

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2016/198521 A1

(43) International Publication Date
15 December 2016 (15.12.2016)

(51) International Patent Classification:

C12N 9/96 (2006.01) *C07K 14/755* (2006.01)
A61K 38/37 (2006.01)

(21) International Application Number:

PCT/EP2016/063155

(22) International Filing Date:

9 June 2016 (09.06.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

15171338.5 10 June 2015 (10.06.2015) EP

(71) Applicant: **NOVO NORDISK A/S** [DK/DK]; Novo Allé,
2880 Bagsværd (DK).

(72) Inventors: **BOLT, Gert**; Novo Nordisk A/S, Novo Allé,
2880 Bagsværd (DK). **KARPF, Ditte Maria**; Novo Nordisk
A/S, Novo Allé, 2880 Bagsværd (DK). **NØRBY, Peder
Lisby**; Novo Nordisk A/S, Novo Allé, 2880 Bagsværd
(DK).

(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))

(54) Title: FVIII FUSION PROTEINS

(57) Abstract: The present invention relates to FVIII fusion molecules and compositions suitable for treatment of blood clotting dis-
eases.



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FVIII FUSION PROTEINS

TECHNICAL FIELD

The present invention relates to treatment and/or prophylaxis of haemophilia using FVIII fusion proteins.

5 BACKGROUND

Haemophilia A is an inherited bleeding disorder: formation of the blood clot in the patients occurs normally but the clot is unstable due to a lack of secondary thrombin formation. The disease is treated by intravenous injection of coagulation factor VIII (FVIII) isolated from blood or produced recombinantly. Current haemophilia treatment
10 recommendations are moving from traditional on-demand treatment towards prophylaxis. The circulatory *in vivo* half life of endogenous FVIII is 12-14 hours and prophylactic treatment with FVIII is thus to be performed several times a week in order to obtain a virtually symptom-free life for the patients.

Recombinant FVIII variants with a prolonged circulatory half life (e.g. heterologous
15 FVIII:Fc fusions) are becoming available for the haemophilia patients but such compounds still need to be administered several times a week. Another frequently used strategy for prolonging FVIII circulatory half life is conjugation of FVIII, employing chemical or enzymatic methods. There is thus a need in the art for improved safe and convenient Haemophilia A therapies offering compounds with prolonged circulatory half life.

20 Miao et al. (Blood, 103: 3412-3419 (2004)) discloses FVIII molecules with B domains of intermediate length (54-269 amino acids). These molecules are reported to be produced in higher yields than FVIII with shorter B domains. One of the FVIII compounds described by Miao et al. has a 226 amino acid B domain (amino acid 741-966) connected to the FVIII LC sequence via a dipeptide QR linker ("226aa/N6"). This compound does not
25 comprise the processing protease recognition site, and therefore 226aa/N6 does not become processed at the B domain-light chain junction.

WO2013160005 discloses that FVIII molecules with a B domain having a length of 100-400 amino acids have an improved bioavailability in connection with subcutaneous FVIII administration.

SUMMARY

The present invention relates to FVIII fusion proteins, comprising a FVIII molecule with a B domain (a full length or a truncated B domain), wherein said FVIII molecule is fused to (at least one) FVIII B domain fusion partner, wherein said FVIII B domain fusion partner(-s) is derived from the FVIII B domain and comprises 100-908 amino acids. Such therapeutic proteins are associated with various advantages.

The present invention furthermore relates to compositions suitable for treatment of blood clotting diseases.

DESCRIPTION

The inventors of the present invention have made the surprising observation that FVIII proteins can be fused with at least one additional (truncated) B domain in order to obtain a therapeutic molecule with advantageous properties.

Definitions

The term "treatment", as used herein, refers to the medical therapy of any human or other vertebrate subject in need thereof. Said subject is expected to have undergone physical examination by a medical practitioner, or a veterinary medical practitioner, who has given a tentative or definitive diagnosis which would indicate that the use of said specific treatment is beneficial to treating a disease in said human or other vertebrate. The timing and purpose of said treatment may vary from one individual to another, according to the subject's health. Thus, said treatment may be prophylactic, palliative, symptomatic and/or curative.

Mode of administration: Compounds and pharmaceutical compositions according to the invention may be administered parenterally, such as e.g. intravenously or extravascularly (such as e.g. intradermally, intramuscularly, subcutaneously, etc). Compounds and pharmaceutical compositions according to the invention may be administered prophylactically and/or therapeutically and/or on demand.

Factor VIII: Factor VIII (FVIII) is a large, complex glycoprotein that is primarily produced by endothelial cells including liver sinusoidal endothelial cells (LSECs) and possibly also hepatocytes. Human FVIII codes for 2351 amino acids, including a signal peptide, and contains several distinct domains as defined by homology. There are three A-domains, a unique B-domain, and two C-domains. The domain order can be listed as NH2-

A1-A2-B-A3-C1-C2-COOH. Small acidic regions C-terminal of the A1 (the a1 region) and A2 (the a2 region) and N-terminal of the A3 domain (the a3 region) play important roles in FVIII interaction with other coagulation proteins, including thrombin and von Willebrand factor (VWF).

- 5 During cellular processing furin cleaves prior to the a3 region. The resulting A1-a1-A2-a2-B chain is termed the heavy chain (HC) while the a3-A3-C1-C2 is termed the light chain (LC). The chains are connected by bivalent metal ion-bindings.

10 **Table 1:** FVIII domains and regions. The numbering of domains, regions and single amino acid residues in the Factor VIII molecule follow the numbering of full length Factor VIII (also if the B-domain is truncated or if a fusion partner (herein a FVIII B domain fusion partner) is added to the molecule).

Domain	Region	Amino acid number *)	Number of amino acid residues
A1		1-336	336
	a1	337-372	36
A2		373-710	338
	a2	711-740	30
B **)		741-1648	908
***)	a3	1649-1689	41
A3		1690-2020	331
C1		2021-2173	153
C2		2174-2332	159
Total FVIII		1-2332	2332

- 15 *) The numbering of domains, regions and single amino acid residues is in accordance with uniprot:P00451. Other FVIII alleles exist as well in human populations.

- 20 **) The nucleotide sequence encoding full length Factor VIII encodes a B domain of 908 amino acid residues. During protein synthesis, the B-domain in full-length FVIII is processed, resulting in a mixture of heavy chain with different length of B-domains attached (Jankowski MA et al. Haemophilia 2007; 13: 30-37). rFVIII with truncated truncated B domains may comprise B domains being significantly shorter than 908 amino acids - one example of a truncated B domain is the 21 amino acid B domain linker according to SEQ ID NO 2.

***) Some naturally occurring FVIII variants comprise an a3 region spanning amino acids 1655-1689 and 1658-1689 (Lind P et al. Eur J Biochem 1995; 232: 19-27). Such FVIII proteins, as well as other naturally occurring FVIII variants, are also part of the present invention.

5

Endogenous FVIII molecules circulate *in vivo* as a pool of molecules with B domains of various sizes, the shortest having C-terminal at position 740, i.e. at the C-terminal of A2-a2, and thus contains no B domain. FVIII molecules with B-domains of different length all maintain procoagulant activity. Upon activation with thrombin, FVIII is cleaved C-terminal of A1-a1 at position 372, C-terminal of A2-a2 at position 740, and between a3 and A3 at position 1689, the latter cleavage releasing the a3 region with concomitant loss of affinity for VWF. The activated FVIII molecule is termed FVIIIa. The activation allows interaction of FVIIIa with phospholipid surfaces like activated platelets and activated factor IX (FIXa), i.e. the tenase complex is formed, allowing efficient activation of factor X (FX) resulting in thrombin generation and ultimately formation of a fibrin-stabilized haemostatic clot.

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"Wildtype(wt)/native FVIII" is the human FVIII molecule derived from the full length sequence as shown in **SEQ ID NO: 1** (amino acid 1-2332). "FVIII" includes natural allelic variants of FVIII that may exist and occur from one individual to another. It follows that FVIII fusion proteins according to the present invention are recombinantly produced proteins (rFVIII), using well known methods of production and purification. The degree and location of glycosylation, tyrosine sulfation and other post-translation modifications of FVIII occurring in the cell may therefore vary, depending on the chosen host cell and its growth conditions.

20

Pharmaceutical compositions according to the present invention may comprise FVIII fusion proteins according to the present invention.

25

Following activation, FVIII fusion proteins herein are capable of functioning in the coagulation cascade in a manner that – on a molar basis - is functionally similar, or equivalent, to wt/endogenous FVIII, inducing the formation of FXa via interaction with FIXa on an activated platelet and supporting the formation of a blood clot. FVIII(a) activity can be assessed *in vitro* using techniques well known in the art. Clot analyses, FX activation assays (often termed chromogenic assays), thrombin generation assays and whole blood thromboelastography are examples of such *in vitro* techniques. FVIII molecules according to the present invention have FVIII activity that is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, 100% or even more than 100% of that of native human FVIII. "Native human FVIII" is herein understood to be either plasma derived purified

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FVIII or recombinant FVIII corresponding to SEQ ID NO 1, or a B domain truncated version thereof.

Endogenous full length FVIII is synthesized as a single-chain precursor molecule. Prior to secretion, the precursor is cleaved into the heavy chain and the light chain.

