, H-M.; Blood. 87:4235-4244, 1996; Downes, K. A., et al., J. Clin. Apheresis. 19:86-89, 2004), or with confounding potential causes of thrombotic microangiopathy such as stem cell (Adachi, T., et al., Int. J. Hematol. 83:415-419, 2006) or solid organ transplants (Pham, P. T., et al., Transplantation. 74:1077-1080, 2002; Mal, H., et al., Transplantation. 81:1628-1632, 2006), systemic lupus erythematosis (Ahmed, S., et al., Am J. Med. 116:786-787, 2004; Rick, M. E., et al., Am. J. Hematol. 75:96-100, 2004; Fujisaki, K., et al., Clin Nephrol. 64:305-310, 2005; Coppo, P., et al., Medicine. 83:233-244, 2004), preeclampsia or HELLP syndrome (Lattuada, A et al., Haematologica. 88:1029-1034, 2003; George, J. N., Curr. Opin. Hematol. 10:339-344, 2003). In such situations, ADAMTS13 testing can facilitate diagnosis and expedite treatment.

[0006] However, the clinical use of ADAMTS13 data requires assays that are rapid, robust, and feasible for most hospital laboratories. Available assays for ADAMTS13 activity can be technically challenging, slow to perform, insensitive to inhibitors, and difficult to automate (Furlan, M., et al., Blood. 87:4223-4234, 1996; Gerritsen, H. E., et al., Thromb Haemost. 82:1386-1369, 1999).

[0007] FRETS-VWF73 is a fluorogenic ADAMTS13 substrate that corresponds to VWF Asp^{1596}-Arg^{1668} (73 residues), replacing Gln^{1599} with 2,3-diaminopropionic acid (A2pr) linked to N-methyl anthranilate (Nma), and Asn^{1610} with A2pr-2,4-dinitrophenyl (Dnp) (Kokame, K., et al., Br. J. Haematol. 129:93-100, 2005). In FRETS-VWF73, Gln^{1599} is replaced with A2pr (Nma), and Asn^{1610} is replaced with A2pr (Dnp). Nma absorbs at 340 nm and emits at 440 nm; nearby Dnp quenches this fluorescence. When the Tyr^{1605}-Met^{1606} bond is cleaved, quenching is relieved, producing a fluorescence signal proportional to product concentration. FRETS-VWF73 assays require <1 hour and have been adapted to measure inhibitors. Results with FRETS-VWF73 and multimeric VWF substrates are generally congruent (Kokame, K., et al., Br. J. Haematol. 129:93-100, 2005; Kremer Hoving a, J. A., et al., J. Thromb. Haemost. 4:1146-1148, 2006; Groot, E., et al., J. Thromb. Haemost. 4:698-699, 2006; Tripodi, A., et al., J. Thromb. Haemost. 6:1534-1541, 2008; Peyvandi, F., et al., J. Thromb. Haemost. 8:631-640, 2019).

[0008] Despite these attributes, FRETS-VWF73 has several limitations. For example, it is chemically synthesized, and expensive (~$10/data point). Furthermore, assay conditions are not physiological (pH 6.0, low ionic strength) (Kokame, K., et al., Br. J. Haematol. 129:93-100, 2005). Nma absorption at 340 nm and emission at 450 nm make assays susceptible to interference from autofluorescence, absorbance and quenching by plasma proteins such as hemoglobin and bilirubin (Meyer, S. C., et al., J. Thromb. Haemost. 5:866-867, 2007). To avoid such problems, in these methods plasma is diluted ≧1:20 in ADAMTS13 assays using FRETS-VWF73 substrate. This dilution limits assay sensitivity to ~3% of normal ADAMTS13 levels, prevents the detection of some inhibitors, and can overestimate ADAMTS13 activity for patients with low titer inhibitors (Kremer Hoving a, J. A., et al., Blood. 115:1500-1511, 2010).

[0009] Current assays of ADAMTS13 involve dilutions of ≧1:20 and cannot detect inhibitors with low affinity and rapid dissociation kinetics. Alternatively, ELISA methods are used that measure antibody bound to immobilized ADAMTS13 and results are reported as "μg antibody/mL," analogous to assays for anti-PF4 antibodies. However, this approach cannot distinguish inhibitory from noninhibitory antibodies that may have no biological significance.

[0010] United States Patent Application 20100240050 of Bhatia, S. N., et al., entitled "Methods and Products For In Vivo Enzyme Profiling." discloses a "pro-diagnostic reagent" comprising "a carrier domain linked to an enzyme susceptible domain which is linked to a signature molecule." A "signature molecule" can be a peptide, nucleic acid, small molecule, fluorophore/quencher, carbohydrate, or particle, such as a peptide comprising a fluorophore and a quencher. However, the carrier domain comprises a particle, for example, a microparticle or a nanoparticle. Although this publication lists ADAMTS13 and VWF as an enzyme and substrate, none of the peptide sequences disclosed in this application appear to derive from VWF or could be used as a target substrate for ADAMTS13.

[0011] U.S. Pat. Nos. 7,468,258 and 7,927,864 to Owen disclose a peptide consisting of a sequence from VWF which further comprises self-quenching fluorophores. This fluorogenic ADAMTS13 substrate relies on the autoquenching properties of the disclosed paired fluorophores as an alternative to fluorophore-quencher combinations.

[0012] However, there is an unmet need for an ADAMSTI3 substrate that can be used with minimal sample dilution.

SUMMARY

[0013] The present inventors have developed fluorogenic substrates which can be used to measure ADAMTS13 activity as well as ADAMTS13 inhibitor activity. An ADAMTS13

substrate of the present teachings comprises an oligopeptide. In some embodiments, an oligopeptide of the present teachings can be encoded in a vector and expressed in a cell or a microorganism such as *E. coli*. An ADAMTS13 substrate of the present teachings can further comprise a fluorophore and a fluorescence quencher.

[0014] In some embodiments, a fluorogenic ADAMTS13 substrate of the present teachings comprises an oligopeptide consisting of no more than 80 amino acids of sequence of von Willebrand Factor (VWF). In various embodiments, the oligopeptide can comprise a scissile tyrosine-methionine (Y-M) peptide, a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction; a C-terminal segment; a fluorophore; and a fluorescence quencher.

[0015] In some configurations, an oligopeptide of an ADAMTS13 substrate can consist of no more than 73 amino acids. In some configurations, an oligopeptide of an ADAMTS13 substrate can consist of no more than 72 amino acids. In some configurations, an oligopeptide of an ADAMTS13 substrate can consist of no more than 71 amino acids.

[0016] In some configurations, the cysteine substitution can be situated 3 amino acids from the scissile Y-M peptide. In some configurations, the cysteine substitution can be a N1610C substitution of a VWF sequence.

[0017] In some configurations, an oligopeptide can comprise a substitution of lysine K 1617 for an amino acid that does not comprise a primary amine on its side chain that can react with amine-reactive reagents (such as a succinimidyl ester or an isothiocyanate). In some configurations, an oligopeptide can comprise a K1617R substitution.

[0018] In some configurations, an oligopeptide can comprise a substitution of glutamic acid E1798 of VWF with glycine, so that the oligopeptide comprises a glycine at its amino terminal.

[0019] In some configurations, a probe can comprise an oligopeptide consisting of a sequence set forth as SEQ ID NO: 1:

```
DREQAPNLVYMVTGCPASDEIKRLPGDIQVVPIGVGPNANVQELERIGW
PNAPILIQDFETLPREAPDLVLQR.
```

[0020] In some configurations, a probe can comprise an oligopeptide consisting of a sequence set forth as SEQ ID NO: 2:

```
GQAPNLVYMVTGCPASDEIRRLPGDIQVVPIGVGPNANVQELERIGWPN
APILIQDFETLPREAPDLVLQR.
```

[0021] In various aspects, an ADAMTS13 substrate of the present teachings can comprise a fluorophore and a fluorescence quencher situated on opposite sides of a scissile Y-M peptide. In various configurations, a fluorophore can have an absorption maximum of >550 nm or >630 nm.

[0022] In various aspects, a fluorophore can have an emission maximum of >600 nm or >650 nm.

[0023] In some aspects, a fluorophore can comprise at least one sulfate.

[0024] In some aspects, a fluorophore can be an Alexa Fluor® 594 maleimide (Life Technologies Corporation, Carlsbad, Calif.) or a DyLight® 633 maleimide (Thermo Fisher Scientific, Rockford, Ill.).

[0025] In various aspects, a quencher can have an absorption maximum of >550 nm or >630 nm.

[0026] In some aspects, a quencher can comprise at least one sulfate.

[0027] In some aspects, a quencher can be a QSY21-succinimidyl ester or an IRDye QC-1 N-hydroxy succinimidyl ester.

[0028] In some aspects, a probe can be soluble in water at >50 μM.

[0029] In some aspects, a probe can be soluble in water at >200 μM.

[0030] In some embodiments, the present teachings include a vector comprising a nucleic acid sequence encoding an oligopeptide of an ADAMTS13 substrate described herein.

[0031] In some aspects, a nucleic acid sequence can further encode an N-terminal His tag.

[0032] In some aspects, a nucleic acid sequence can further comprise a sequence encoding thioredoxin.

[0033] In some aspects, a nucleic acid sequence can further comprise a sequence encoding an oligopeptide further comprises a sequence encoding a Tobacco Etch Virus (TEV) protease cleavage site.

[0034] In some aspects, a vector can be a plasmid or a virus, such as a bacteriophage.

[0035] In some embodiments, the present teachings include methods of determining presence, absence or quantity of ADAMTS13 activity in a sample.

[0036] In some embodiments, the inventors disclose assays for detecting and quantifying activity of inhibitors of ADAMTS13. In some configurations, an assay of the present teachings can be substantially more sensitive than alternative existing assays for detecting inhibitors of ADAMTS13.

[0037] In various configurations, methods of the present teachings can comprise: forming a mixture comprising a sample and a probe as described herein; and measuring fluorescence at one or more time points after forming the mixture.

[0038] In some configurations, the sample can be a blood sample, a serum sample, or a plasma sample.

[0039] In some configurations, the sample can be undiluted or diluted less than 20-fold in an assay of ADAMTS13 activity or ADAMTS13 inhibitor activity. In some configurations, the sample can be diluted less than 2-fold. In some configurations, the sample can be heparinized plasma. In some configurations, the sample can be citrated plasma. In some configurations, the sample can be serum. In some configurations, the sample can be plasma anticoagulated with any protease inhibitor that does not interfere with ADAMTS13 activity.

[0040] In some aspects, an ADAMTS13 assay or ADAMTS13 inhibitor assay using a probe of the present teachings can be insensitive to >20 mg/dL (250 μM) conjugated bilirubin.

[0041] In some configurations, sensitivity of assays for inhibitors of ADAMTS13 activity can be increased by using recombinant ADAMTS13 (including concentrated recombinant ADAMTS13) as the enzyme source instead of pooled normal plasma.

[0042] In some embodiments, the present teachings include methods of producing a probe described herein.

[0043] In some configurations, these methods can comprise expressing, in a cell, an oligopeptide encoded by a vector described herein. Alternatively, an oligopeptide can be generated by chemical means such as, for example but not limited

to, the solid-phase method of Merrifield, R., J. Am. Chem. Soc. 85: 2149-2154, 1963. In various configurations, an oligopeptide can be digested with His-tagged TEV protease to yield an oligopeptide comprising VWF sequence comprising a cysteine as described herein. In various configurations, an oligopeptide comprising a cysteine can be reacted with a fluorophore comprising a maleimide. In various configurations, an oligopeptide can comprise a primary amine such as the N-terminal primary amine which can be reacted with a fluorescence quencher comprising a succinimidyl ester or other amine-reactive moiety such as, without limitation, an isothiocyanate.

[0044] Alternatively, an oligopeptide can be generated by chemical means such as, for example but not limited to, the solid-phase method of Merrifield, R., J. Am. Chem. Soc. 85: 2149-2154, 1963. In various configurations, an oligopeptide can be digested with His-tagged TEV protease to yield an oligopeptide comprising VWF sequence comprising a cysteine as described herein. In various configurations, an oligopeptide comprising a cysteine can be reacted with a fluorescence quencher comprising a maleimide. In various configurations, an oligopeptide can comprise a primary amine such as the N-terminal primary amine which can be reacted with a fluorophore comprising a succinimidyl ester or other amine-reactive moiety such as, without limitation, an isothiocyanate.