- 5 Recombinant FVIII can be produced by means of two different strategies. Either the heavy chain with or without the B-domain and the light chain are synthesized individually as two different polypeptide chains (two-chain strategy) or the B domain-truncated FVIII is synthesized as a single precursor polypeptide chain (single-chain strategy) that is cleaved into the heavy and light chains in the same way as the full-length FVIII precursor. In a B
- 10 domain-truncated FVIII (precursor) fusion polypeptide according to the invention, produced by the single-chain strategy, the heavy and light chain moieties are often separated by a linker. To minimize the risk of introducing immunogenic epitopes, the sequence of the B domain linker is preferably derived from the FVIII B-domain. The C-terminal part of the linker contains a furin recognition site resulting in intracellular processing into a heavy and a light
- 15 chain. In the B domain of full length FVIII, amino acid 1644-1648 constitutes this recognition site. The thrombin cleavage site leading to removal of the linker during activation of B domain-truncated FVIII is located in the $\alpha 2$ region C-terminal to the A2 domain. Thus, the size and amino acid sequence of the linker is unlikely to influence its removal from the remaining FVIII molecule by thrombin activation. Deletion/truncation of the B domain is often
- 20 considered to be an advantage for recombinant production of FVIII. Nevertheless, parts of the B domain can be included in the B domain linker without reducing the productivity.

SEQ ID NO: 1: wt human FVIII (Ser750 residue shown in bold and underline)

ATRRYYLGAVELSWDYMQSDLGELPVDARFPFPRVPKSFPFNTSVVYKKTFLVEFT

25 DHLFNIAKPRPPWMGLLGPTIQAEVYDTVITLKNMASHPVSLHAVGVSYWKASEGAHEYDD
QTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALL
VCREGLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGY
VNRSPLGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL
MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVR

30 DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGR
KYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLIIIFKNQASRPYNIYPHGITDVRP
LYSRRLPKGKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYSSFVNMERDLASGLI
GPLLICYSKESVDQQRGNQIMSDKRNVLFSVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQA
SNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPF

35 SGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYEDSYEDISAYLLSKN

NAIEPRFSQNSRHPSTRQKQFNATTIPENDIEKTPWFAHRTMPKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESGLQL
 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
 LFGKKSSPLTESGGPLSLSEENNSKLLSGLMNSQESSWGKNVSTESGRLFKGKRAHG
 5 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMFLPES
 ARWIQRTHGKNSLNSGQGPSKQLVSLGPEKSVGQNFLSEKNKVVVGKGFTKDVGLKE
 MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHVTGTGNFMKNLF
 LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
 10 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 PAASYRKKDSGVQESSHFLQGAKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVEN
 TVLPKPDLPKTSKGVELLPKVHIYQKDLFPTETSNNGSPGHLDLVEGSLLQGTEGAIKWNEAN
 RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPKTAFAKKKDTILSL
 15 NACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQSDQEEID
 YDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLNRNAQS
 GSVPPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFY
 SSLISYEEDQRQGAEPKRFVKNETKTYFWKVQHMHMAPTKDEFDCKAWAYFSDVDLEKD
 VHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQME
 20 DPTFKENYRFHAINGYIMDTLPGLVMAQDQRRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEE
 YKMALYNLYPGVFETVEMLPKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHI
 RDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGKTKGARQKFS
 SLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNFNPPIIARYIRLHPHTHSIR
 STLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAW
 25 RPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGK
 VKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

The B domain in FVIII spans amino acids 741-1648 of **SEQ ID NO: 1**. The B domain
 undergoes endo-proteolysis at several different sites, generating large heterogeneity in
 30 circulating plasma FVIII molecules as explained above and in Jankowski et al, Haemophilia
 2007; 13: 30-37 and D'Amici et al, Electrophoresis 2010; 31: 2730-2739 . While the B-
 domain plays a role in intracellular expression of FVIII, the exact extracellular function of the
 heavily glycosylated B domain, if any, is unknown. What is known is that the B domain is
 dispensable for FVIII activity in the coagulation cascade. Recombinant FVIII is thus
 35 frequently produced in the form of B domain-/truncated variants. In one embodiment, the

FVIII fusion protein is produced by an expression vector encoding a FVIII molecule comprising a 21 amino acid residue linker (B domain linker) sequence with the following sequence: **SEQ ID NO 2:** SFSQNSRHPSQNPPVLKRHQR. An O-glycan is attached to the underlined S in SEQ ID NO 2 – this residue corresponds to position S750 in SEQ ID NO1. In

5 another embodiment, the FVIII fusion protein herein comprises a linker sequence with the following sequence: **SEQ ID NO: 3:** SFSQNSRHPSQNPPVLKRHQ. In another embodiment, the FVIII fusion protein herein comprises a linker sequence with the following sequence:

SEQ ID NO: 4: FSQNSRHPSQNPPVLKRHQR. In another embodiment, FVIII fusion protein herein are B domain/truncated FVIII variants comprising an O-glycan attached to the Ser 750
10 residue shown in SEQ ID NO 1.

FVIII fusion proteins herein may be FVIII molecules comprising the full B domain, or it may be comprise a FVIII molecule with a truncated B domain. FVIII molecules according to the invention comprise 908, 4-908, 10-908, 15-908, 4-900, 5-900, 10-900, 15-900, 20-900, 25-900, 50-900, 100-900, 200-900, 300-900, 400-900, 500-900, 600-900, 700-900, 10-700,
15 15-700, 20-700, 25-700, 50-700, 100-700, 200-700, 300-700, 400-700, 500-700, 600-700, 10-500, 15-500, 20-500, 25-500, 50-500, 100-500, 200-500, 300-500, 400-500, 10-400, 15-400, 20-400, 25-400, 50-400, 100-400, 200-400, 300-400, 10-300, 15-300, 20-300, 25-300, 50-300, 100-300, 200-300, 10-250, 15-250, 20-250, 25-250, 50-250, 100-250, 200-250, 10-200, 15-200, 20-200, 25-200, 50-200, 100-200, 10-100, 15-100, 20-100, 25-100, 50-100, 10-
20 50, 15-50, 20-50, 10-25, 10-20, 10-15, 15-25, 15-20, 20-21, 20-25, or 20-30 amino acids. As explained herein, such FVIII molecules are fused to a fusion partner derived from the FVIII B domain – a FVIII B domain fusion partner.

The inventors of the present invention have made the surprising observation that fusion of a FVIII molecule with a FVIII B domain fusion partner, as defined herein, results in a
25 FVIII fusion protein with a surprisingly long *in vivo* circulatory half life ($T_{1/2}$). The FVIII part of this fusion protein may be a B domain truncated FVIII molecule comprising a B domain linker with an amino acid sequence selected from SEQ ID No 2, 3, or 4. The FVIII B domain fusion partner has a size from 100-908, 100-900, 100-800, 100-700, 100-600, 100-500, 100-400 amino acids, preferably 150-650, more preferably 150-600, more preferably 150-550,
30 more preferably 150-500, more preferably 150-450, more preferably 150-400, more preferably 150-350, more preferably 200-700, more preferably 200-600, more preferably 200-500, more preferably 200-400, more preferably 200-300, and most preferably about 200 to 250 amino acids. Such FVIII fusion proteins may or may not comprise the Ser750 residue according to SEQ ID NO 1. A simple and safe way of obtaining therapeutic FVIII molecules
35 with advantageous properties is thus provided with the present invention.

FVIII B domain fusion partner: The FVIII B domain fusion partner according to the present invention is derived from the FVIII B domain. Preferably, the only modification compared to the wt FVIII B domain is that the FVIII B domain fusion partner may be truncated, i.e. a region has been removed/deleted relative to the wt FVIII B domain. In other embodiments, two, three or more truncations were made in the FVIII B domain fusion partner – relative to the wt FVIII B domain. In some embodiments, minor amino acid modifications (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 point mutations in the form of amino acid alterations, -additions and/or -deletions) may be present in the FVIII B domain fusion partner – relative to the corresponding positions in the wt FVIII B domain. The FVIII fusion proteins according to the present invention are thus structurally similar to the native FVIII molecule in that the fusion partner is derived from the FVIII B domain rather than being derived from a heterologous molecule. FVIII fusion proteins according to the present invention can thus be said to be a homologous type of FVIII fusion protein with a desirable safety profile compared to heterologous FVIII fusion molecules. Safety in use of therapeutic FVIII molecules is particularly important in order to reduce the risk of developing patient FVIII inhibitors (FVIII neutralizing antibodies).

The FVIII B domain fusion partner/B domain fusion partner according to the present invention can be regarded as an “additional FVIII B domain” having a size of 100-908 amino acids as described above. In some embodiments, the FVIII fusion proteins according to the invention may even comprise more than one FVIII B domain fusion partners, such as e.g. two, three, or even four FVIII B domain fusion partners fused head-to-tail and/or fused to or within different domains of the FVIII molecule. FVIII molecules according to the present invention may thus comprise a FVIII molecule fused to two, three, or four identical or different FVIII B domain fusion partners.

The inventors have herein made the surprising discovery that the FVIII *in vivo* circulatory half life can be increased by fusing a FVIII derived B domain fusion partner to a FVIII molecule. This effect may be caused by an ability of this additional FVIII B domain(-s) to interfere with various FVIII clearance receptors. An explanation of the apparent ability of FVIII B domain fusion partners to interfere with FVIII clearance may be that the FVIII B domain tends to fold in a less structured and compact way compared to other proteins - thus mimicking the effect of large bulky groups frequently attached to therapeutic proteins to prolong the half life thereof, such as e.g. polymeric groups (e.g. poly ethylen glycol, poly sialic acid, dextran, starch, heparosan, etc.), XTENTM polypeptides, etc. It thus follows that FVIII B domain fusion partners according to the present invention may have a relatively low

tendency to form secondary and/or tertiary protein structures such as e.g. alpha helix, beta sheets and folded domains. Secondary structures can be measured by e.g. circular dichroism (CD). Another feature of the FVIII B domain is a tendency to be heavily glycosylated with N-glycans but likely also several O-glycans – this structure may provide the B domain with a large hydrodynamic volume in a similar manner as e.g. PEG polymers, polysaccharide polymers, etc.

Some FVIII B domain fusion partners according to the present invention may furthermore have the advantage of being relatively homogenous in structure – in the production cell line as well as in *in vivo* circulation. An example thereof is the 226 amino acid B domain fusion partner corresponding to SEQ ID NO 11 – this FVIII B domain fusion partner tends to avoid endo-proteolytical processing. The tendency to undergo this processing can be analysed and quantified using established techniques such as e.g. HPLC, SDS-PAGE, etc.

SEQ ID no. 5 (FVIII amino acid 741-769):

SFSQNSRHPSTRQKQFNATTIPENDIEKT

SEQ ID no. 6 (FVIII amino acid 741-794):

SFSQNSRHPSTRQKQFNATTIPENDIEKTPWFAHRTMPKIQNVSSDLLMLL

SEQ ID no. 7 (FVIII amino acid 741-857):

SFSQNSRHPSTRQKQFNATTIPENDIEKTPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL

SEQ ID no. 8 (FVIII amino acid 741-903):

SFSQNSRHPSTRQKQFNATTIPENDIEKTPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSS

SEQ ID no. 9 (FVIII amino acid 741-914):

SFSQNSRHPSTRQKQFNATTIPENDIEKTPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYD

SEQ ID no. 10 (FVIII amino acid 741-954):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMN

5

SEQ ID no. 11 (FVIII amino acid 741-966) "226" amino acid B domain:

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSS

10

SEQ ID no. 12 (FVIII amino acid 741-968):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTE

15

SEQ ID no. 13 (FVIII amino acid 741-1003):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVISLTKTNKT

20

SEQ ID no. 14 (FVIII amino acid 741-1009):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVISLTKTNKTSNNSAT

25

30

SEQ ID no. 15 (FVIII amino acid 741-1018):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT

35

LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGP

SEQ ID no. 16 (FVIII amino acid 741-1020):

5 SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSL

10

SEQ ID no. 17 (FVIII amino acid 741-1070):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
 15 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTS

SEQ ID no. 18 (FVIII amino acid 741-1079):

20 SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 25 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQ

SEQ ID no. 19 (FVIII amino acid 741-1206):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
 30 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGFTKDVGLKE
 35 MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQ

SEQ ID no. 20 (FVIII amino acid 741-1230):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
5 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
ARWIQRTHGKNSLNSGQGSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
10 MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
LL

SEQ ID no. 21 (FVIII amino acid 741-1261):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
15 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
20 ARWIQRTHGKNSLNSGQGSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRT

SEQ ID no. 22 (FVIII amino acid 741-1301):

25 SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
30 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
ARWIQRTHGKNSLNSGQGSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNT

SEQ ID no. 23 (FVIII amino acid 741-1309):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
5 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
10 LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQ

SEQ ID no. 24 (FVIII amino acid 741-1386):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
15 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMFLPES
20 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRS
25

SEQ ID no. 25 (FVIII amino acid 741-1395):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
30 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
35 LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE

KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTL
TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSS

SEQ ID no. 26 (FVIII amino acid 741-1444):

5 SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
10 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSEFFKMLFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTL
15 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
PAASYRKKDSGVQESSHFLQGAKNNLS

SEQ ID no. 27 (FVIII amino acid 741-1440):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
20 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSEFFKMLFLPES
25 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTL
TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
30 PAASYRKKDSGVQESSHFLQGAKK

SEQ ID no. 28 (FVIII amino acid 741-1461):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
35 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHFRPQLHHSGDMVFTPESEGLQL

RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
 5 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGFTKDVGLKE
 MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
 LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 10 PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGS

SEQ ID no. 29 (FVIII amino acid 741-1514):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPMKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPEGLQL
 15 RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGFTKDVGLKE
 20 MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
 LLSTRQNVESYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE
 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGS
 25 TVLPKPDLPKTSKGVELLPKVHIYQKDLFPTETSNNGS

SEQ ID no. 30 (FVIII amino acid 741-1524):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPMKIQNVSSDLLMLLRQ
 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPEGLQL
 30 RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDDTT
 LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
 ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGFTKDVGLKE
 35 MVFPSSRNLFNLNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF

LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTL
 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVEN
 5 TVLPKPDLPKTS GKV ELLPKVHIYQKDLFPTETSNGSPGHLDLVEGS

SEQ ID no. 31 (FVIII amino acid 741-1554):

FSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSSDLLMLLRQS
 PTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHFRPQLHHSGDMVFTPESGLQLR
 10 LNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDRTL
 FGKKSSPLTESGGPLSLSEENND SKLLESGLMNSQESSWGKNVSTESGRLFKGKRAHGP
 ALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTPLI
 HDRMLMDKNATALRLNHMSNKTTSKKNMEMVQQKKEGPIPPDAQNPDM SFFKMLFLPESA
 RWIQRTHGKNSLNSGQGSPKQLVSLGPEKSV EGQNFLSEKNKVVVGKGEFTKDVGLKEM
 15 VFPSSRNFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLFL
 STRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVEKY
 ACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTLT
 QIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 AASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVENT
 20 VLPKPDLPKTS GKV ELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEANR
 PGKVPFLRVATESS

SEQ ID no. 32 (FVIII amino acid 741-1595):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSSDLLMLLRQ
 25 SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSSEMTHFRPQLHHSGDMVFTPESGLQL
 RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDRT
 LFGKKSSPLTESGGPLSLSEENND SKLLESGLMNSQESSWGKNVSTESGRLFKGKRAHG
 PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVT
 LIHDRMLMDKNATALRLNHMSNKTTSKKNMEMVQQKKEGPIPPDAQNPDM SFFKMLFLPES
 30 ARWIQRTHGKNSLNSGQGSPKQLVSLGPEKSV EGQNFLSEKNKVVVGKGEFTKDVGLKE
 MVFPSSRNFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF
 LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
 KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETEEKRIIVDDTSTQWSKNMKHLTPSTL
 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
 35 PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVEN

TVLPKPDLPKTSKGVELLPKVHIYQKDLFPTETSNNGSPGHLDLVEGSLLQGTEGAIKWNEAN
RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPKTAFAKKK

SEQ ID no. 33 (FVIII amino acid 741-1643):

5 SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNSKLLSGLMNSQESSWGKNVSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
10 LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGKNFMKNLF
LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
15 TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
PAASYRKKDSGVQESSHFLQGAKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVEN
TVLPKPDLPKTSKGVELLPKVHIYQKDLFPTETSNNGSPGHLDLVEGSLLQGTEGAIKWNEAN
RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPKTAFAKKKDTILSL
NACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVL

20

SEQ ID no. 34 (FVIII amino acid 741-1648):

SFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTMPKIQNVSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESEGLQL
RLNEKLGTTAATELKKLDFKVSSTSNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
25 LFGKKSSPLTESGGPLSLSEENNSKLLSGLMNSQESSWGKNVSTESGRLFKGKRAHG
PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP
LIHDRMLMDKNATALRLNHMSNKTTSKNNMEMVQQKKEGPIPPDAQNPDMSSFFKMLFLPES
ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE
MVFPSSRNLFNLNDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGKNFMKNLF
30 LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRKHTAHFSKKGEEENLEGLGNQTKQIVE
KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL
TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL
PAASYRKKDSGVQESSHFLQGAKNNLSLAILTLEMTGDQREVGS LGTSATNSVTYKKVEN
TVLPKPDLPKTSKGVELLPKVHIYQKDLFPTETSNNGSPGHLDLVEGSLLQGTEGAIKWNEAN

RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPKTAFFKKKDTILSL
NACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHRQR

Fusion proteins: Fusion proteins according to the present invention are proteins
5 created through the in-frame joining of two or more DNA sequences which originally encoded
FVIII and the FVIII B domain fusion partner. Translation of the fusion protein DNA sequence
will result in a single protein sequence which may have functional properties derived from
each of the original proteins or peptides. DNA sequences encoding fusion proteins may be
created by standard molecular biology methods such as overlapping PCR or DNA ligation –
10 the FVIII B domain fusion partner according to the present invention can be inserted at any
position and in any domain or region as long as FVIII activity is preserved. The resulting
fusion protein DNA sequence may be inserted into an appropriate expression vector that
supports the heterologous fusion protein expression in a standard host organism.