The present disclosure includes the following aspects, without limitation:

1. A probe comprising:

[0045] an oligopeptide consisting of no more than 80 amino acids of sequence of von Willebrand Factor (VWF), said oligopeptide comprising a scissile Y-M peptide, a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction, and a C-terminal segment;

[0046] a fluorophore; and

[0047] a fluorescence quencher,

wherein the fluorophore and the fluorescence quencher are bound to the oligopeptide on opposite sides of the scissile Y-M peptide.

2. A probe in accordance with aspect 1, wherein the oligopeptide consists of no more than 73 amino acids.

3. A probe in accordance with aspect 1, wherein the oligopeptide consists of no more than 71 amino acids.

4. A probe in accordance with aspect 1, wherein the cysteine substitution is situated 3 amino acids from the scissile Y-M peptide.

5. A probe in accordance with aspect 1, wherein the cysteine substitution is a N1610C substitution of a VWF sequence.

6. A probe in accordance with aspect 1, wherein the oligopeptide comprises a substitution of lysine K1617 for an amino acid that does not comprise a primary amine on its side chain.

7. A probe in accordance with aspect 1, comprising a K1617R substitution.

8. A probe in accordance with aspect 1, wherein the oligopeptide consists of the sequence set forth as SEQ ID NO: 2,

GQAPNLVYMVTGCPASDEIRRLPGDIQVVPIGVGPNANVQELERIGWPN

APILIQDFETLPREAPDLVLQR.

9. A probe in accordance with aspect 1, wherein the fluorophore has an absorption maximum >550 nm.

10. A probe in accordance with aspect 1, wherein the fluorophore has an emission maximum >600 nm.

11. A probe in accordance with aspect 1, wherein the fluorophore has an absorption maximum >630 nm.

12. A probe in accordance with aspect 1, wherein the fluorophore has an emission maximum >650 nm.

13. A probe in accordance with aspect 1, wherein the fluorophore comprises at least one sulfate.

14. A probe in accordance with aspect 1, wherein the fluorophore is Alexa Fluor 594 maleimide.

15. A probe in accordance with aspect 1, wherein the fluorophore is DyLight 633 maleimide.

16. A probe in accordance with aspect 1, wherein the quencher has an absorption maximum >550 nm.

17. A probe in accordance with aspect 1, wherein the quencher has an absorption maximum >630 nm.

18. A probe in accordance with aspect 1, wherein the quencher comprises at least one sulfate.

19. A probe in accordance with aspect 1, wherein the quencher is QSY21-succinimidyl ester.

20. A probe in accordance with aspect 1, wherein the quencher is IRDye QC-1 N-hydroxy succinimidyl ester.

21. A probe in accordance with aspect 1, wherein the quencher is attached to the oligopeptide at the amino terminal of the oligopeptide.

22. A probe in accordance with aspect 1, wherein the fluorophore is attached to the oligopeptide at the cysteine.

23. A probe in accordance with aspect 1, wherein the probe is soluble in water at >50 μM.

24. A vector comprising a nucleic acid sequence encoding an oligopeptide of aspect 1.

25. A vector in accordance with aspect 24, further comprising a nucleic acid sequence encoding an N-terminal His tag.

26. A vector in accordance with aspect 24, wherein the nucleic acid sequence encoding an oligopeptide further comprises a sequence encoding thioredoxin.

27. A vector in accordance with aspect 24, wherein the nucleic acid sequence encoding an oligopeptide further comprises a sequence encoding a Tobacco Etch Virus (TEV) protease cleavage site.

28. A vector in accordance with aspect 24, wherein the vector is a plasmid.

29. A method of determining presence, absence or quantity of ADAMTS13 activity in a sample, comprising:

[0048] forming a mixture comprising a sample and a probe of aspect 1; and

[0049] measuring fluorescence at one or more time points after forming the mixture,

wherein the sample is diluted less than 20-fold.

30. A method in accordance with aspect 29, wherein the sample is a serum sample.

31. A method in accordance with aspect 30, wherein the serum sample is an undiluted serum sample.

32. A method in accordance with aspect 30, wherein the serum sample is a concentrated serum sample.

33. A method in accordance with aspect 29, wherein the sample is a plasma sample.

34. A method in accordance with aspect 33, wherein the plasma sample is an undiluted plasma sample.

35. A method in accordance with aspect 33, wherein the plasma sample is a concentrated plasma sample.

36. A method of determining presence, absence or quantity of ADAMTS13 inhibitor activity in a sample, comprising:

[0050] forming a mixture comprising a sample, a source of ADAMTS13, and a probe of aspect 1; and

[0051] measuring fluorescence at one or more time points after forming the mixture.

37. A method in accordance with aspect 36, further comprising inactivating ADAMTS13 activity endogenous to the sample prior to forming the mixture.
38. A method in accordance with aspect 37, wherein the inactivating ADAMTS13 activity comprises heating the sample.
39. A method in accordance with aspect 38, wherein the heating the sample comprises heating the sample to about 56° C. for about 30 min.
40. A method in accordance with aspect 36, wherein the source of ADAMTS13 is selected from the group consisting of normal plasma, recombinant ADAMTS13, and a combination thereof.
41. A method in accordance with aspect 36, wherein the sample is a serum sample.
42. A method in accordance with aspect 41, wherein the serum sample is an undiluted serum sample.
43. A method in accordance with aspect 41, wherein the serum sample is a concentrated serum sample.
44. A method in accordance with aspect 36, wherein the sample is a plasma sample.
45. A method in accordance with aspect 44, wherein the plasma sample is an undiluted plasma sample.
46. A method in accordance with aspect 44, wherein the plasma sample is a concentrated plasma sample.
47. A method of producing a probe of aspect 1, comprising:
[0052] expressing in a cell, an oligopeptide encoded by the vector of aspect 24;
[0053] digesting the oligopeptide with His-tagged TEV protease to yield an oligopeptide comprising VWF sequence comprising a cysteine of aspect 5; and
[0054] reacting the cysteine with a fluorophore comprising a maleimide, and reacting a primary amine of the oligopeptide such as the N-terminal primary amine with a fluorescence quencher comprising a succinimidyl ester.

BRIEF DESCRIPTION OF THE DRAWINGS

[0055] FIG. **1** illustrates an optimized fluorogenic ADAMTS13 substrate.
[0056] FIG. **2** illustrates FRETS-rVWF71 assays.
[0057] FIG. **3** illustrates preparation of FRETS-rVWF71.
[0058] FIG. **4** illustrates ADAMTS13 activity toward FRETS-rVWF71.
[0059] FIG. **5** illustrates effect of bilirubin and hemoglobin on FRETS-rVWF71.
[0060] FIG. **6** illustrates kinetics of FRETS-rVWF71 cleavage by plasma ADAMSTI3.
[0061] FIG. **7** illustrates plasma concentration dependence of FRETS-rVWF71 assays.
[0062] FIG. **8** illustrates plasma ADAMTS13 activity of healthy controls.

DETAILED DESCRIPTION

[0063] The present inventors have developed fluorogenic substrates which can be used to measure ADAMTS13 activity. An ADAMTS13 substrate of the present teachings comprises an oligopeptide which can be encoded in a vector and expressed in a cell or a microorganism such as *E. coli*. ADAMTS13 substrate of the present teachings can further comprise a fluorophore and fluorescence quencher.
[0064] The present inventors have shown that the substrate, FRETS-rVWF71, can be cleaved by ADAMTS13 and that it can be used as an ADAMTS13 substrate without significant interference in undiluted serum or plasma. Contact between a probe of the present teachings and ADAMTS13 can lead to cleavage of the probe which can result in a positive signal, i.e., an increase in fluorescence. In various configurations, the increase in fluorescence can be quantified and can serve as a measure of ADAMTS13 activity comprised by a sample. Furthermore, the quantifiable fluorescence signal can be used to measure ADAMTS13 inhibitor activity in a sample.
[0065] In various configurations, FRETS-rVWF71 allows ADAMTS13 activity and ADAMTS13 inhibitor activity assays to be performed in undiluted plasma or serum. In various configurations of the present teachings, an assay using FRETS-rVWF71 can be approximately 8-fold more sensitive than an assay using FRETS-VWF73, when using equal concentrations of plasma as the enzyme source. In various configurations, FRETS-rVWF71 can be used in undiluted plasma, which can increase sensitivity another 30-fold relative to FRETS-VWF73, or 240-fold overall. Because the substrate can be used in undiluted plasma, it can be used to detect ADAMTS13 inhibitors that have not been possible to detect with FRETS-VWF73.
[0066] In some embodiments, the inventors disclose assays for detecting and quantifying activity of inhibitors of ADAMTS13. In some configurations, an assay of the present teachings can be substantially more sensitive than alternative assays for detecting inhibitors of ADAMTS13. In some configurations, an assay of the present teachings can detect inhibitor activity at <0.5 "Bethesda-like Units" (BU) per mL. For example, a sample from a subject with TTP and undetectable ADAMTS13 activity, and in which a FRETS-VWF73-based assay reported an inhibitor titer ≦1 U/ml. The same sample tested with a FRETS-rVWF71-based assay of the present teachings revealed an inhibitor titer of 6 U/ml.
[0067] In some configurations, sensitivity to inhibitors can be increased by using concentrated recombinant ADAMTS13 as the enzyme source instead of pooled normal plasma.
[0068] In some embodiments, an ADAMTS13 substrate of the present teachings can be a probe comprising an oligopeptide consisting of no more than 80 amino acids of sequence of von Willebrand Factor (VWF), wherein said oligopeptide comprises a scissile Y-M peptide, a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction, and a C-terminal segment; a fluorophore; and a fluorescence quencher. In some configurations, an oligopeptide of an ADAMTS13 substrate can consist of no more than 73 amino acids. In some configurations, an oligopeptide of an ADAMTS13 substrate can consist of no more than 71 amino acids.
[0069] In some configurations, the cysteine substitution can be situated 3 amino acids from the scissile Y-M peptide. In some configuration s, the cysteine substitution can be a N1610C substitution of a VWF sequence, wherein the entire sequence of human VWF is:

(Genbank GenBank: AAB59458.1).

(SEQ ID NO: 3)

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  1  miparfagvl lalalilpgt lcaegtrgrs starcslfgs dfvntfdgsm ysfagycsyl