Fusion proteins may furthermore contain a linker or spacer peptide sequence that
15 separates the protein or peptide parts which define the fusion protein. The linker or spacer
peptide sequence may facilitate the correct folding of the individual protein or peptide parts
and may make it more likely for the individual protein or peptide parts to retain their individual
functional properties. Linker or spacer peptide sequences may be inserted into fusion protein
DNA sequences during the in frame assembly of the individual DNA fragments that make up
20 the complete fusion protein DNA sequence i.e. during overlapping PCR or DNA ligation.
Examples of linker sequences according to the invention comprising repetitive GS residues
include e.g.: (GS)_n, (GGGS)_n, (GGGGGS)_n, (GGGS)_n(GGG), etc. The linker can thus be very
short, e.g. 1, 2, 3, 4, 5, 6, 7, or 8 amino acids or somewhat longer, e.g. 5-10, 5-15, 5-20, 5-
30, 5-50, 10-15, 10-20, 10-30, 10-40, 10-50, 15-20, 15-30, 15-40, 15-50 amino acids.

Pharmaceutical compositions:

The present invention provides compositions and formulations comprising one or
more FVIII fusion proteins of the invention, formulated together with one or more
pharmaceutically acceptable carrier(-s).

30 Accordingly, one object of the invention is to provide a pharmaceutical formulation
comprising a protein according to the invention present in a concentration from 0.25 mg/ml to
250 mg/ml, and wherein said formulation has a pH from 2.0 to 10.0. The formulation may
further comprise one or more of a buffer system, a preservative, a tonicity agent, a chelating
agent, a stabilizer, or a surfactant, as well as various combinations thereof. The use of

preservatives, isotonic agents, chelating agents, stabilizers and surfactants in pharmaceutical compositions is well-known to the skilled person. Reference may be made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In one embodiment, the pharmaceutical formulation is an aqueous formulation. Such a formulation is typically a solution or a suspension, but may also include colloids, dispersions, emulsions, and multi-phase materials. The term "aqueous formulation" is defined as a formulation comprising at least 50% w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50 % w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50 %w/w water.

In another embodiment, the pharmaceutical formulation is a freeze-dried formulation, to which the physician or the patient adds solvents and/or diluents prior to use. In a further aspect, the pharmaceutical formulation comprises an aqueous solution.

Pharmaceutical compositions according to the present invention are preferably suitable for intravenous (IV) and/or extravascular administration (e.g. subcutaneous (sc) or intradermal administration) in prophylactic/therapeutic treatment of haemophilia.

"Haemophilia": Haemophilia/hemophilia/blood clotting diseases is a group of hereditary genetic disorders that impair the body's ability to control blood clotting or coagulation ("bleeding disorders"), which is used to stop bleeding when a blood vessel is broken. Haemophilia A (clotting factor VIII deficiency) is the most common form of the disorder, present in about 1 in 5,000–10,000 male births.

List of embodiments:

It is understood that all aspects and embodiments of the invention can be combined and that they are not to be understood in any limiting way.

Embodiment 1: A FVIII fusion protein, comprising a FVIII molecule with a B domain (a full length or a truncated B domain), wherein said FVIII molecule is fused to a FVIII B domain fusion partner, wherein said FVIII B domain fusion partner is derived from the FVIII B domain and comprises 100-908 amino acids.

Embodiment 2: A FVIII fusion protein according to the invention, wherein said FVIII molecule is a B domain truncated molecule with a B domain comprising 0-908, 4-908 or 15-25 amino acids. The FVIII B domain may be selected from the list consisting of SEQ ID no 2, 3, and 4.

Embodiment 3: A FVIII fusion protein according to the invention, wherein the FVIII B domain fusion partner is a truncated FVIII B domain.

Embodiment 4: A FVIII fusion protein according to the invention, wherein said FVIII molecule is a B domain truncated FVIII molecule comprising a B domain amino acid
5 sequence selected from the group consisting of SEQ ID NO 2, SEQ ID NO 3, and SEQ ID NO 4.

Embodiment 5: A FVIII fusion protein according to the invention, wherein said FVIII B domain fusion partner is fused to the C-terminal end of the FVIII B domain.

Embodiment 6: A FVIII fusion protein according to the invention, wherein said FVIII
10 B domain fusion partner is fused to the N-terminal end of the FVIII light chain.

Embodiment 7: A FVIII fusion protein according to the invention, wherein the FVIII B domain fusion partner is fused to the C-terminal end of the FVIII light chain. The FVIII B domain fusion partner can also be inserted into one or more domains within the heavy chain and/or the light chain as long as the resulting fusion protein maintains FVIII activity.

Embodiment 8: A FVIII fusion protein according to the invention, wherein said FVIII
15 B domain fusion partner comprises the amino acid sequence selected from the list consisting of: SEQ ID No 5, SEQ ID No 6, SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, SEQ ID No 10, SEQ ID No 11, SEQ ID No 12, SEQ ID No 13, SEQ ID No 14, SEQ ID No 15, SEQ ID No 16, SEQ ID No 17, SEQ ID No 18, SEQ ID No 19, SEQ ID No 20, SEQ ID No 21, SEQ ID No
20 22, SEQ ID No 23, SEQ ID No 24, SEQ ID No 25, SEQ ID No 26, SEQ ID No 27, SEQ ID No 28, SEQ ID No 29, SEQ ID No 30, SEQ ID No 31, SEQ ID No 32, SEQ ID No 33, and SEQ ID No 34.

Embodiment 9: A FVIII fusion protein according to the invention, wherein said FVIII B domain fusion partner has a low degree of secondary and tertiary structures. Secondary
25 and tertiary structure can be measured by e.g. Circular Dichroism (CD). Based on e.g. a CD wavelength spectrum from 190-260 nm, wherein the fingerprint of the FVIII B domain fusion partner resembles that of a disordered polypeptide having a signal minimum of 204 nm. Preferably, the FVIII B domain fusion partner is homogenous in structure in *in vivo* circulation upon administration to the patient in need thereof. An example of a protein according to the
30 invention having a homogenous structure is a FVIII protein fused to the FVIII B domain fusion partner according to SEQ ID NO 11.

Embodiment 10: A pharmaceutical composition comprising a FVIII fusion protein according to the invention.

Embodiment 11: A method for making a FVIII fusion protein according to the
35 invention, wherein said method comprises the step of incubating a host cell under

appropriate conditions, wherein said host cell comprises an expression vector that encodes a FVIII fusion protein according to the invention.

Embodiment 12: A nucleotide molecule encoding a FVIII fusion protein according to the invention.

5 Embodiment 13: An expression vector comprising a nucleotide molecule according to the invention.

Embodiment 14: A host cell comprising an expression vector according to the invention.

10 Embodiment 15: A FVIII fusion protein according to the invention, or a pharmaceutical formulation according to the invention, for use as a medicament for treatment of haemophilia.

Embodiment 16: A FVIII fusion protein according to the invention, or a pharmaceutical composition according to the invention, for treatment of haemophilia by subcutaneous administration.

15 Embodiment 17: A FVIII fusion protein according to the invention, or a pharmaceutical formulation according to the invention, for treatment of haemophilia by intravenous administration.

Embodiment 18: A FVIII fusion protein according to the invention, wherein said protein comprises the amino acid sequence selected from the list consisting of: SEQ ID NO
20 35, SEQ ID NO 36, and SEQ ID NO 37.

EXAMPLES

25 While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

EXAMPLE 1**Expression constructs encoding FVIII with a B domain sequence fused to the light chain C-terminus**

5 For generation of plasmids encoding FVIII with a B domain sequence fused to the light chain C-terminus, we utilized a plasmid named #1742 consisting of the mammalian expression vector pTT5 (Durocher et al., Nucleic Acids Research, Vol 30, No 2 edition 9 (2002)) with an insert encoding the "F8-500-C2-linked-GS BDD FVIII" molecule. Starting at the N-terminus, the F8-500 protein is synthesized from an expression vector encoding a B domain truncated
10 FVIII molecule comprising a heavy chain a 21 amino acid B domain linker (SFSQNSRHPSQNPPVLKRHQR – SEQ ID NO 2), and the FVIII light chain (amino acids 1649-2332 of full-length wild-type human FVIII). The sequence of the 21 amino acid linker is derived from the FVIII B domain and consists of amino acids 741-750 and 1638-1648 of full length wild-type human FVIII. The F8-500-C2-linked-GS BDD FVIII molecule is identical to
15 F8-500 except that a glycine-serine (GS) dipeptide has been added to the C-terminus of the FVIII light chain. This has been done by adding a Bam HI restriction site encoding the GS dipeptide immediately upstream to the stop signal in the F8-500 coding open reading frame. Immediately down-stream to the same stop signal, the #1742 plasmid contains a Not I restriction site. Thus, DNA encoding fusion partners can be introduced at the C-terminus of
20 the FVIII molecule encoded by plasmid #1742 by insertion of DNA encoding the fusion partner in Bam HI and Not I digested plasmid #1742.

A PCR fragment of FVIII encoding DNA encoding FVIII amino acid 741-966 followed by a stop codon was amplified with an oligonucleotide forward primer containing a Bam HI
25 restriction site and an oligonucleotide reverse primer containing a Not I site. Bam HI and Not I digested PCR fragment was ligated into Bam HI and Not I digested plasmid #1742 giving rise to plasmid #2149. Thus, plasmid #2149 consists of pTT5 with insert encoding F8-500-C2-linked-G-FVIII(741-966) (SEQ ID no 35). This FVIII molecule consists of the F8-500 protein described above with a G residue followed by FVIII residue 741-966 fused to the C
30 terminus of the FVIII light chain.