 61  laggcqkrsf siigdfqngk rvslsvylge ffdihlfvng tvtqgdqrvs mpyaskglyl

121  eteagyykls geaygfvari dgsgnfqvll sdryfnktcg lcgnfnifae ddfmtqegtl
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181 tsdpydfans walssgeqwc erasppsssc nissgemqkg lweqcqllks tsvfarchpl
241 vdpepfvalc ektlcecagg lecacpalle yartcaqegm vlygwtdhsa cspvcpagme
301 yrqcvspcar tcqslhinem cqercvdgcs cpegqlldeg lcvestecpc vhsgkryppg
361 tslsrdcntc icrnsqwics neecpgeclv tgqshfksfd nryftfsgic qyllardcqd
421 hsfsivietv qcaddrdavc trsvtvrlpg lhnslvklkh gagvamdgqd iqlpllkgdl
481 riqhtvtasv rlsygedlqm dwdgrgrllv klspvyagkt cglcgnyngn qgddfltpsg
541 laeprvedfg nawklhgdcq dlqkqhsdpc alnprmtrfs eeacavltsp tfeachravs
601 plpylrncry dvcscsdgre clcgalasya aacagrgvrv awrepgrcel ncpkgqvylq
661 cgtpcnltcr slsypdeecn eaclegcfcp pglymdergd cvpkaqcpcy ydgeifqped
721 ifsdhhtmcy cedgfmhctm sgvpgsllpd avlssplshr skrslscrpp mvklvcpadn
781 lraeglectk tcqnydlecm smgcvsgclc ppgmvrhenr cvalercpcf hqgkeyapge
841 tvkigcntcv crdrkwnctd hvcdatcsti gmahyltfdg lkylfpgecq yvlvqdycgs
901 npgtfrilvg nkgcshpsvk ckkrvtilve ggeielfdge vnvkrpmkde thfevvesgr
961 yiilllgkal svvwdrhlsi svvlkqtyqe kvcglcgnfd giqnndltss nlqveedpvd
1021 fgnswkvssq cadtrkvpld sspatchnni mkqtmvdssc riltsdvfqd cnklvdpepy
1081 ldvciydtcs cesigdcacf cdtiaayahv caqhgkvvtw rtaticpqsc eernlrengy
1141 ecewrynsca pacqvtcqhp eplacpvqcv egchahcppg kildellqtc vdpedcpvce
1201 vagrrfasgk kvtlnpsdpe hcqichcdvv nltceacqep gglvvpptda pvspttlyve
1261 disepplhdf ycsrlldlvf lldgssrlse aefevlkafv vdmmerlris qkwvrvavve
1321 yhdgshayig lkdrkrpsel rriasqvkya gsqvastsev lkytlfqifs kidrpeasri
1381 alllmasqep qrmsrnfvry vqglkkkkvi vipvgigpha nlkqirliek qapenkafvl
1441 ssvdeleqqr deivsylcdl apeappptlp phmaqvtvgp gllgvstlgp krnsmvldva
1501 fvlegsdkig eadfnrskef meeviqrmdv gqdsihvtvl qysymvtvey pfseaqskgd
1561 ilqrvreiry qggnrtntgl alrylsdhsf lvsqgdreqa pnlvymvtgn pasdeikrlp
1621 gdiqvvpigv gpnanvqele rigwpnapil iqdfetlpre apdlvlqrcc sgeglqiptl
1681 spapdcsqpl dvillldgss sfpasyfdem ksfakafisk anigprltqv svlqygsitt
1741 idvpwnvvpe kahllslvdv mqreggpsqi gdalgfavry ltsemhgarp gaskavvilv
1801 tdvsvdsvda aadaarsnrv tvfpigigdr ydaaqlrila gpagdsnvvk lqriedlptm
1861 vtlgnsflhk lcsgfvricm dedgnekrpg dvwtlpdqch tvtcqpdgqt llkshrvncd
1921 rglrpscpns qspvkveetc gcrwtcpcvc tgsstrhivt fdgqnfkltg scsyvlfqnk
1981 eqdlevilhn gacspgarqg cmksievkhs alsvelhsdm evtvngrlvs vpyvggnmev
2041 nvygaimhev rfnhlghift ftpqnnefql qlspktfask tyglcgicde ngandfmlrd
2101 gtvttdwktl vqewtvqrpg qtcqpileeq clvpdsshcq vlllplfaec hkvlapatfy
2161 aicqqdschq eqvceviasy ahlcrtngvc vdwrtpdfca mscppslvyn hcehgcprhc
2221 dgnvsscgdh psegcfcppd kvmlegscvp eeactqcige dgvqhqflea wvpdhqpcqi
2281 ctclsgrkvn cttqpcptak aptcglceva rlrqnadqcc peyecvcdpv scdlppvphc
2341 erglqptltn pgecrpnftc acrkeeckrv sppscpphrl ptlrktqccd eyecacncvn
2401 stvscplgyl astatndcgc ttttclpdkv cvhrstiypv gqfweegcdv ctctdmedav
2461 mglrvaqcsq kpcedscrsg ftyvlhegec cgrclpsace vvtgsprgds qsswksvgsq
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2521 waspenpcli necvrvkeev fiqqrnvscp qlevpvcpsg fqlscktsac cpscrcerme

2581 acmlngtvig pgktvmidvc ttcrcmvqvg visgfklecr kttcnpcplg ykeennt gec

2641 cgrclptact iqlrggqimt lkrdetlqdg cdthfckvne rgeyfwekrv tgcppfdehk

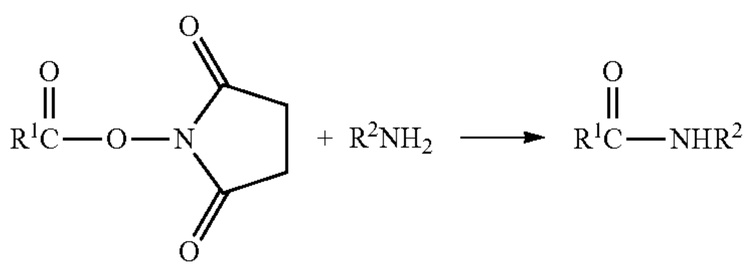
2701 claeggkimk ipgtccdtce epecnditar lqyvkvgsck sevevdihyc qgkcaskamy

2761 sidindvqdq csccsptrte pmqvalhctn gsvvyhevln ameckcsprk csk

[0070] In some aspects, an oligopeptide of the present teachings can comprise, prior to derivatization, a thiol (—SH) such as a cysteine, for example a N1610C substitution (FIG. **1**). In various configurations, a thiol such as that of a cysteine can be derivatized with a thiol-reactive moiety such as a maleimide moiety of a fluorophore or a fluorescence quencher. In some aspects, prior to derivatization, an oligopeptide of the present teachings can have a single thiol; in various configurations, this thiol can be available for derivatization.

[0071] In some aspects, an oligopeptide of the present teachings can comprise, prior to derivatization, at least one amine such as an amino terminal primary amine, or an amino acid comprising a primary amine such as a lysine. An amine of an oligopeptide can be can be available for derivatization with an amine-reactive moiety such as an N-hydroxysuccinimide (NHS) moiety of a fluorophore or a fluorescence quencher. In some aspects, prior to derivatization, an oligopeptide can have a single primary amine that is available for derivatization. In some aspects, an oligopeptide of the present teaching can include a substitution of a lysine of wild type VWF, such as a lysine K1617 for an amino acid that does not comprise a primary amine on its side chain, such as a K1617R substitution (FIG. **1**).

[0072] In some configurations, an oligopeptide can comprise a substitution of E1598G, which can provide an amino terminal end after release from a precursor. These mutations can result in an oligopeptide having one thiol and one primary amine, which can be available for addition of one fluorophore and one quencher to the oligopeptide (FIG. **1**). In various embodiments, these reactive sites can be located on opposite sides of a protease cleavage target site such as the Y-M peptide of von Willebrand Factor. In various configurations, the succinimidyl ester and the maleimide can react through the reactions below, wherein R^1 can be a fluorophore or fluorescence quencher and R^2 can be an oligopeptide.



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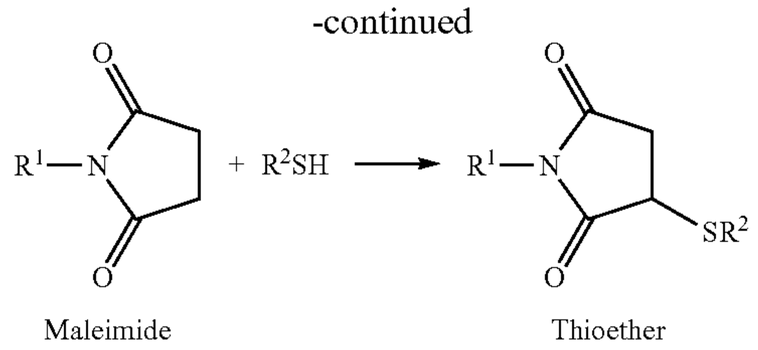


[0073] Accordingly, as used herein, oligopeptides reacted with such reactive precursors may not include a reactive moiety such as a succinimide in a final product. In various configurations of the present teachings, prior to cleavage of an oligopeptide comprising both a fluorophore and a quencher, fluorescence is quenched. When the Tyr1605-Met1606 bond is cleaved, quenching is relieved, thereby resulting in a fluorescence signal. In various configurations, the fluorescence signal can be proportional to product concentration. In various configurations, a fluorescence signal can be detected by means well known to skilled artisans, such as but not limited to fluorescence spectrometry.

[0074] In some configurations, a probe can comprise an oligopeptide consisting of a sequence set forth as SEQ ID NO: 1:

DREQAPNLVYMVTGNPASDEIKRLPGDIQVVPIGVGPNANVQELERIGW

PNAPILIQDFETLPREAPDLVLQR.

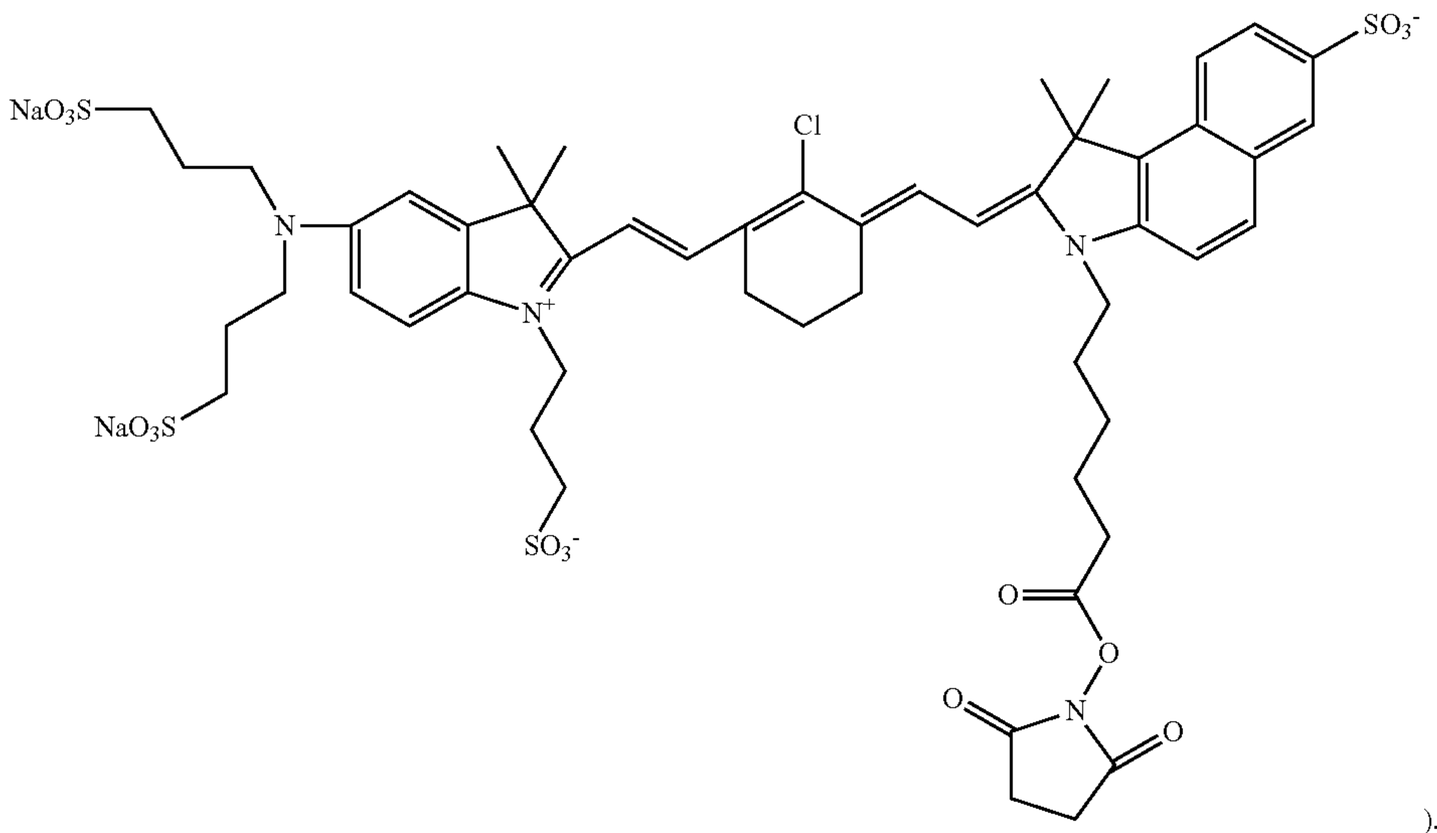
[0075] In some configurations, a probe can comprise an oligopeptide consisting of a sequence set forth as SEQ ID NO: 2 and as FRETS-rVWF71 (FIG. **1**):

GQAPNLVYMVTGCPASDEIRRLPGDIQVVPIGVGPNANVQELERIGWPN

APILIQDFETLPREAPDLVLQR.

[0076] In various aspects, a fluorophore can have an absorption maximum of >550 nm or >630 nm. In various aspects, a fluorophore can have an emission maximum of >600 nm or >650 nm. In some aspects, a fluorophore, prior to reaction with an oligopeptide, can be, without limitation, an Alexa Fluor® 594 maleimide (Life Technologies Corp. Carlsbad, Calif.) (abs 590 nm, em 617 nm, ϵ 96,000) or a DyLight® 633 maleimide (Thermo, abs 638 nm, em 658 nm, ϵ 170,000).