Subsequently, the Bam HI/Not I fragment from plasmid #2149 was transferred to plasmid #1679. The latter plasmid consists of pTT5 with insert encoding the F8-500-C2-linked-(GGGS)₆ BDD FVIII molecule. The F8-500-C2-linked-(GGGS)₆ BDD FVIII molecule is
35 identical to F8-500 except that a (GGGS)₆ peptide has been added to the C-terminus of the

FVIII light chain. The six nucleotides immediately upstream to the stop signal constitute a Bam HI restriction site. Immediately down-stream to the same stop signal, the #1742 plasmid contains a Not I restriction site. Thus, DNA encoding fusion partners can be introduced at the C-terminus of the FVIII molecule encoded by plasmid #1679 by insertion of DNA encoding the fusion partner in Bam HI and Not I digested plasmid #1679. Insertion of the Bam HI/Not I fragment from plasmid #2149 into plasmid #1679 resulted in plasmid #2185 consisting of pTT5 with insert encoding encoding F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) (SEQ ID no 36). This FVIII molecule consists of the F8-500 protein described above with a (GGGS)5-GGG linker followed by FVIII residue 741-966 fused to the C terminus of the FVIII light chain.

SEQ ID no. 35 (F8-500-C2-linked-G-FVIII(741-966)) - described in Example 1:

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRPVKSFPFNTSVVYKKTLFVEFTDHLFNIA
 KPRPPWMGLLGPTIQAEVYDTVITLKNMASHPVSLHAVGVSYWKASEGAHEYDDQTSQRE
 KEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGS
 LAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLP
 GLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQF
 LLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSP
 SFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLV LAPDDRSYKSQYLNNGPQRIGRKYKKVRF
 MAYTDETFKTREAIQHESGILGPLLYGEVGD TLLIIFKNQASRPYNIYPHGITDVRPLYSRRLP
 KGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYSSFVNMERDLASGLIGPLLCY
 KESVDQQRGNQIMSDKRNVLFSVFDENRSWYL TENIQRFLPNPAGVQLEDPEFQASNIMHSI
 NGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKVMYEDTLTLFPFSGETVF
 MSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPR
 SFSQNSRHP SQNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPR
 SFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYR
 GELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPKRFVVKPNE
 TKTYFWKVQH HMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLL VCHTNTLNPAHGRQV
 TVQEFAFFTIFDETKSWYFTENMERNCRAPCN IQMEDPTFKENYRFHAINGYIMDTLPGLV
 MAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGI
 WRVECLIGEHLHAGMSTFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYS
 GSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNS
 TGTLMVFFGNVDSSGIKHNFNPPIIARYIRLHP THYSIRSTLRMELMGCDLNSCSMPLGMES
 KAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKV TG
 VTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVVFQGNQDSFTPVVNSLDPPLLT

RYLRIHPQSWVHQIALRMEVLGCEAQDLYGSFSQNSRHPSTRQKQFNATTIPENDIEKTD
WFAHRTMPKIQNVSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNLSLSE
MTHFRPQLHHSGDMVFTPESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAA
GTDNTSSLGPPSMPVHYDSQLDITLFGKKSSPLTESGGPLSLSEENNSKLLSGLMNSQE
5 SSWGKNVSS

SEQ ID no. 36 (F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966)) - described in Example 1:

ATTRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSVVYKKTLFVEFTDHLFNIA
10 KPRPPWMGLLGPTIQAEVYDTVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQRE
KEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGS
LAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLP
GLIGCHRSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQF
LLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNPS
15 SFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLV LAPDDR SYKSQYLNNGPQRIGRKYKKVRF
MAYTDETFKTREAIQHESGILGPLLYGEVGDITLLIFKNQASRPYNIYPHGITDVRPLYSRRLP
KGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSFVNMERDLASGLIGPLLCY
KESVDQQRGNQIMSDKRNVLFSVFDENRSWYL TENIQRFLPNPAGVQLEDPEFQASNIMHSI
NGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDITLTFPFSGETVF
20 MSMENPGLWLWGCHNSDFRNRGMTALLKVSSCDKNTGDYEDSYEDISAYLLSKNNAIEPR
SFSQNSRHPSQNPPVLKRHQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPR
SFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYR
GELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPKRFVKNPNE
TKTYFWKVQHMAPTKDEFDCKAWAYFSDVDLEKDVHSGLIGPLLCHTNTLNPAHGRQV
25 TVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHANGYIMDTLPGLV
MAQDQIRWYLLSMGSNENIHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGI
WRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYS
GSINAWSTKEPFSWIKVDLLAPMIIHGKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNS
TGTLMVFFGNVDSSGIKHNFNPPIIARYIRLHPHTHYSIRSTLRMELMGCDLNSCSMPLGMES
30 KAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTG
VTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGKVVFQGNQDSFTPVVNSLDPPLLT
RYLRIHPQSWVHQIALRMEVLGCEAQDLYGGGSGGGSGGGSGGGSGGGSGGGSGFSQNS
RHPSTRQKQFNATTIPENDIEKTDWFAHRTMPKIQNVSSDLLMLLRQSPTPHGLSLSDL
QEAKYETFSDDPSPGAIDSNNLSLSEMTFRPQLHHSGDMVFTPESGLQLRLNEKLGTTAAT

ELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTTLFGKKSSPLTES
GGPLSLSEENNSKLLSGLMNSQESSWGKNVSS

SEQ ID no. 37 (F8-500-LCN-FVIII-(741-966)) - described in Example 8:

5 ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRPVKSFPFNTSVVYKKTLFVEFTDHLFNIA
KPRPPWMGLLGPTIQAEVYDTVITLKNMASHPVSLHAVGVSYWKASEGAEYDDQTSQRE
KEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALLVCREGS
LAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGYVNRSLP
GLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLLMDLGQF
10 LLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDVVRFDDDNSP
SFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLV LAPDDRSYKSQYLNNGPQRIGRKYKKVRF
MAYTDETFKTREAIQHESGILGPLLYGEVGD TLLIIFKNQASRPYNIYPHGITDVRPLYSRRLP
KGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYSSFVNMERDLASGLIGPLLCY
KESVDQQRGNQIMSDKRNVLFSVFDENRSWYL TENIQRFLPNPAGVQLEDPEFQASNIMHSI
15 NGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPFSGETVF
MSMENPGLWLIGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKNNAIEPR
SFSQNSRHPSQNPPVLKRHQRSFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPM
PKIQNVSSDLLMLLRQSPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQL
HHS GDMVFTPESGLQLRLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSL
20 GPPSMPVHYDSQLDTTLFGKKSSPLTESGGPLSLSEENNSKLLSGLMNSQESSWGKNV
SSEITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDY
GMSSSPHVLNRNAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDN
IMVTFRNQASRPYSFYSSLISYEEDQRQGAEPKKNFVKPNETKTYFWKVQH HMAPTKDEFD
CKAWAYFSDVDLEKDVHSGLIGPLL VCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTE
25 NMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQIRWYLLSMGSNENIH
SIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLV
YSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLA
PMIIHGKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKH NIF
NPPIIARYIRLHPHTHYSIRSTLRMELMGCDLN SCSMPLGMESKAISDAQITASSYFTNM FATW
30 SPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLIS
SSQDGHQWTLFFQNGKV KVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVL
GCEAQDLY

EXAMPLE 2**Transient expression of FVIII with a B domain sequence fused to the light chain C-terminus**

5 HKB11 cells at a density of $0.9 - 1.1 \times 10^6$ were transfected with a complex of plasmid (0.7 mg/l or 1.0 mg/l) and the transfection agent, 293Fectin (Invitrogen) (1.0 ml/l or 1.4 ml/l). The transfection complex was prepared by diluting the plasmid and the transfection separately, mixing the two solutions, and incubating the mixture at room temperature for 20 minutes. The complex mixture was added to the cell suspension and the suspension was incubated in
10 shaker incubator for 4 or 5 days at 36.5 °C or 37 °C and at 5 % or 8 % CO₂. Cell culture supernatants were analysed by chromogenic FVIII assay as described in Example 3 and/or filtered through a 0,22 µm membrane filter and utilized for purification of FVIII variant as described in Example 4.

15 EXAMPLE 3**FVIII:C in cell culture supernatants measured by chromogenic assay**

The FVIII activity (FVIII:C) of the rFVIII compound was evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and a FVIII
20 standard (Coagulation reference, Technoclone) were diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1 % BSA, pH 7.3, with preservative). Fifty µl of samples, standards, and buffer negative control were added to 96-well microtiter plates (Spectraplates MB, Perkin Elmer). All samples were tested diluted 1:100, 1:400, 1:1600, and 1:6400. The factor IXa/factor X reagent, the phospholipid reagent and CaCl₂ from the Coatest SP kit were mixed
25 5:1:3 (vol:vol:vol) and 75 µl of this added to the wells. After 15 min incubation at room temperature, 50 µl of the factor Xa substrate S-2765/thrombin inhibitor I-2581 mix was added and the reactions were incubated 5 min at room temperature before 25 µl 1 M citric acid, pH 3, was added. The absorbance at 405 nm was measured on an Envision microtiter plate reader (Perkin Elmer) with absorbance at 620 nm used as reference wavelength. The value
30 for the negative control was subtracted from all samples and a calibration curve prepared by linear regression of the absorbance values plotted vs. FVIII concentration. The specific activity was calculated by dividing the activity in each supernatant with the FVIII antigen concentration determined by ELISA. The ELISA was a sandwich ELISA using polyclonal sheep anti-FVIII antibody for catching and the same but HRP-conjugated antibody for
35 detection (Matched-Pair Antibody Set for ELISA of human Factor VIII antigen, Affinity

Biologicals, cat. no. F8C-EIA). Purified FVIII "F8-500" was used as calibrator. Results shown in Table 2 demonstrate that FVIII activity of the fusion protein according to the invention is maintained.

5 **Table 2. Specific activities of FVIII molecules in supernatant from transiently transfected HKB11**

FVIII molecule	Specific activity (mU/ng)
F8-500	14.5
F8-500-C2-linked-G-FVIII(741-966) SEQ ID NO 35	9.2*
F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) SEQ ID NO 36	8.3*

*The specific activity could potentially be underestimated due to overestimation of the concentration of FVIII with a B domain fusion partner as additional epitopes in the B-domain fusion partner may be included in the measurement (polyclonal antibodies are used in the ELISA).

EXAMPLE 4

Purification of FVIII molecules

15 Purification of F8-500 from the supernatant of transiently transfected HKB11 cells was carried out by a three step purification method using: Affinity chromatography on a VIIISelect column (GE Health Care) followed by affinity chromatography on an F25 column (Novo Nordisk A/S) and an anionic exchange chromatography column, Poros 50 HQ (GE Health Care).

20 A volume of 1700 ml cell culture media as pumped onto a VIIISelect column (2.6 x 3.5 cm, Vol= 7 ml) at 3 ml/min. Prior to the application the column was equilibrated with a buffer consisting of: 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 250 mM NaCl, pH=7.3. The column was washed with a buffer consisting of: 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1.5 M NaCl, pH=7.3. F8-500 was eluted with a buffer consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 1M ammonium acetate, 6.5 M 1,2-propanediol, pH=7.3.

30 The eluted F8-500 protein ended up in a pool volume of 14 ml which was diluted with 140 ml buffer consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, pH=7.3 and

thereafter pumped onto a F25 affinity column (2 x 2.5 cm, Vol= 5 ml). Prior to the application the column was equilibrated with a buffer consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 150 mM NaCl, 1 M glycerol, pH=7.3. The column was washed with a buffer consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 650 mM NaCl, pH=7.3. The bound FVIII protein was eluted with 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 2.5 M NaCl, 50% ethylenglycol, pH=7.3.

The eluted F8-500 protein ended up in a pool volume of 10 ml. This pool was diluted with 290 ml buffer consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, pH=7.3 and pumped onto a Poros 50 HQ column (0.5 x 6 cm, Vol= 1.2 ml). Prior to the application the column was equilibrated with buffer A: consisting of 20 mM imidazole, 10 mM CaCl₂, 0.02% Tween 80, 50 mM NaCl, 1 M glycerol pH=7.3. After application the column was eluted with a linear gradient from buffer A to buffer B over 5 column volumes. Buffer B is identical to buffer A except for the NaCl which is 1 M in buffer B. The eluted F8-500 protein ended up in a pool of 1 ml.

The overall yield using this 3 step purification procedure was close to 40%. The purity of the purified F8-500 protein was analysed by HPLC and SDS-gel electrophoresis essentially as previously described (Thim, L. et al. Haemophilia 16 (2010), 349-359).

Purification of F8-500-C2-linked-(GGGS)₅-GGG-FVIII(741-966) from the supernatant of transiently transfected HKB11 cells was carried out by a two-step purification method using affinity chromatography on a VIIISelect column (GE Health Care) followed by an anionic exchange chromatography column, Poros 50 HQ (GE Health Care). This purification method was identical to the method described above for purification of F8-500 with two exceptions: 1) The F25 affinity chromatography step is omitted and 2) in the anionic exchange chromatographic step on the Poros 50 HQ column the elution from the column is carried out with a gradient over 10 column volumes instead of 5 column volumes.

The overall yield using this 2 step purification procedure was close to 50%. The purity of the from the supernatant of transiently transfected HKB11 cells was analysed by HPLC and SDS-gel electrophoresis essentially as previously described (Thim, L. et al. Haemophilia 16 (2010), 349-359).

EXAMPLE 5**FVIII:C in purified samples measured by chromogenic assay**

The FVIII activity (FVIII:C) of the rFVIII compound was evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) were diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1 % BSA, pH 7.3, with preservative). Fifty μ l of samples, standards, and buffer negative control were added to 96-well microtiter plates (Nunc) in duplicates. The factor IXa/factor X reagent, the phospholipid reagent and CaCl_2 from the Coatest SP kit were mixed 5:1:3 (vol:vol:vol) and 75 μ l of this added to the wells. After 15 min incubation at room temperature 50 μ l of the factor Xa substrate S-2765/thrombin inhibitor I-2581 mix was added and the reactions incubated 10 min at room temperature before 25 μ l 1 M citric acid, pH 3, was added. The absorbance at 415 nm was measured on a Spectramax microtiter plate reader (Molecular Devices) with absorbance at 620 nm used as reference wavelength. The value for the negative control was subtracted from all samples and a calibration curve prepared by curve fitting of the absorbance values plotted vs. FVIII concentration. The specific activity was calculated by dividing the activity of the samples with the protein concentration determined by HPLC. For HPLC, the concentration of the sample was determined by integrating the area under the peak in the chromatogram corresponding to the light chain and compare with the area of the same peak in a parallel analysis of a wild-type rFVIII, where the concentration was determined by amino acid analyses. The results shown in Table 3 demonstrate the FVIII with a B domain sequence fused to the light chain C-terminus has FVIII activity.

Table 3. Specific activities of purified FVIII molecules

Compound	Specific activity measured by chromogenic assay (example 5) (IU/mg)	Specific activity measured by one-stage clot assay (Example 6) (IU/mg)
F8-500	14944	13365
F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) SEQ ID NO 36	16506	10982

EXAMPLE 6**FVIII:C in purified samples measured by one-stage clot assay**

FVIII:C of the rFVIII compounds was further evaluated in a one-stage FVIII clot assay as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) were diluted in HBS/BSA buffer (20 mM hepes, 150 mM NaCl, pH 7.4 with 1 % BSA) to approximately 10 U/ml followed by 10-fold dilution in FVIII-deficient plasma containing VWF (Dade Behring). The samples were subsequently diluted in HBS/BSA buffer. The APTT clot time was measured on an ACL300R or an ACL5000 instrument (Instrumentation Laboratory) using the single factor program. FVIII-deficient plasma with VWF (Dade Behring) was used as assay plasma and SynthASil, (HemosIL™, Instrumentation Laboratory) as aPTT reagent. In the clot instrument, the diluted sample or standard is mixed with FVIII-deficient plasma, aPTT reagents at 37°C. Calcium chloride is added and time until clot formation is determined by turbidity. The FVIII:C in the sample is calculated based on a standard curve of the clot formation times of the dilutions of the FVIII standard. The results are shown in table 3.

EXAMPLE 7**Pharmacokinetic evaluation of FVIII with a B domain sequence fused to the light chain C-terminus**

The pharmacokinetics of F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) and F8-500 both produced from transiently transfected HKB11 cells were evaluated in FVIII deficient mice (FVIII KO mice) and in VWF deficient mice (VWF KO mice).

FVIII deficient mice (FVIII exon 16 knock out mice with mixed C57Bl/6 and SV129 background, bred at Taconic M&B, (B6.129S4-F8tm1Kaz/J) and VWF-deficient mice (VWF exon 4 + 5 KO mice with C57Bl/6 background ((B6.129S2-VWFtm1Wgr/J) bred at Charles River, Germany) were included in the study. The VWF deficient mice had app. 13% of normal FVIII chromogenic activity, while the FVIII-KO mice had no detectable FVIII chromogenic activity.

A mixture of male and female mice (approximately 1:1) with an approximate weight of 25 grams and age range of 15-28 weeks were used. The mice received a single i.v. dose of either of the two FVIII variants (280 IU/kg) in the tail vein (n=6). Blood was taken using non-

coated capillary glass tubes from the orbital plexus at regular time points in the interval of 0.08-3 or 0.08-7 h post-administration, for compound F8-500-C2-linked-(GGGS)6-FVIII(741-966) and F8-500 respectively, in VWF deficient mice and at 5, 24, 30 or 5, 24, 48 h post administration for compound F8-500 and F8-500-C2-linked-(GGGS)6-FVIII(741-966),
5 respectively, in FVIII deficient mice. Three blood samples were taken from each mouse, and 2 to 63 samples were obtained at each time point. Blood was immediately stabilized with sodium citrate and diluted in four volumes FVIII Coatest SP buffer before 5 min centrifugation at 4000g. Plasma obtained from diluted blood was frozen on dry ice and kept at -80°C prior to analysis in the FVIII chromogenic activity assay (plasma from FVIII deficient mice) and in a
10 FVIII antigen based LOCI assay (plasma from VWF KO mice).