[0077] In various aspects, a quencher can have an absorption maximum of >550 nm or >630 nm. In some aspects, a quencher, prior to reaction with an oligopeptide, can be, without limitation, a QSY21-succinimidyl ester (abs 661 nm, ϵ 90,000) or an IRDye QC-1 N-hydroxy succinimidyl ester (LI-COR, Lincoln, Nebr.; abs 737 nm, ϵ 96,000,



).

[0078] In some aspects, a substrate of the present teachings can comprise a fluorophore or quencher that comprises at least one sulfate. In some aspects, a substrate of the present teachings can be soluble in water at concentrations >50 μM, or greater than that of FRETS-VWF73.

[0079] In some embodiments, the present teachings include a vector comprising a nucleic acid sequence encoding an oligopeptide of an ADAMTS13 substrate described herein. In some aspects, a nucleic acid sequence can further encode a His tag such as an N-terminal His tag. In some aspects, a nucleic acid sequence can further comprise a sequence encoding thioredoxin. In some aspects, a nucleic acid sequence can further comprise a sequence encoding a Tobacco Etch Virus (TEV) protease cleavage site. In some aspects, a vector can be a plasmid or a virus, such as a bacteriophage.

[0080] In some embodiments, the present teachings include methods of determining presence, absence or quantity of ADAMTS13 activity in a sample. In various configurations, such methods can comprise: forming a mixture comprising a sample and a probe as described herein and measuring fluorescence at one or more time points after forming the mixture.

[0081] In some embodiments, the present teachings include methods of determining presence, absence or quantity of ADAMTS13 inhibitor activity in a sample. In various configurations, such methods can comprise: forming a mixture comprising a sample and a probe as described herein and measuring fluorescence at one or more time points after forming the mixture. Such methods can further comprise including in the mixture ADAMTS13 which can be obtained from a source such as, without limitation, ADAMTS13 produced in a microorganism using recombinant methods, or ADAMTS13 present in serum or plasma, such as normal pooled serum or plasma.

[0082] In some embodiments, the present teachings include methods of producing a probe described herein. In some configurations, these methods can comprise expressing, in a cell, an oligopeptide encoded by the vector of described herein, digesting the oligopeptide with His-tagged TEV protease to yield an oligopeptide comprising VWF sequence comprising a cysteine as described herein and reacting the cysteine with a fluorophore comprising a maleimide, and reacting a primary amine of the oligopeptide such as the N-terminal primary amine with a fluorescence quencher comprising a succinimidyl ester.

[0083] In some embodiments, the present teachings include methods of producing a probe described herein. In some configurations, these methods can comprise expressing, in a cell, an oligopeptide encoded by the vector of described herein, digesting the oligopeptide with His-tagged TEV protease to yield an oligopeptide comprising VWF sequence comprising a cysteine as described herein and reacting the cysteine with a fluorescence quencher comprising a maleimide, and reacting a primary amine of the oligopeptide such as the N-terminal primary amine with a fluorophore comprising a succinimidyl ester or another amine-reactive moiety such as an isothiocyanate.

EXAMPLES

[0084] The following Examples are intended to be illustrative of various aspects of the present teachings and are not intended to be limiting of any aspect. While some of examples may include conclusions about the way the invention may function, the inventors do not intend to be bound by those conclusions, but put them forth only as possible explanations. Unless indicated by use of past tense, presentation of an example does not imply that an experiment or procedure was, or was not, conducted, or that results were, or were not, actually obtained.

[0085] The methods and compositions described herein utilize laboratory techniques well known to skilled artisans, and can be found in laboratory manuals such as Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Spector, D. L. et al., Cells: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998; Ausubel, F. M., et al., ed., Current Protocols in Molecular Biology, Wiley Interscience, 2003; Nagy, A., et al., Manipulating the Mouse Embryo: A Laboratory Manual (Third Edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003; Hedrickson et al., Organic Chem-

istry 3rd edition, McGraw Hill, New York, 1970; Carruthers, W., and Coldham, I., Modern Methods of Organic Synthesis (4th Edition), Cambridge University Press, Cambridge, U.K., 2004; Graham Solomons T. W., et al., Organic Chemistry 9th edition, Wiley, John & Sons, Incorporated, 2007.

[0086] As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context indicates otherwise. Methods of administration of pharmaceuticals and dosage regimes can be determined according to standard principles of pharmacology well known skilled artisans, using methods provided by standard reference texts such as Remington: the Science and Practice of Pharmacy (Alfonso R. Gennaro ed. 19th ed. 1995); Hardman, J. G., et al., Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, McGraw-Hill, 1996; and Rowe, R. C., et al., Handbook of Pharmaceutical Excipients, Fourth Edition, Pharmaceutical Press, 2003.

[0087] These and all other publications cited in this disclosure are incorporated herein by reference, each in its entirety.

Example 1

[0088] This example illustrates a substrate oligopeptide.

[0089] The inventors designed a chimeric construct in plasmid pET-32 Xa/LIC (Novagen, Billerica, Mass.) encoding an N-terminal His-tag, thioredoxin, Tobacco Etch Virus (TEV) protease cleavage site, a Gly residue, and VWF (Gln^{1599}-Arg^{1668}). Two mutations were made in the VWF segment: N1610C introduced a thiol group for alkylation, and K1617R removed an amino group that competes with the peptide N-terminus for chemical modification. This oligopeptide was expressed in IPTG-induced BL21 cells, purified on Ni-NTA-agarose, and digested with His-tagged TEV protease (made by the present inventors). TEV protease and thioredoxin were removed on Ni-NTA-agarose, and the C-terminal peptide (FIG. **1**) was purified by C18 reverse phase HPLC. >100 mg of this peptide was prepared, sufficient for thousands of assays.

[0090] In FIG. **1**, the scissile Y-M bond is indicated (triangle) and the C-terminal segment of VWF domain A2 which can promote efficient cleavage by ADAMTS13 consists of the carboxy terminal sequence EAPDLVLQR (underlined). Residues mutated in von Willebrand Disease type 2A, associated with increased proteolysis, are circled. FRETS-rVWF71 has the mutations E1598G, N1610C, and K1617R (boxed). Cys1610 is modified with DyLight 633 and the N-terminus is modified with IRDye QC-1.

Example 2

[0091] This example illustrates fluorescence donors and quenchers.

[0092] The present inventors have tested several combinations of donor and quencher groups chosen for lack of interference from blood proteins. For example, modification at Cys^{1610} with Alexa Fluor 594 maleimide (abs 590 nm, em 617 nm, ϵ 96,000) and at N-terminal. Gly with QSY21-succinimidyl ester (abs 661 nm, ϵ 90,000) gave superior fluorescence quenching and sensitivity compared to FRETS-VWF73, but this substrate had poor solubility. In one embodiment, a substrate of the present teachings is modified at Cys^{1610} with DyLight 633-maleimide (Thermo, abs 638 nm, em 658 nm, ϵ 170,000), and at N-terminal Gly with IRDye QC-1 N-hydroxysuccinimidyl ester (LI-COR, abs 737 nm, ϵ 96,000). These dyes incorporate sulfate groups and have markedly increased water solubility. After RP-HPLC purification the doubly-labeled substrate (FRETS-rVWF71) is soluble at >50 μM. These dyes absorb/emit in the near-infrared, which does not overlap with the spectrum of blood proteins, hemoglobin, or bilirubin.

Example 3

[0093] This example illustrates ADAMTS13 activity assays.

[0094] Preliminary studies show that paired samples of citrated plasma and serum have the same ADAMTS13 activity, which is stable for extended times at 4° C. or −20° C. These findings are consistent with published data (Gerritsen, H. E., et al., Blood. 98:1654-1661, 2001; Furlan, M., et al., Blood. 87:4223-4234, 1996).

[0095] In these experiments, the FRETS-rVWF71 substrate was utilized in a microtiter format (FIG. **2**), which is compatible with fluorescence plate readers that are supported by laboratory equipment suppliers and available in many clinical hemostasis laboratories, such as the BIO-TEK FLx800 Fluorometer from DiaPharma (West Chester, Ohio) or Technoclone (Vienna). Reactions were performed in 200 μL 5 mM Bis-Tris, pH 6, 25 mM $CaCl_2$, at room temperature with added plasma as shown (FIG. **2**). Fluorescence was monitored in a $Victor^2$ V microplate reader (Perkin Elmer Life Sciences, Boston, Mass.) with 638 nm excitation filter and 658 nm emission filter.

[0096] Using 1 μM substrate with varying amounts of plasma or serum and optimal filters for each substrate, ΔF/min for VWF-rFRETS71 is 8-fold greater compared to VWF-FRETS73. In addition, assays with VWF-rFRETS71 are linear with time and linear with enzyme up to >95% serum or plasma added, with no significant background fluorescence or interference from blood constituents. The combination of stronger fluorescence signal and compatibility with undiluted plasma make FRETS-rVWF71 ~250-fold more sensitive than FRETS-VWF73.

Example 4

[0097] This example illustrates ADAMTS13 inhibitor assays.

[0098] In this prophetic example, activity of ADAMTS13 inhibitors is examined. In these experiments, antibody source can be patient serum, optionally heat-treated to inactivate endogenous ADAMTS13 (56° C., 30 min) (Zheng, X. L., et al., Blood. 103:4043-4049, 2004). The ADAMTS13 source is normal pooled serum (or plasma), or recombinant ADAMTS13 to further increase sensitivity. In these assays, a fixed amount of ADAMTS13 (5 μL) and variable amounts of inhibitor (90 μL serum and serial dilutions) are mixed and preincubated (15 min). FRETS-rVWF71 is added (5 μL) and rates of product generation are analyzed by standard methods to yield an inhibitor titer in "Bethesda-like" units. The assay permits the detection of inhibitors at <<0.5 "BU" per mL.

Example 5

[0099] This prophetic example illustrates an inhibitor assay based on FRETS-rVWF71 cleavage in minimally diluted Li^+-heparin plasma.

[0100] We have designed an inhibitor assay based on FRETS-rVWF71 cleavage in minimally diluted Li^+-heparin plasma. The inhibitor source is patient plasma, optionally heat-treated to inactivate endogenous ADAMTS13 (56° C.,

30 min). Equal amounts of pooled normal plasma and inhibitor (and serial dilutions) are mixed and preincubated. Samples (1000) are assayed with FRETS-rVWF71 (200 μl final volume) and rates of product generation are analyzed to yield an inhibitor titer in "Bethesda-like" units.

Example 6

[0101] This example illustrates ADAMTS13 activity assays.

[0102] In these experiments (FIG. **7**A), reactions were performed in 200 μL 50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 100 μg/ml BSA, 0.05% Tween 20, at 30° C. with added plasma, in a Perkin Elmer Victor2V plate reader with 635/15 nm excitation and 660/10 nm emission filters. FIG. **7**B shows initial slope vs. PNP standard curve.

[0103] After RP-HPLC purification, the doubly-labeled substrate "FRETS-rVWF71" is soluble at >200 μM. In addition, assays with FRETS-rVWF71 were insensitive to >20 mg/dL (250 μM) conjugated bilirubin, which is incompatible with FRETS-VWF73 (Meyer, S. C., et al., J. Thromb. Haemost. 5: 866-867, 2007).

[0104] In these experiments, samples were analyzed from 100 healthy controls. The mean ADAMTS13 activity was 1.11 U/mL with SD 021 U/dL (referenced to local pooled normal plasma). Intra-assay and inter-assay coefficients of variation were <3%. Results obtained with FRETS-rVWF71 and FRETS-V WF73 were congruent, with a coefficient of variation of 3.8% for healthy controls assayed by both methods.

Example 7

[0105] This example illustrates preparation of fluorogenic FRETS-rVWF71 substrate.

[0106] In these experiments, a precursor substrate peptide was expressed from a plasmid that encodes thioredoxin, a His-tag, a TEV cleavage site, and VWF Gln^{1599}-Arg^{1668}. The mutation D1610C introduced a unique Cys, and the mutation K1617R removed a primary amine that otherwise would have competed with subsequent chemical modification of the N-terminus. The expressed chimeric protein was readily soluble and was purified by chromatography on Ni-NTA agarose (FIG. **3**) with a yield of >50 mg/L of LB culture medium. Removal of thioredoxin by cleavage with His-tagged TEV protease gave the product rVWF71, which has an extra Gly before VWF residues Gln^{1599}-Arg^{1668}. Peptide rVWF71 was purified by Ni-NTA chromatography to remove His-tagged thioredoxin and TEV protease, followed by reverse-phase HPLC (FIG. **3**) to yield >10 mg rVWF71/L of LB culture medium.

[0107] FIG. **3**A shows polyacrylamide 4-12% gel electrophoresis of intermediates and purified FRETS-rVWF71. Lanes correspond to: 1, protein markers; 2, thioredoxin-VWF71 fusion protein eluted from Ni-NTA agarose; 3, after cleavage by TEV protease; 4, unbound products after rechromatography on Ni-NTA agarose; 5, after purification by HPLC; 6, rVWF71 peptide modified with DyLight 633 and purified by HPLC; 7, purified FRETS-rVWF71; 8, purified FRETS-rVWF71. Except for lanes 6 and 7, which are unstained, gels were stained with Simply Blue SafeStain.

[0108] FIG. **3**B shows purification of rVWF71 peptide. In these experiments, thioredoxin-VWF71 fusion protein was digested with TEV protease, chromatographed on Ni-NTA agarose to remove His-tagged TEV protease and thioredoxin, and purified by HPLC on a C18 column in 0.1% TFA developed with a 20-90% acetonitrile gradient. The eluate was monitored for absorbance at 280 nm. The peak at 20 min corresponds to panel A, lane 5.

[0109] FIG. **3**C shows purification of singly modified rVWF71. In these experiments, DyLight 633-rVWF71 was purified by HPLC on a C18 column in 50 mM TEAA, pH 6.0, developed with a 20-45% acetonitrile gradient. The eluate was monitored for absorbance at 280 nm and 627 nm, and for emission at 658 nm after excitation at 635 nm. The inset shows an expanded view of the peak at 23 min with traces labeled by wavelength.

[0110] FIG. **3**D shows purification of FRETS-rVWF71. In these experiments, after reaction with IRDye QC-1 N-hydroxy-succinimidyl ester, FRETS-rVWF71 was purified by HPLC as described for DyLight 633-rVWF71 (panel C) with additional monitoring of absorbance at 819 nm. The inset shows an expanded view of the peak at 21 min with traces labeled by wavelength.

[0111] In these experiments, DyLight 633 maleimide (Thermo Scientific, Waltham, Mass.) 1 mg/100 μL in dimethyl sulfoxide (DMSO) was added dropwise with stirring to peptide VWF71 (10 mg) in ≦2 mL of 100 mM sodium phosphate, pH 7.1, in the dark, and stirred overnight at room temperature. The product DyLight 633-rVWF71 was purified by HPLC on C18 using the TEAA/acetonitrile solvent system as described for the purification of VWF71. DyLight 633-rVWF71 was lyophilized and desalted on a small column of PD-10 in ≦2 mL of 100 mM sodium phosphate, pH 7.9. IRDye QC-1 N-hydroxysuccinimide ester 0.5 mg in 100 μL DMSO was added dropwise with stirring and the solution was stirred overnight at room temperature protected from light. The product FRETS-rVWF71 was purified by reverse phase HPLC, lyophilized, dissolved in ≦0.5 mL of deionized water and applied onto a column (7×230 mm) of Amberlite IR120 sodium form. FRETS-rVWF71 product was eluted with deionized water and lyophilized or concentrated by ultrafiltration to ≧250 μM. The concentration was verified by amino acid analysis (The Protein Chemistry Laboratory, Texas A&M University). Working stocks stored at −20° C. were thawed and refrozen repeatedly over several months without any change in stability or chemical properties.

[0112] The rVWF71 was modified with DyLight 633 maleimide in ~70% yield and purified by HPLC (FIG. **3**). The rVWF71 had been reduced with 10 mM dithiothreitol before purification by HPLC and buffer exchange to maximize the efficiency of alkylation by DyLight 633 maleimide. The N-terminal Gly of DyLight 633-rVWF71 was modified with IRDye QC-1 N-hydroxy-succinimidyl ester in ~90% yield and purified by HPLC. The final FRETS-rVWF71 product was converted into the sodium salt, which was stable and soluble at >250 μM.

[0113] Absorbance maxima for FRETS-rVWF71 were observed at 627 nm and 819 nm, consistent with the presence of both dyes. Upon excitation at 635 nm, the fluorescence emission at 660 nm of uncleaved FRETS-rVWF71 and fully cleaved FRETS-rVWF71 showed little dependence on pH, varying <7% between pH 5 and pH 10.

Example 8

[0114] This example illustrates the optimization of FRET-rVW71 cleavage by plasma ADAMSTS13.

[0115] In these experiments, matched samples of serum, and plasma anticoagulated with sodium citrate, Li^+-heparin

and Na^+-heparin were obtained from volunteer healthy donors with informed consent according to a human studies protocol approved by the Washington University Institutional Review Board.

[0116] Assays were performed at 30° C. Samples (100 μL) of plasma or serum, diluted as necessary in assay buffer, were pipetted in duplicate in 96 well white microplates (Optiplate-96, PerkinElmer, Waltham, Mass.). Reaction was initiated by addition of 100 μL substrate in assay buffer. Cleavage of FRETS-rVWF71 was detected as an increase in fluorescence compared to control reactions without added enzyme at 2 min intervals using a Victor^{2}V Multilabel Counter (PerkinElmer, Waltham, Mass.) or Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek, Winooski, Vt.) equipped with 635±10 nm excitation and 660±10 nm emission filters. Initial velocities were determined by fitting progress curves to the polynomial $\Delta F=A+Bt+Ct^2$, where ΔF is the change in fluorescence, t is time, A is the y-intercept, B is the initial velocity (slope), and the term Ct^2 accounts for any decline in velocity due to substrate consumption or photobleaching.

[0117] ADAMTS13 assays with the FRETS-VWF73 substrate (Kokame, K., et al. Br. J. Haematol. 129:93-100, 2005) (Peptide International, Louisville, Ky.) were performed according to the manufacturer's instructions. Hemoglobin was prepared by hypotonic lysis of red blood cells from a voluntary donor and quantified with Drabkin's reagent (Sigma, St. Louis, Mo.).

[0118] Optimal conditions for cleavage of FRETS-rVWF71 were similar to those reported for ADAMTS13 cleavage of other substrates (Kokame, K., et al. Br. J. Haematol. 129:93-100, 2005; Tsai, H. M., et al. Blood. 87:4235-44, 1996; Anderson, P. J., et al. J. Biol. Chem. 281:850-7, 2006). Activity was maximal at pH 6, very low ionic strength, and 5-10 mM $CaCl_2$ (FIG. **4**). Under conditions of physiological ionic strength (150 mM NaCl) and pH 7.4, the rate of reaction was decreased ~50%.

[0119] To optimize compatibility with minimally diluted plasma samples, the standard assay buffer was made 50 mM HEPES, pH 7.4, 150 mM NaCl, and 10 mM $CaCl_2$, supplemented with 0.05% Tween-20 and 400 μg/mL bovine serum albumin. Under these conditions, assays of serum or plasma anticoagulated with citrate, Li+-heparin or Na+-heparin gave comparable results. Values for citrated plasma were decreased as expected from dilution by the citrate anticoagulant solution (FIG. **4**). Li+-heparin plasma is commonly used for clinical chemistry assays and therefore was selected for ADAMTS13 assay development.

[0120] Bilirubin interferes with ADAMTS13 assays that use the FRETS-VWF73 substrate because bilirubin absorbs light at the same wavelengths as the chromophores in the substrate (Hoving a, J. A. K., et al. J. Thromb. Haemost. 5:866-7, 2007). However, the spectrum of bilirubin does not overlap with DyLight 633 and IRDye QC-1, and bilirubin ≦20 mg/dL) did not inhibit ADAMTS13 activity assays with FRETS-rVWF71 (FIG. **5**). Hemoglobin absorbs at 550 nm and also interferes with FRETS-VWF73 assays. In addition, hemoglobin directly inhibits ADAMTS13 regardless of the assay method (Studt, J. D., et al. Blood. 105:542-4, 2005). As expected, hemoglobin ≦20 g/L) did not affect the detection of FRETS-rVWF71 cleavage products, but did inhibit ADAMTS13 activity with an IC50 of 10-15 g/L (FIG. **5**).

Example 9

[0121] This example illustrates the kinetics of FRETS-rVWF71 cleavage.

[0122] In these experiments, FRETS-rVWF71 cleavage by plasma ADAMTS13 (50 μL) was assessed in 200 μL reactions containing 50 mM HEPES, pH 7.4, 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Tween-20, and varying concentrations of FRETS-rVWF71. The concentration of ADAMTS13 in PNP is ~1.03 μg/ml (Feys, H. B., et al. J. Thromb. Haemost 4:955-62, 2006), or ~6 nM for a molecular mass of 170 kDa. For each concentration of substrate, the relationship between product fluorescence and concentration was determined directly by cleaving FRETS-rVWF71 to completion with excess recombinant ADAMTS13 MDTCS (Feys, H. B., et al. J. Thromb. Haemost. 7:2088-95, 2009) and measuring the fluorescence. Control assays contained plasma that was completely deficient in ADAMTS13. The initial velocities (nM/min) as a function of substrate concentration were fitted to the Michealis-Menten equation by nonlinear regression analysis (Prism, GraphPad).

[0123] Plasma ADAMTS13 cleaved FRETS-rVWF71 with a K_m of 1.8 μM and a k_{cat} of 6.8 min^{-1} at 30° C. (FIG. **6**). For comparison, ADAMTS13 cleaves FRETS-VWF73 in the same buffer with a K_m of 3.2 μM and a k_{cat} of 58 min^{-1} at 37° C. (Anderson, P. J., et al. J. Biol. Chem. 281:850-7, 2006). Therefore, the larger dyes of FRETS-rVWF71 do not impair substrate binding to ADAMTS13 but decrease the rate of catalysis ~8-fold.

Example 10

[0124] This example demonstrates FRETS-rVWF71 assay performance.

[0125] Progress curves for cleavage of FRETS-rVWF71 by plasma ADAMTS13 were approximately linear with time for at least 60 minutes (FIG. **7**A). To account for small time-dependent decreases in reaction rate with high concentrations of enzyme, initial rates were obtained by fitting to a second order polynomial. Reaction rate increased approximately linearly with the volume of added plasma. For maximum sensitivity reactions were performed with 100 μL plasma in a reaction volume of 200 μL. Standard calibration curves were constructed with PNP (FIG. **7**B).

[0126] Results with FRETS-rVWF71 and FRETS-VWF73 assayed under standard conditions (Kokame, K., et al. Br. J. Haematol. 129:93-100, 2005) correlated well with an inter-assay CV of 3.8%. Using 1 μM substrate and 5 μL PNP, the change in fluorescence (ΔF/min) for FRETS-rVWF71 (50 mM HEPES, pH 7.4, 150 mM NaCl) was 8-fold greater than for FRETS-VWF73 (5 mM Bis-Tris, pH 6).

[0127] The FRETS-rVWF71 assay for ADAMTS13 activity has been adapted to measure autoantibody inhibitors of ADAMTS13 in a manner analogous to the measurement of factor VIII inhibitors in "Bethesda-like" units.

[0128] Plasma samples with ADAMTS13 protease activity <10% were tested for the presence of an ADAMTS13 inhibitor. Plasma samples were serially diluted two-fold with assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, and 0.05% Tween-20) to yield 8 dilutions. In duplicate, PNP (50 μL) was mixed with an equal volume (50 μL) of undiluted test plasma and each serially diluted sample. Control conditions included PNP mixed with an equal volume of cleavage buffer. The microplate containing the samples was sealed with adhesive film (Sealplate, Excel Scientific, Victorville,

Calif.) and incubated at 37° C. for 1 h. Reaction was initiated by addition of 100 μL of assay buffer containing 2 μM FRETS-rVWF71. Fluorescence emission was monitored and initial reaction rates were determined.