Briefly, the FVIII chromogenic activity assay was analysed using CoatestSP FVIII kit, Chromogenix (#82408663). Calibration was done using purified FVIII (N8-SRM standard, batch 307.7008.09.2, Novo Nordisk) diluted appropriately in Coatest buffer to produce the
15 following calibrator points 0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mU/ml. Minimal required dilution of plasma samples was 1:80. All samples were analysed in duplicates in four different dilutions (1:80, 1:240, 1:720 and 1:2160) and the result was reported as mean of dilutions within the calibration range plus %CV of these results. QC samples: Control plasma N (ORKE 41, Siemens Health care diagnostics product GmbH) diluted appropriately extra
20 high, high, medium and low level. In short the assay was carried out by mixing 50 µl calibrator or sample with 75 µl FIX mixture (FIXa/X, phospholipid and CaCl₂ reagent from the kit mixed 5+1+3). After 15 min incubation time, 50 µl Xa substrate (S-2765) was added and the enzymatic reactions were stopped after 10-15 min by addition of 25 µl stop buffer. All samples, calibrators and QC-samples were analysed in duplicates. Absorbance was read at
25 405 nm with 620 nm as reference using Tecan Infinite reader M200, and Magellan 6.6 for data transformation. The lower limit of quantification (LLOQ) in the plasma was 34 mU/ml. The calibration curve was fitted using 4 Parametric logistic (4PL Marquardt) in Magellan and sample results were obtained by interpolation from the calibration curve. Interpolation and estimation of %CV of duplicates was done by Magellan. %CV of double replicates should be
30 <10, %CV of double samples in different dilutions should be <25.

The FVIII antigen based LOCI assay was a homogenous bead based assay. LOCI reagents included two latex bead reagents and a biotinylated anti-rFVIII light chain antibody (hF8-4F11, Novo Nordisk). One of the bead reagents was a generic reagent (D-beads) and was
35 coated with streptavidin and contains a photosensitive dye. The second bead reagent (A-

beads) was coated with another anti-FVIII light chain antibody (hF8-4F45, Novo Nordisk). 5 µl diluted sample/calibrator/control was added to A-Screenplate-384 followed by 15 µl mixture of biotin-hF8-4F11, hF8-4F45-A-beads. Incubation at RT for 1 hour before 30 µl D-beads was added. Incubation for 30 minutes before reading. During the assay the three reactants combined with FVIII or FVIII analogues to form a bead-aggregate-immune complex. Illumination of the complex released singlet oxygen from the D-beads which channels into the A-beads and triggered chemiluminescence which was measured in the EnVision plate reader. The amount of light generated was proportional to the antigen concentration of FVIII. The LLOQ in plasma was 100 IU/L.

Pharmacokinetic analysis of mean plasma concentration versus time dataset for the VWF deficient mice was carried out by non-compartmental methods (NCA) using WinNonlin. For the FVIII deficient mice, the evaluation of the half-life was based on log-linear regression of the individual plasma concentrations versus time using SAS JMP.

Table 4 and 5 list the FVIII activity levels at the specified time points for the two compounds dosed to VWF and FVIII deficient mice. Table 6 and 7 list the estimated pharmacokinetic parameters. In both of the mice strains F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) has a slower elimination, i.e. a longer $t_{1/2}$ as compared to F8-500.

Table 4: FVIII activity in FVIII-KO mice (FVIII chromogenic activity in IU/ml), na=not available

FVIII	FVIII activity in samples from mice ¹ (IU/mL)			
	5 h	24 h	30 h	48 h
F8-500	2.31	0.22	0.12	na
	1.93	0.13	0.04	na
	1.84	0.13	0.06	na
	2.53	0.2	0.11	na
	2.86	0.37	0.22	na
	2.82	0.38	0.22	na
F8-500-C2-linked- (GGGS)5-GGG- FVIII(741-966) SEQ ID NO 36	2.72	0.63	na	0.14
	2.91	0.71	na	0.13
	2.40	0.51	na	0.12
	2.89	0.75	na	0.23

	2.27	0.36	na	0.04
	2.86	0.40	na	0.07

1) each line represent data from one individual mouse

Table 5: FVIII activity in VWF deficient mice (antigen concentrations in IU/L). na= not available, < indicates FVIII activity below (LLOQ)

FVIII	FVIII antigen levels in samples from mice ¹ (IU/L)							
	0.08 h	0.33 h	0.5 h	0.66 h	1 h	2 h	3 h	4 h
F8-500-C2-linked-(GGGS)5-GGG-FVIII (741-966) SEQ ID NO 36	2700	1280	na	1050	1810	580	439	910
	2720	1280	na	1100	1920	585	476	950
F8-500	1250	499	250	na	114	na	na	na
	970	338	154	na	<	na	na	na
	1280	460	359	na	<	na	na	na

5 1) each line represent data from one individual mouse

Table 6: Estimated pharmacokinetic parameters after i.v. administration to VWF deficient mice (based on FVIII antigen data)

Compound	C _{max}	t _½	CL	MRT
	(IU/L)	(h)	(mL/h/kg)	(h)
F8-500	1167	0.36	537	0.4
F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) SEQ ID NO 36	2710	2.4	38.3	4.1

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Table 7: Estimated t_½ and 95% CI on the estimated t_½ after i.v. administration to FVIII deficient mice (based on chromogenic activity data)

	t _½	95% CI
	(h)	(h)
F8-500	5.6	5.1-6.2
F8-500-C2-linked-(GGGS)5-GGG-FVIII(741-966) SEQ ID NO 36	9.4	8.2-11

EXAMPLE 8**Expression construct encoding FVIII with a B domain sequence fused to the light chain N-terminus**

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The mammalian expression vector pGB445 with insert encoding the FVIII molecule F8-500-LCN-FVIII(741-966) was generated as described in the following. The construction of pGB445 was carried out by combining DNA sequences from pJSV331 and pGB368.

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pJSV331 consists of pQMCF-5 with insert encoding F8-500. pGB368 consists of pTT5 with insert encoding amino acid 741-966 of human FVIII. A PCR product containing the entire nucleotide sequence of pJSV331 was generated from pJSV331 using Phusion U DNA polymerase (Thermo Fisher Scientific), the forward primer

AGATCACUCGTACTACTCTTCAGTCTGA (**SEQ ID no 38**), and the reverse primer

AGCTCCGUTGATGGCGTTTCAAGACC (**SEQ ID no 39**). In this PCR product, the pJSV331

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sequence starts with the codon encoding amino acid 1649 at the N-terminus of the FVIII light chain and ends with the codon encoding amino acid 1648 at the C-terminus of the FVIII

heavy chain. Likewise, a PCR product containing the nucleotide sequence encoding

FVIII(741-966) was generated from pGB368 using Phusion U DNA polymerase, the forward

primer ACGGAGCUTTAGCCAGAACAGCAGAC (**SEQ ID no 40**), and the reverse primer

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AGTGATCUCGGAGGACACGTTCTTGCC (**SEQ ID no 41**). The two overlapping PCR

products were joined at both ends with the Uracil-Specific Excision Reagent enzyme (New

England Biolabs) giving rise to the pGB445 plasmid consisting of pQMCF-5 with insert

encoding F8-500-LCN-FVIII(741-966). This molecule consists of the F8-500 FVIII molecule

described in Example 1 fused to amino acid 741-966 of FVIII at the N-terminus of the FVIII

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light chain (SEQ ID no 37).

EXAMPLE 9**Generation of concentrated cell culture supernatants containing FVIII with a B domain sequence fused to the light chain N-terminus**

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Portions of 1×10^7 CHOEBNALT85 cells in 700 ml 1:1 CD CHO and SFM II medium

(Thermo Fisher Scientific) were electroporated with 10 mg pGB445 in a Gene Pulser Cuvette

(BioRad) using a GenePulser Xcell (Biorad). Subsequently, the cells were transferred to 125

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ml Erlenmeyer flasks with 30 ml of the above growth medium and incubated in a shaker

incubator at 36.5 °C and 8 % CO₂. Five days after transfection, the culture volumes were increased 20 % by addition of CHO CD Efficient Feed B (Thermo Fisher Scientific), and the incubation temperature was reduced to 30 °C. After 5 days of incubation at 30 °C, cell culture supernatants were collected and pooled. Proteins in the cell culture supernatant were concentrated by centrifugation of the supernatant in Ultra-15 Centrifugal Filter Devices with 50 K Nominal Molecular Weight Limit (Amicon) leading to approximately 10 fold reduction in the cell culture supernatant volume. The concentrated cell culture supernatants were frozen with 20 mM imidazol pH 7.0 (Sigma) and 0.2 % Tween 80 (Merck) and utilized for pharmacokinetic analysis of F8-500-LCN-FVIII(741-966) (Example 10). Cell culture supernatant was analysed by chromogenic FVIII assay as described in Example 3 and a specific activity of 5.8 mU/ng was calculated. The specific activity of F8-500-LCN-FVIII(741-966) could potentially be underestimated due to overestimation of the concentration of FVIII with a B domain fusion partner as additional epitopes in the B-domain fusion partner may be included in the measurement (polyclonal antibodies are used in the ELISA).

EXAMPLE 10

Pharmacokinetic study of FVIII with a B domain sequence fused to the light chain N-terminus in concentrated cell culture supernatant

Pharmacokinetic study of the F8-500-LCN-FVIII-(741-966) FVIII molecule was carried out essentially as described in Example 7, except that the FVIII compound was administered as concentrated cell culture supernatant.

Blood was taken using non-coated capillary glass tubes from the orbital plexus at 0.08, 0.5, 1, 3, 5, 18, 24, 30 and 48 h post-dose from FVIII deficient mice.

Pharmacokinetic analysis of plasma FVIII activity levels versus time dataset was modelled using the non-linear mixed effects approach (Phoenix NLME 1.3).