[0129] The inhibitor titer was determined by fitting the initial reaction rates to a four parameter logistic model or sigmoidal dose response equation by nonlinear regression (Prism):

$$\text{Rate}(D)=\text{Max}+(\text{Max}-\text{Min})/(1+10\hat{}((\log T-\log D)*H))$$

[0130] Where Rate(D) is the initial rate with test plasma at dilution D, Max is the maximum rate in the absence of test plasma, Min is the rate with no active enzyme (fixed at 0), H is the Hill slope, and T is the inhibitor titer.

[0131] For selected patient samples, ADAMST13 activity and inhibitor titer were measured with FRETS-VWF73 at the BloodCenter of Wisconsin reference laboratory.

[0132] A fixed amount of ADAMTS13 in PNP (100 μL) was preincubated with serially diluted plasma samples and assayed for ADAMTS13 activity. Reaction rates were analyzed according to a model for competitive sigmoidal dose-response inhibition to obtain the dilution of plasma at which ADAMTS13 activity is decreased by 50%, and the inverse of the dilution is the inhibitor titer (FIG. **7**C). This assay design allows the detection of inhibitors with a titer <0.5 U/ml.

Example 11

[0133] This example illustrates ADAMTS13 assays in healthy donors and patients with TTP.

[0134] In these experiments, frozen Li^+-heparin plasma samples were obtained from 100 healthy controls (Biological Specialty Corp., Colmar, Pa.) deidentified except for demographic information on gender, age, and ethnicity. Pooled normal Li^+-heparin plasma (PNP) for assay standardization was prepared from at least 35 donors. For each healthy control, 25 μL and 100 μL plasma samples were assayed in duplicate for ADAMST13 activity. Up to 20 healthy controls were assayed per 96 well microplate. For cross-validation, ≧3 samples were randomly selected from each assayed batch and reanalyzed with the subsequent batch. A standard curve was constructed from duplicate assays of PNP 5 μL, 25 μL, 50 μL, 75 μL and 100 μL. ADAM ST13 activity of randomly selected controls samples was determined by the FRETS-VWF73 method.

[0135] Li^+-heparin plasma was obtained from individuals suspected to have TPP with their informed consent according to a human studies protocol approved by the Washington University Institutional Review Board. Anticoagulated blood was centrifuged at 2000×g for 10 min at 10° C., and the blood cells debris were discarded. The supernatant was assayed immediately and the remainder stored in aliquots at −80° C.

[0136] ADAMSTI3 activity was assayed in Li^+-heparin plasmas from 96 healthy controls (FIG. **8**). Using a PNP standard prepared from 35 donors, the mean ADAMTS13 activity was 107±18% (SD). Intra-assay and inter-assay coefficients of variation were less <2%. Mean ADAMTS13 activity was not significantly different based on gender or ethnicity (FIG. **8**). There was no significant relationship between ADAMTS13 activity and age.

[0137] ADAMTS13 assays using both FRETS-rVWF71 and FRETS-VWF73 substrates were performed on samples from several patients with idiopathic TTP (Table 1). Both substrates gave consistent results for ADAMTS13 activity, although FRETS-rVWF71 assays were 20-fold more sensitive. Three patients did not have detectable ADAMTS13 inhibitors by either assay. For the remaining patients, inhibitor assays with FRETS-rVWF71 gave titers 1.4-fold to >5-fold higher than inhibitor assays with FRETS-VWF73. This difference in sensitivity for inhibitors reflects the 1:20 dilution of plasma required for assays with FRETS-VWF73.

TABLE 1

Comparison of ADAMTS13 assays for patients with TTP

	ADAMTS13 activity (%)		ADAMTS13 Inhibitor (units/ml)	
Patient	FRETS-rVWF71	FRETS-VWF73	FRETS-rVWF71	FRETS-VWF73
UPN 323	7.6	<5	3.8	1.0
UPN 330	1.8	<5	4.5	1.8
UPN 333	2.2	<5	1.9	0.9
UPN 334	0.2[a]	<5[b]	23[a]	3.6[b]
UPN 335	10	<5	<0.4	<0.4
UPN 336	1.8	<5	3.1	0.6
UPN 337	1.2	<5	<0.4	<0.4
UPN 339	30	14	<0.4	<0.4
UPN 340	1.9	<5	<0.4	1.8
UPN 342	9.5	7	2.7	1.9
UPN 346	3.3	<5	ND	ND

[a]Sample of Sep. 5, 2011.
[b]Sample of Sep. 13, 2011.
ND, not done.

Example 12

[0138] This example illustrates construction of a vector of the present teachings.

[0139] To construct a plasmid comprising a sequence encoding amino acid residues Gln1599-Arg1668 (VWF70) of von Willebrand factor (VWF), DNA sequence of VWF was amplified from pSVHvWF1 (Matsushita, T., and Sadler, J. E. J. Biol. Chem. 270:13406-14, 1995) using primers with Ligation Independent Cloning overhangs (Aslanidis, C. and de Jong, P. J. Nucleic. Acids. Res. 18:6069-74, 1990):

```
Forward,
                                                    (SEQ ID NO: 4)
GGTAATGAGGGTCGCGAGAACCTTTATTTCCAGGGCCAGGCGCCC

Reverse,
                                                    (SEQ ID NO: 5)
AGAGGAGAGTTAGAGCCTCACCTCTGCAGCACCAGGTC
```

The forward primer encodes a tobacco etch virus (TEV) protease cleavage site and the reverse primer introduces a stop codon. The PCR product was purified and ligated into pET-32 Xa/LIC (Novagen, Billerica, Mass.) to yield a plasmid that encodes thioredoxin, a His-tag, a TEV cleavage site, a Gly residue, and VWF Gln1599-Arg1668. The mutations D1610C and K1617R were introduced using a site-directed mutagenesis kit (Stratagene, Santa Clara, Calif.) to yield plasmid pET32XaTEVvWF70. The sequence was confirmed by ABI BigDye V3.1 terminator cycle sequencing.

Example 13

[0140] This example illustrates recombinant peptide preparation.

[0141] In these experiments, plasmid pET32XaTEVvWF70 was transformed into *E. coli* BL21

(DE3). Single colonies were inoculated in 5 mL of LB medium containing 50 μg/mL ampicillin and grown for 5 h at 37° C. with shaking at 250 rpm. While maintaining the antibiotic and growth conditions, the 5 mL culture was transferred into 50 mL of LB for 1 h, after which the 50 mL culture was transferred into 1 L of LB. When the culture reached optical density at 600 nm of ~0.6-0.7, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM and incubation was continued for 4 hours. The cell pellet was obtained by centrifugation at 8,000×g for 15 min at 4° C. and stored at −80° C. if not processed immediately.

[0142] The cell pellet was thawed in water at room temperature for 10 min and resuspended in 10 mL/g pellet of B-PER Protein Extraction Reagent (Pierce), 10 μL/mL benzonase (Sigma, St. Louis, Mo.) 10 μL/mL, Halt Protease Inhibitor Cocktail (Pierce) and 1 mM phenylmethylsulfonyl fluoride. The suspension was incubated for 15 min at room temperature with gentle shaking (Orbitron Rotator I, Boekel Scientific). The supernatant was recovered by centrifugation at 20,000×g for 15 min at 4° C., diluted with an equal volume of His-binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 10 mM imidazole), and applied to a 5 ml column of nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Agarose Beads Technologies, Spain) equilibrated with His-binding buffer. The Ni-NTA column was washed with 50 mL of His-binding buffer and bound protein was eluted with 300 mM imidazole in His-binding buffer. The eluted protein was concentrated to 10 mL by ultrafiltration and dialyzed in a Slide-A-Lyzer cassette (MWCO 7,000, Pierce) overnight with two buffer changes against TEV protease cleavage buffer (50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 0.5 mM EDTA).

[0143] The protein concentration was determined by the BCA method (BioRad, Hercules, Calif.) and the affinity tags cleaved by adding 1 mg of His-tagged TEV protease (Kapust, R. B. and Waugh, D. S. Protein. Sci. 8:1668-74, 1999) per 10 mg of protein and incubation for 16-20 h at room temperature. The solution was dialyzed overnight against His-binding buffer in a Slide-A-Lyzer cassette (MWCO 3,000), and cleaved affinity tags and TEV protease were removed by adsorption on Ni-NTA agarose. Dithiothreitol was added to 10 mM and the unbound product was further purified by HPLC on a C18 column (300 Å, 5 μm, 150×10 mm, GraceVydac) equilibrated with buffer A (50 mM triethylammonium acetate (TEAA), pH 6.0) and developed at 2 mL/min with 3 min of 35% buffer B (60% acetonitrile/40% 50 mM TEAA, pH 6.0) followed by a linear gradient of 35%-75% buffer B for 35 min. Alternatively, the column was equilibrated with 0.1% trifluoroacetic acid (TFA) and developed at 2 mL/min with 20% buffer C (0.092% TFA, 90% acetonitrile) for 5 min, followed by a linear gradient of 20% to 90% buffer C for 35 min. Fractions containing pure VWF71 peptide were lyophilized using a Speed Vac concentrator (Savant), dissolved in a minimum volume of 100 mM sodium phosphate, pH 7.1, and desalted on a small column of PD-10 in the same buffer.

SEQUENCE LISTING

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Ile Gly Val Gly Pro Asn Ala Asn Val Gln Glu Leu Glu Arg Ile Gly
        35                  40                  45

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Ala Arg Cys Ser Leu Phe Gly Ser Asp Phe Val Asn Thr Phe Asp Gly
35 40 45

Ser Met Tyr Ser Phe Ala Gly Tyr Cys Ser Tyr Leu Leu Ala Gly Gly
50 55 60

Cys Gln Lys Arg Ser Phe Ser Ile Ile Gly Asp Phe Gln Asn Gly Lys
65 70 75 80

Arg Val Ser Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu
85 90 95

Phe Val Asn Gly Thr Val Thr Gln Gly Asp Gln Arg Val Ser Met Pro
100 105 110

Tyr Ala Ser Lys Gly Leu Tyr Leu Glu Thr Glu Ala Gly Tyr Tyr Lys
115 120 125

Leu Ser Gly Glu Ala Tyr Gly Phe Val Ala Arg Ile Asp Gly Ser Gly
130 135 140

Asn Phe Gln Val Leu Leu Ser Asp Arg Tyr Phe Asn Lys Thr Cys Gly
145 150 155 160

Leu Cys Gly Asn Phe Asn Ile Phe Ala Glu Asp Asp Phe Met Thr Gln
165 170 175

Glu Gly Thr Leu Thr Ser Asp Pro Tyr Asp Phe Ala Asn Ser Trp Ala
180 185 190

Leu Ser Ser Gly Glu Gln Trp Cys Glu Arg Ala Ser Pro Pro Ser Ser
195 200 205

Ser Cys Asn Ile Ser Ser Gly Glu Met Gln Lys Gly Leu Trp Glu Gln
210 215 220

Cys Gln Leu Leu Lys Ser Thr Ser Val Phe Ala Arg Cys His Pro Leu
225 230 235 240

Val Asp Pro Glu Pro Phe Val Ala Leu Cys Glu Lys Thr Leu Cys Glu
245 250 255

Cys Ala Gly Gly Leu Glu Cys Ala Cys Pro Ala Leu Leu Glu Tyr Ala
260 265 270

Arg Thr Cys Ala Gln Glu Gly Met Val Leu Tyr Gly Trp Thr Asp His
275 280 285

Ser Ala Cys Ser Pro Val Cys Pro Ala Gly Met Glu Tyr Arg Gln Cys
290 295 300

Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His Ile Asn Glu Met
305 310 315 320

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Cys Gln Glu Arg Cys Val Asp Gly Cys Ser Cys Pro Glu Gly Gln Leu
                325                 330                 335

Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
            340                 345                 350

Ser Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser Arg Asp Cys Asn
        355                 360                 365

Thr Cys Ile Cys Arg Asn Ser Gln Trp Ile Cys Ser Asn Glu Glu Cys
    370                 375                 380

Pro Gly Glu Cys Leu Val Thr Gly Gln Ser His Phe Lys Ser Phe Asp
385                 390                 395                 400

Asn Arg Tyr Phe Thr Phe Ser Gly Ile Cys Gln Tyr Leu Leu Ala Arg
                405                 410                 415

Asp Cys Gln Asp His Ser Phe Ser Ile Val Ile Glu Thr Val Gln Cys
            420                 425                 430

Ala Asp Asp Arg Asp Ala Val Cys Thr Arg Ser Val Thr Val Arg Leu
        435                 440                 445

Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His Gly Ala Gly Val
    450                 455                 460

Ala Met Asp Gly Gln Asp Ile Gln Leu Pro Leu Leu Lys Gly Asp Leu
465                 470                 475                 480

Arg Ile Gln His Thr Val Thr Ala Ser Val Arg Leu Ser Tyr Gly Glu
                485                 490                 495

Asp Leu Gln Met Asp Trp Asp Gly Arg Gly Arg Leu Leu Val Lys Leu
            500                 505                 510

Ser Pro Val Tyr Ala Gly Lys Thr Cys Gly Leu Cys Gly Asn Tyr Asn
        515                 520                 525

Gly Asn Gln Gly Asp Asp Phe Leu Thr Pro Ser Gly Leu Ala Glu Pro
    530                 535                 540

Arg Val Glu Asp Phe Gly Asn Ala Trp Lys Leu His Gly Asp Cys Gln
545                 550                 555                 560

Asp Leu Gln Lys Gln His Ser Asp Pro Cys Ala Leu Asn Pro Arg Met
                565                 570                 575

Thr Arg Phe Ser Glu Glu Ala Cys Ala Val Leu Thr Ser Pro Thr Phe
            580                 585                 590

Glu Ala Cys His Arg Ala Val Ser Pro Leu Pro Tyr Leu Arg Asn Cys
        595                 600                 605

Arg Tyr Asp Val Cys Ser Cys Ser Asp Gly Arg Glu Cys Leu Cys Gly
    610                 615                 620

Ala Leu Ala Ser Tyr Ala Ala Ala Cys Ala Gly Arg Gly Val Arg Val
625                 630                 635                 640

Ala Trp Arg Glu Pro Gly Arg Cys Glu Leu Asn Cys Pro Lys Gly Gln
                645                 650                 655

Val Tyr Leu Gln Cys Gly Thr Pro Cys Asn Leu Thr Cys Arg Ser Leu
            660                 665                 670

Ser Tyr Pro Asp Glu Glu Cys Asn Glu Ala Cys Leu Glu Gly Cys Phe
        675                 680                 685

Cys Pro Pro Gly Leu Tyr Met Asp Glu Arg Gly Asp Cys Val Pro Lys
    690                 695                 700

Ala Gln Cys Pro Cys Tyr Tyr Asp Gly Glu Ile Phe Gln Pro Glu Asp
705                 710                 715                 720

Ile Phe Ser Asp His His Thr Met Cys Tyr Cys Glu Asp Gly Phe Met
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-continued

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Leu Ser Ser Pro Leu Ser His Arg Ser Lys Arg Ser Leu Ser Cys Arg
        755                 760                 765

Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp Asn Leu Arg Ala Glu
    770                 775                 780

Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr Asp Leu Glu Cys Met
785                 790                 795                 800

Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro Pro Gly Met Val Arg
                805                 810                 815

His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys Pro Cys Phe His Gln
            820                 825                 830

Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys Ile Gly Cys Asn Thr
        835                 840                 845

Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr Asp His Val Cys Asp
    850                 855                 860

Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr Leu Thr Phe Asp Gly
865                 870                 875                 880

Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr Val Leu Val Gln Asp
                885                 890                 895

Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile Leu Val Gly Asn Lys
            900                 905                 910

Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys Arg Val Thr Ile Leu
        915                 920                 925

Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly Glu Val Asn Val Lys
    930                 935                 940

Arg Pro Met Lys Asp Glu Thr His Phe Glu Val Val Glu Ser Gly Arg
945                 950                 955                 960

Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser Val Val Trp Asp Arg
                965                 970                 975

His Leu Ser Ile Ser Val Val Leu Lys Gln Thr Tyr Gln Glu Lys Val
            980                 985                 990

Cys Gly Leu Cys Gly Asn Phe Asp  Gly Ile Gln Asn Asn  Asp Leu Thr
        995                 1000                1005

Ser Ser  Asn Leu Gln Val Glu  Glu Asp Pro Val Asp  Phe Gly Asn
    1010                1015                1020

Ser Trp  Lys Val Ser Ser Gln  Cys Ala Asp Thr Arg  Lys Val Pro
    1025                1030                1035

Leu Asp  Ser Ser Pro Ala Thr  Cys His Asn Asn Ile  Met Lys Gln
    1040                1045                1050

Thr Met  Val Asp Ser Ser Cys  Arg Ile Leu Thr Ser  Asp Val Phe
    1055                1060                1065

Gln Asp  Cys Asn Lys Leu Val  Asp Pro Glu Pro Tyr  Leu Asp Val
    1070                1075                1080

Cys Ile  Tyr Asp Thr Cys Ser  Cys Glu Ser Ile Gly  Asp Cys Ala
    1085                1090                1095

Cys Phe  Cys Asp Thr Ile Ala  Ala Tyr Ala His Val  Cys Ala Gln
    1100                1105                1110

His Gly  Lys Val Val Thr Trp  Arg Thr Ala Thr Leu  Cys Pro Gln
    1115                1120                1125

Ser Cys  Glu Glu Arg Asn Leu  Arg Glu Asn Gly Tyr  Glu Cys Glu
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    1130                1135                1140

Trp Arg  Tyr Asn Ser Cys Ala  Pro Ala Cys Gln Val  Thr Cys Gln
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His Pro  Glu Pro Leu Ala Cys  Pro Val Gln Cys Val  Glu Gly Cys
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His Ala  His Cys Pro Pro Gly  Lys Ile Leu Asp Glu  Leu Leu Gln
    1175                1180                1185

Thr Cys  Val Asp Pro Glu Asp  Cys Pro Val Cys Glu  Val Ala Gly
    1190                1195                1200

Arg Arg  Phe Ala Ser Gly Lys  Lys Val Thr Leu Asn  Pro Ser Asp
    1205                1210                1215

Pro Glu  His Cys Gln Ile Cys  His Cys Asp Val Val  Asn Leu Thr
    1220                1225                1230

Cys Glu  Ala Cys Gln Glu Pro  Gly Gly Leu Val Val  Pro Pro Thr
    1235                1240                1245

Asp Ala  Pro Val Ser Pro Thr  Thr Leu Tyr Val Glu  Asp Ile Ser
    1250                1255                1260

Glu Pro  Pro Leu His Asp Phe  Tyr Cys Ser Arg Leu  Leu Asp Leu
    1265                1270                1275

Val Phe  Leu Leu Asp Gly Ser  Ser Arg Leu Ser Glu  Ala Glu Phe
    1280                1285                1290

Glu Val  Leu Lys Ala Phe Val  Val Asp Met Met Glu  Arg Leu Arg
    1295                1300                1305

Ile Ser  Gln Lys Trp Val Arg  Val Ala Val Val Glu  Tyr His Asp
    1310                1315                1320

Gly Ser  His Ala Tyr Ile Gly  Leu Lys Asp Arg Lys  Arg Pro Ser
    1325                1330                1335

Glu Leu  Arg Arg Ile Ala Ser  Gln Val Lys Tyr Ala  Gly Ser Gln
    1340                1345                1350

Val Ala  Ser Thr Ser Glu Val  Leu Lys Tyr Thr Leu  Phe Gln Ile
    1355                1360                1365

Phe Ser  Lys Ile Asp Arg Pro  Glu Ala Ser Arg Ile  Ala Leu Leu
    1370                1375                1380

Leu Met  Ala Ser Gln Glu Pro  Gln Arg Met Ser Arg  Asn Phe Val
    1385                1390                1395

Arg Tyr  Val Gln Gly Leu Lys  Lys Lys Lys Val Ile  Val Ile Pro
    1400                1405                1410

Val Gly  Ile Gly Pro His Ala  Asn Leu Lys Gln Ile  Arg Leu Ile
    1415                1420                1425

Glu Lys  Gln Ala Pro Glu Asn  Lys Ala Phe Val Leu  Ser Ser Val
    1430                1435                1440

Asp Glu  Leu Glu Gln Gln Arg  Asp Glu Ile Val Ser  Tyr Leu Cys
    1445                1450                1455

Asp Leu  Ala Pro Glu Ala Pro  Pro Pro Thr Leu Pro  Pro His Met
    1460                1465                1470

Ala Gln  Val Thr Val Gly Pro  Gly Leu Leu Gly Val  Ser Thr Leu
    1475                1480                1485

Gly Pro  Lys Arg Asn Ser Met  Val Leu Asp Val Ala  Phe Val Leu
    1490                1495                1500

Glu Gly  Ser Asp Lys Ile Gly  Glu Ala Asp Phe Asn  Arg Ser Lys
    1505                1510                1515
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Glu Phe  Met Glu Glu Val Ile  Gln Arg Met Asp Val  Gly Gln Asp
    1520                 1525                 1530

Ser Ile  His Val Thr Val Leu  Gln Tyr Ser Tyr Met  Val Thr Val
    1535                 1540                 1545

Glu Tyr  Pro Phe Ser Glu Ala  Gln Ser Lys Gly Asp  Ile Leu Gln
    1550                 1555                 1560

Arg Val  Arg Glu Ile Arg Tyr  Gln Gly Gly Asn Arg  Thr Asn Thr
    1565                 1570                 1575

Gly Leu  Ala Leu Arg Tyr Leu  Ser Asp His Ser Phe  Leu Val Ser
    1580                 1585                 1590

Gln Gly  Asp Arg Glu Gln Ala  Pro Asn Leu Val Tyr  Met Val Thr
    1595                 1600                 1605

Gly Asn  Pro Ala Ser Asp Glu  Ile Lys Arg Leu Pro  Gly Asp Ile
    1610                 1615                 1620

Gln Val  Val Pro Ile Gly Val  Gly Pro Asn Ala Asn  Val Gln Glu
    1625                 1630                 1635

Leu Glu  Arg Ile Gly Trp Pro  Asn Ala Pro Ile Leu  Ile Gln Asp
    1640                 1645                 1650

Phe Glu  Thr Leu Pro Arg Glu  Ala Pro Asp Leu Val  Leu Gln Arg
    1655                 1660                 1665

Cys Cys  Ser Gly Glu Gly Leu  Gln Ile Pro Thr Leu  Ser Pro Ala
    1670                 1675                 1680

Pro Asp  Cys Ser Gln Pro Leu  Asp Val Ile Leu Leu  Leu Asp Gly
    1685                 1690                 1695

Ser Ser  Ser Phe Pro Ala Ser  Tyr Phe Asp Glu Met  Lys Ser Phe
    1700                 1705                 1710

Ala Lys  Ala Phe Ile Ser Lys  Ala Asn Ile Gly Pro  Arg Leu Thr
    1715                 1720                 1725

Gln Val  Ser Val Leu Gln Tyr  Gly Ser Ile Thr Thr  Ile Asp Val
    1730                 1735                 1740

Pro Trp  Asn Val Val Pro Glu  Lys Ala His Leu Leu  Ser Leu Val
    1745                 1750                 1755

Asp Val  Met Gln Arg Glu Gly  Gly Pro Ser Gln Ile  Gly Asp Ala
    1760                 1765                 1770

Leu Gly  Phe Ala Val Arg Tyr  Leu Thr Ser Glu Met  His Gly Ala
    1775                 1780                 1785

Arg Pro  Gly Ala Ser Lys Ala  Val Val Ile Leu Val  Thr Asp Val
    1790                 1795                 1800

Ser Val  Asp Ser Val Asp Ala  Ala Ala Asp Ala Ala  Arg Ser Asn
    1805                 1810                 1815

Arg Val  Thr Val Phe Pro Ile  Gly Ile Gly Asp Arg  Tyr Asp Ala
    1820                 1825                 1830

Ala Gln  Leu Arg Ile Leu Ala  Gly Pro Ala Gly Asp  Ser Asn Val
    1835                 1840                 1845

Val Lys  Leu Gln Arg Ile Glu  Asp Leu Pro Thr Met  Val Thr Leu
    1850                 1855                 1860

Gly Asn  Ser Phe Leu His Lys  Leu Cys Ser Gly Phe  Val Arg Ile
    1865                 1870                 1875

Cys Met  Asp Glu Asp Gly Asn  Glu Lys Arg Pro Gly  Asp Val Trp
    1880                 1885                 1890

Thr Leu  Pro Asp Gln Cys His  Thr Val Thr Cys Gln  Pro Asp Gly
    1895                 1900                 1905
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Gln Thr  Leu Leu Lys Ser His  Arg Val Asn Cys Asp  Arg Gly Leu
    1910                 1915                 1920