Table 8 lists the FVIII activity levels at the specified time points for the compound dosed to FVIII deficient mice while table 9 lists the estimated pharmacokinetic parameters. From these data it is apparent that F8-500-LCN-FVIII-(741-966) has a terminal half-life that is 1.4 (statistically significant) longer compared to N8 (FVIII).

Table 8: FVIII activity in FVIII-KO mice (FVIII chromogenic activity in IU/ml).

FVIII	FVIII activity in samples from mice ¹ (IU/mL)								
Sampling time points (h)	0.08	1	2	4	6	18	24	30	48
F8-500-LCN-FVIII-(741-966)	2.31	2.60	1.74	1.20	1.15	0.57	0.36	0.11	0.07
	2.33	2.56	1.98	1.18	1.19	0.71	0.36	0.11	0.11
	2.50	2.72	1.80	1.23	1.19	0.81	0.37	0.12	0.15

Table 9: Estimated pharmacokinetic parameters including 95% CI (based on FVIII chromogenic activity data)

Compound	C _{max}	t _{1/2} [*]	MRT
	(IU/L)	(h)	(h)
F8-500-LCN-FVIII-(741-966)	2.7 (2.4-3.1)	9.8 [*] (8.8-11)	13 (12-15)
N8 (FVIII)	2.4 (2.1-2.7)	7.1 [#] (6.3-7.9)	10 (9.1-11)

*: Terminal half-life, based on a 2-compartment model

#: Half-life, based on a 1-compartment model

EXAMPLE 11

Secondary and tertiary structure analysis of a B-domain fragment comprising 226 amino acid residues.

The FVIII B domain fusion partner according to SEQ ID no. 11 was expressed with a C-terminal HPC4-tag (EDQVDPRLIDGK – **SEQ ID NO 42**).

The resulting protein was expressed and purified essentially as previously described using anti- HPC4 affinity chromatography.

Tryptophan fluorescence: FVIII(741-966)-HPC4 was prepared at a concentration of 5 µM in the following buffer, 20 mM HEPES, 100 mM NaCl, 5mM EDTA, 0.005% Tween80, pH 7.5. Also, a denatured sample was prepared similarly but with the addition of 6 M guanadinium hydrochloride. 200 µl of sample was loaded to a quartz cuvette with a 1 cm path length and measured on a SpectraMax M2e platereader (Molecular Devices Ltd, UK) with the following

settings: excitation wavelength of 280nm, emission wavelengths of 300-450 nm with steps of 1 nm, sensitivity of 6 and a temperature of 21°C. $\lambda_{\max}$ was determined by polynomial fitting in KaleidaGraph 4.5.

5 Circular dichroism: The secondary structure fingerprint was measured with Far-UV circular dichroism (CD). FVIII(741-966)-HPC4 was prepared to 13.8 mg/ml protein in 20 mM HEPES, 100 mM NaCl, 5mM EDTA, 0.005% Tween80, pH 7.5 and loaded to a rectangular quartz CD cuvette with a 0.01 mm light path. With a Chirascan CD spectrophotometer (Applied Photophysics, UK) wavelength spectra from 190-260 nm were measured with steps of 1 nm, 10 a bandwidth of 1 nm at 25 °C. 3 consecutive scans was carried out and averaged the corresponding buffer scan was subtracted. For the full range (190-260nm) the noise was below 500V, i.e. data quality was good.

To evaluate whether a thermal unfolding transition could be observed, 1 mm quartz cuvettes 15 were used due to improper heating of 0.01 mm cuvettes. Due to high noise from the buffer in a 1 mm cuvette, the buffer was diluted four times and the protein concentration was diluted 100 times. This way, acceptable signal to noise was acquired from 200-260 nm. The sample was measured at 25, 50, 70 and 90°C using the settings described above. No visual aggregation was observed upon melting.

20 Tryptophan (Trp) fluorescence is a sensitive reporter of tertiary structure as the wavelength corresponding to the highest intensity ($\lambda_{\max}$) shifts when the polarity of its near-environment changes. Trp residues located in a folded environment (i.e. hydrophobic) have a $\lambda_{\max}$ of ~334 nm. When Trp is solvent-exposed (i.e. in a hydrophilic environment), the $\lambda_{\max}$ red-shifts to 25 ~355 nm. For FVIII(741-966)-HPC4 a $\lambda_{\max}$ of 355 nm is found suggesting that this residue is in a hydrophilic environment. As Trp residues are often engaged in hydrophobic interactions, this data suggest that the two Trp residues are located in disordered regions of the polypeptide. Addition of chemical denaturant, 6 M GdmCl, which leads to complete unfolding and a subsequent maximum red-shift, also leads to a $\lambda_{\max}$ of 355 nm, confirming that the Trp 30 residues in FVIII(741-966)-HPC4 are fully solvent-exposed.

A secondary structure fingerprint of FVIII(741-966)-HPC4 was obtained with Far-UV circular dichroism (CD) spectroscopy. Based on wavelength spectrum from 190-260 nm, the fingerprint resembles that of a disordered polypeptide having a signal minimum of 204 nm. This minima does not correspond to standard minima of α -helical structure ($\lambda_{\min}$ =218 nm) and 35 β -sheet structure ($\lambda_{\min}$ =208 nm and 222 nm) suggesting that the disordered conformation is

predominant. However, evaluation of the Far-UV CD spectrum of FVIII(741-966)-HPC4 as a function of temperature indicates that there is a thermal unfolding transition occurring at higher temperatures. A dichroic point is identified when overlaying Far-UV CD spectra obtained at 25, 50, 70 and 90°C suggesting that it is a two-state thermal unfolding transition.

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In conclusion, Far-UV CD and Trp fluorescence analysis suggest that there is a substantial degree of disorder in FVIII(741-966)-HPC4. However, based on thermal unfolding followed with Far-UV CD, some persistent structure appear to be present in FVIII(741-966)-HPC4.

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CLAIMS

1. A FVIII fusion protein comprising a FVIII molecule with a B domain, wherein said FVIII molecule is fused to a FVIII B domain fusion partner, wherein said FVIII B domain fusion partner is derived from the FVIII B domain and comprises 100-908 amino acids.

2. A FVIII fusion protein according to claim 1, wherein said FVIII molecule is a B domain truncated FVIII molecule with a B domain comprising 15-25 amino acids.

3. A FVIII fusion protein according to any one of the preceding claims, wherein the FVIII B domain fusion partner is a truncated FVIII B domain.

4. A FVIII fusion protein according to any one of the preceding claims, wherein said FVIII molecule is a B domain truncated FVIII molecule comprising a B domain amino acid sequence selected from the group consisting of SEQ ID NO 2, SEQ ID NO 3, and SEQ ID NO 4.

5. A FVIII fusion protein according to any one of the preceding claims, wherein said FVIII B domain fusion partner is fused to the C-terminal end of the FVIII B domain.

6. A FVIII fusion protein according to any one of the preceding claims, wherein said FVIII B domain fusion partner is fused to the N-terminal end of the FVIII light chain.

7. A FVIII fusion protein according to any one of the preceding claims, wherein the FVIII B domain fusion partner is fused to the C-terminal end of the FVIII light chain.

8. A FVIII fusion protein according to any one of the preceding claims, wherein said FVIII B domain fusion partner comprises the amino acid sequence selected from the group consisting of: SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 21, SEQ ID NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 32, SEQ ID NO 33, and SEQ ID NO 34.

9. A FVIII fusion protein according to any one of the preceding claims, wherein said FVIII B domain fusion partner has a low degree of secondary and/or tertiary structures.

5 10. A FVIII fusion protein according to any one of the preceding claims, wherein said protein has a homogenous structure.

11. A pharmaceutical composition comprising a FVIII fusion protein according to any one of claims 1-10.

10 12. A FVIII fusion protein according to any one of claims 1-10, or a pharmaceutical formulation according to claim 11, for use as a medicament for treatment of haemophilia.

13. A FVIII fusion protein according to any one of claims 1-10, or a pharmaceutical composition according to claim 11, for treatment of haemophilia by subcutaneous
15 administration.

14. A FVIII fusion protein according to any one of claims 1-10, or a pharmaceutical formulation according to claim 11, for treatment of haemophilia by intravenous
20 administration.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/063155

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/96 A61K38/37 C07K14/755
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K C07K

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

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

EPO-Internal, BIOSIS, EMBL, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/023894 A1 (BIOGEN IDEC INC [US]) 19 February 2015 (2015-02-19) claims 1,53,54,69,72,82,83 -----	1-4,6-14
X	US 2006/122376 A1 (CHAPMAN BARBARA [US] ET AL) 8 June 2006 (2006-06-08) paragraph [0010]; example 7 -----	1-5,8-11
X	WO 2013/160005 A1 (NOVO NORDISK AS [DK]) 31 October 2013 (2013-10-31) page 4, lines 12-19; claim 1 -----	1,3,4, 9-14
X	US 5 595 886 A (CHAPMAN BARBARA [US] ET AL) 21 January 1997 (1997-01-21) column 2, lines 30-54 ----- -/--	1-5,8-11

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

14 September 2016

Date of mailing of the international search report

04/10/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Obel, Nicolai

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/063155

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/101284 A1 (NOVO NORDISK AS [DK]; BOLT GERT [DK]; KJAERGAARD KRISTIAN [DK]; NOERBY) 25 August 2011 (2011-08-25) page 1, line 31 - page 2, line 2; claims 1,2,5,6 -----	1-14
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PCT/EP2016/063155

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