Arg Pro  Ser Cys Pro Asn Ser  Gln Ser Pro Val Lys  Val Glu Glu
    1925                 1930                 1935

Thr Cys  Gly Cys Arg Trp Thr  Cys Pro Cys Val Cys  Thr Gly Ser
    1940                 1945                 1950

Ser Thr  Arg His Ile Val Thr  Phe Asp Gly Gln Asn  Phe Lys Leu
    1955                 1960                 1965

Thr Gly  Ser Cys Ser Tyr Val  Leu Phe Gln Asn Lys  Glu Gln Asp
    1970                 1975                 1980

Leu Glu  Val Ile Leu His Asn  Gly Ala Cys Ser Pro  Gly Ala Arg
    1985                 1990                 1995

Gln Gly  Cys Met Lys Ser Ile  Glu Val Lys His Ser  Ala Leu Ser
    2000                 2005                 2010

Val Glu  Leu His Ser Asp Met  Glu Val Thr Val Asn  Gly Arg Leu
    2015                 2020                 2025

Val Ser  Val Pro Tyr Val Gly  Gly Asn Met Glu Val  Asn Val Tyr
    2030                 2035                 2040

Gly Ala  Ile Met His Glu Val  Arg Phe Asn His Leu  Gly His Ile
    2045                 2050                 2055

Phe Thr  Phe Thr Pro Gln Asn  Asn Glu Phe Gln Leu  Gln Leu Ser
    2060                 2065                 2070

Pro Lys  Thr Phe Ala Ser Lys  Thr Tyr Gly Leu Cys  Gly Ile Cys
    2075                 2080                 2085

Asp Glu  Asn Gly Ala Asn Asp  Phe Met Leu Arg Asp  Gly Thr Val
    2090                 2095                 2100

Thr Thr  Asp Trp Lys Thr Leu  Val Gln Glu Trp Thr  Val Gln Arg
    2105                 2110                 2115

Pro Gly  Gln Thr Cys Gln Pro  Ile Leu Glu Glu Gln  Cys Leu Val
    2120                 2125                 2130

Pro Asp  Ser Ser His Cys Gln  Val Leu Leu Leu Pro  Leu Phe Ala
    2135                 2140                 2145

Glu Cys  His Lys Val Leu Ala  Pro Ala Thr Phe Tyr  Ala Ile Cys
    2150                 2155                 2160

Gln Gln  Asp Ser Cys His Gln  Glu Gln Val Cys Glu  Val Ile Ala
    2165                 2170                 2175

Ser Tyr  Ala His Leu Cys Arg  Thr Asn Gly Val Cys  Val Asp Trp
    2180                 2185                 2190

Arg Thr  Pro Asp Phe Cys Ala  Met Ser Cys Pro Pro  Ser Leu Val
    2195                 2200                 2205

Tyr Asn  His Cys Glu His Gly  Cys Pro Arg His Cys  Asp Gly Asn
    2210                 2215                 2220

Val Ser  Ser Cys Gly Asp His  Pro Ser Glu Gly Cys  Phe Cys Pro
    2225                 2230                 2235

Pro Asp  Lys Val Met Leu Glu  Gly Ser Cys Val Pro  Glu Glu Ala
    2240                 2245                 2250

Cys Thr  Gln Cys Ile Gly Glu  Asp Gly Val Gln His  Gln Phe Leu
    2255                 2260                 2265

Glu Ala  Trp Val Pro Asp His  Gln Pro Cys Gln Ile  Cys Thr Cys
    2270                 2275                 2280

Leu Ser  Gly Arg Lys Val Asn  Cys Thr Thr Gln Pro  Cys Pro Thr
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    2285                2290                2295

Ala Lys  Ala Pro Thr Cys Gly  Leu Cys Glu Val Ala  Arg Leu Arg
    2300                2305                2310

Gln Asn  Ala Asp Gln Cys Cys  Pro Glu Tyr Glu Cys  Val Cys Asp
    2315                2320                2325

Pro Val  Ser Cys Asp Leu Pro  Pro Val Pro His Cys  Glu Arg Gly
    2330                2335                2340

Leu Gln  Pro Thr Leu Thr Asn  Pro Gly Glu Cys Arg  Pro Asn Phe
    2345                2350                2355

Thr Cys  Ala Cys Arg Lys Glu  Glu Cys Lys Arg Val  Ser Pro Pro
    2360                2365                2370

Ser Cys  Pro Pro His Arg Leu  Pro Thr Leu Arg Lys  Thr Gln Cys
    2375                2380                2385

Cys Asp  Glu Tyr Glu Cys Ala  Cys Asn Cys Val Asn  Ser Thr Val
    2390                2395                2400

Ser Cys  Pro Leu Gly Tyr Leu  Ala Ser Thr Ala Thr  Asn Asp Cys
    2405                2410                2415

Gly Cys  Thr Thr Thr Thr Cys  Leu Pro Asp Lys Val  Cys Val His
    2420                2425                2430

Arg Ser  Thr Ile Tyr Pro Val  Gly Gln Phe Trp Glu  Glu Gly Cys
    2435                2440                2445

Asp Val  Cys Thr Cys Thr Asp  Met Glu Asp Ala Val  Met Gly Leu
    2450                2455                2460

Arg Val  Ala Gln Cys Ser Gln  Lys Pro Cys Glu Asp  Ser Cys Arg
    2465                2470                2475

Ser Gly  Phe Thr Tyr Val Leu  His Glu Gly Glu Cys  Cys Gly Arg
    2480                2485                2490

Cys Leu  Pro Ser Ala Cys Glu  Val Val Thr Gly Ser  Pro Arg Gly
    2495                2500                2505

Asp Ser  Gln Ser Ser Trp Lys  Ser Val Gly Ser Gln  Trp Ala Ser
    2510                2515                2520

Pro Glu  Asn Pro Cys Leu Ile  Asn Glu Cys Val Arg  Val Lys Glu
    2525                2530                2535

Glu Val  Phe Ile Gln Gln Arg  Asn Val Ser Cys Pro  Gln Leu Glu
    2540                2545                2550

Val Pro  Val Cys Pro Ser Gly  Phe Gln Leu Ser Cys  Lys Thr Ser
    2555                2560                2565

Ala Cys  Cys Pro Ser Cys Arg  Cys Glu Arg Met Glu  Ala Cys Met
    2570                2575                2580

Leu Asn  Gly Thr Val Ile Gly  Pro Gly Lys Thr Val  Met Ile Asp
    2585                2590                2595

Val Cys  Thr Thr Cys Arg Cys  Met Val Gln Val Gly  Val Ile Ser
    2600                2605                2610

Gly Phe  Lys Leu Glu Cys Arg  Lys Thr Thr Cys Asn  Pro Cys Pro
    2615                2620                2625

Leu Gly  Tyr Lys Glu Glu Asn  Asn Thr Gly Glu Cys  Cys Gly Arg
    2630                2635                2640

Cys Leu  Pro Thr Ala Cys Thr  Ile Gln Leu Arg Gly  Gly Gln Ile
    2645                2650                2655

Met Thr  Leu Lys Arg Asp Glu  Thr Leu Gln Asp Gly  Cys Asp Thr
    2660                2665                2670
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His Phe  Cys Lys Val Asn Glu  Arg Gly Glu Tyr Phe  Trp Glu Lys
    2675                 2680                 2685

Arg Val  Thr Gly Cys Pro Pro  Phe Asp Glu His Lys  Cys Leu Ala
    2690                 2695                 2700

Glu Gly  Gly Lys Ile Met Lys  Ile Pro Gly Thr Cys  Cys Asp Thr
    2705                 2710                 2715

Cys Glu  Glu Pro Glu Cys Asn  Asp Ile Thr Ala Arg  Leu Gln Tyr
    2720                 2725                 2730

Val Lys  Val Gly Ser Cys Lys  Ser Glu Val Glu Val  Asp Ile His
    2735                 2740                 2745

Tyr Cys  Gln Gly Lys Cys Ala  Ser Lys Ala Met Tyr  Ser Ile Asp
    2750                 2755                 2760

Ile Asn  Asp Val Gln Asp Gln  Cys Ser Cys Cys Ser  Pro Thr Arg
    2765                 2770                 2775

Thr Glu  Pro Met Gln Val Ala  Leu His Cys Thr Asn  Gly Ser Val
    2780                 2785                 2790

Val Tyr  His Glu Val Leu Asn  Ala Met Glu Cys Lys  Cys Ser Pro
    2795                 2800                 2805

Arg Lys  Cys Ser Lys
    2810
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ggtaatgagg gtcgcgagaa cctttatttc cagggccagg cgccc 45

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agaggagagt tagagcctca cctctgcagc accaggtc 38

What is claimed is:

1. A probe comprising:

an oligopeptide consisting of no more than 80 amino acids of sequence of von Willebrand Factor (VWF), said oligopeptide comprising a scissile Y-M peptide, a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction, and a C-terminal segment;

a fluorophore; and

a fluorescence quencher,

wherein the fluorophore and the fluorescence quencher are bound to the oligopeptide on opposite sides of the scissile Y-M peptide.

2. A probe in accordance with claim **1**, wherein the cysteine substitution is a substitution of an amino acid located at least 3 amino acids from the scissile Y-M peptide.

3. A probe in accordance with claim **1**, wherein the cysteine substitution is a N1610C substitution of a VWF sequence.

4. A probe in accordance with claim **1**, wherein the oligopeptide comprises a substitution of lysine K1617 for an amino acid that does not comprise a primary amine on its side chain.

5. A probe in accordance with claim **1**, wherein the oligopeptide consists of the sequence set forth as SEQ ID NO: 2.

6. A probe in accordance with claim **1**, wherein the fluorophore has an absorption maximum >550 nm.

7. A probe in accordance with claim **1**, wherein the fluorophore has an emission maximum >600 nm.

8. A probe in accordance with claim **1**, wherein the fluorophore is selected from the group consisting of Alexa Fluor 594 maleimide and DyLight 633 maleimide.

9. A probe in accordance with claim **1**, wherein the quencher has an absorption maximum >550 nm.

10. A probe in accordance with claim **1**, wherein at least one of the fluorophore and the quencher comprises at least one sulfate.

11. A probe in accordance with claim **1**, wherein the quencher is selected from the group consisting of QSY21-succinimidyl ester and IRDye QC-1 N-hydroxy succinimidyl ester.

12. A probe in accordance with claim **1**, wherein the probe is soluble in water at >50 μM.

13. A vector comprising a nucleic acid sequence encoding an oligopeptide consisting of no more than 80 amino acids of sequence of von Willebrand Factor (VWF), said oligopeptide comprising a scissile Y-M peptide, a cysteine substitution located from 1 to 12 amino acids from the scissile Y-M in the carboxy terminal direction, and a C-terminal segment.

14. A vector in accordance with claim **13**, wherein the nucleic acid sequence encoding an oligopeptide further comprises a sequence selected from the group consisting of a sequence encoding thioredoxin and a sequence encoding a tobacco etch virus (TEV) protease cleavage site.

15. A method of determining presence, absence or quantity of ADAMTS13 activity in a sample, comprising:

forming a mixture comprising a sample and a probe of claim **1**; and

measuring fluorescence at one or more time points after forming the mixture,

wherein the sample is diluted less than 20-fold.

16. A method in accordance with claim **15**, wherein the sample is selected from the group consisting of a serum sample, an undiluted serum sample, a concentrated serum sample, a plasma sample, an undiluted plasma sample and a concentrated plasma sample.

17. A method of determining presence, absence or quantity of ADAMTS13 inhibitor activity in a sample, comprising:

forming a mixture comprising a sample, a source of ADAMTS13, and a probe of claim **1**; and

measuring fluorescence at one or more time points after forming the mixture.

18. A method in accordance with claim **17**, further comprising inactivating ADAMTS13 activity endogenous to the sample prior to forming the mixture.

19. A method in accordance with claim **17**, wherein the source of ADAMTS13 is selected from the group consisting of normal plasma, recombinant ADAMTS13 and a combination thereof.

20. A method in accordance with claim **17**, wherein the sample is selected from the group consisting of a serum sample, an undiluted serum sample, a concentrated serum sample, a plasma sample, an undiluted plasma sample and a concentrated plasma sample.

* * * * *