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A Robust Production Platform for Recombinant Factor VIII

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Abstract

For hemophilia A, factor VIII (FVIII) replacement is still the standard of care for both acute bleeding episodes and prophylaxis. While plasma-derived FVIII products are still available, approximately 75% of the hemophilia communities use recombinant human FVIII products (rhFVIII). However, due to its large molecular weight and complicated post-translational modification, rhFVIII remains as one of the most challenging therapeutic proteins to produce, which leads to the unnecessarily high costs for vulnerable patients and their families. To address this issue, we recently explored if von Willebrand factor (vWF) chaperone would enhance the rhFVIII expression by co-expressing a B-domain–deleted (BDD) rhFVIII with a modified vWF fragment. Using this approach, the rhFVIII production yield has been increased significantly (>2000IU/ml) in lab-scale culture as well as in large-scale production (200L). The purified rhFVIII displayed similar molecular characteristics to commercial rhFVIII products including thrombin cleavage, glycosylation, tyrosine sulfation and binding affinity to vWF. This production platform has also been used successfully for recombinant porcine FVIII and for a new, long-acting FVIII in development. We believe that, using this platform, rhFVIII can become economically competitive and will have a major impact on hemophilia A prophylaxis and on-demand therapy once the product is on the market.

Keywords: Recombinant FVIII Production, Von Willebrand Factor, Fc Fusion, Hemophilia Therapy, Robust Platform

1. Introduction

For decades, factor VIII replacement therapy has been the gold standard for prophylaxis and treatment of hemophilia A including using full-length and B-domain-deleted (BDD) variants, as well as long-acting (LA) FVIII. Other therapies, such as non-factor and gene therapies, have also been developed to address various medical needs for hemophilia patients. Unfortunately, all of these therapies are almost equally expensive based on the cost analysis of Institute for Clinical and Economic Review (ICER), which translated to a market that is failing people with hemophilia for accessing affordable therapy due to escalating costs [1]. Therefore, alternatives that are cost effective for hemophilia patients are definitely needed.

Recombinant FVIII (rFVIII) is considered as a difficult-to-express protein. The impact factors of efficient production of rFVIII were studied extensively, such as mRNA as well as rFVIII proteins instability, low transport rate from ER to Golgi and the binding of the expressed protein in cell culture media with negatively-charged cell surfaces [2-5]. In circulation, plasma von Willebrand factor (VWF) acts as a chaperone protein maintaining the stability of the plasma coagulation FVIII and modulating its plasma clearance and cellular interactions [6,7]. Addition of exogenous plasma-derived vWF to cell culture and co-expression FVIII with full-length vWF were investigated for whether vWF would improve the FVIII stability during the production. Unfortunately, only a few folds of increase in FVIII expression were observed, and the absolute productivity is incredibly low [3,8,9]. A method for increasing production of recombinant BDD-FVIII by use of truncated vWF has been described in a patent [10]. This method increased expression of FVIII by about 10 folds, making commercial production at reduced cost a real possibility. Unfortunately, the method presents a few unknowns, namely, (a) that the use of wild-type vWF fragments appears to result in reduced recovery of FVIII during chromatography, likely due to multimerization/aggregation of FVIII/vWF complexes (Barnett, personal communication), and (b) it is unclear if the high expression levels achieved by this method (~1000IU/ml) resulted in any untoward post-translational modification changes to FVIII. To this end, we have developed a modified and improved version of this expression platform that permits excellent FVIII expression (>2500IU/ml) and recovery that is comparable, biochemically and pharmacokinetically, to other commercial FVIII products.

2. Materials and Methods

2.1. Construction of Dual Expression Vector

Human BDD-FVIII (GenBank Acc. No. ABV90867) and four truncated vWF variant Fc fusion cDNAs were commercially synthesized and sequence-verified (GenScript, Piscataway, NJ). Both gene fragments were then inserted into a pEE12.4–based protein expression plasmid vector with CMV promoters and a L-methionine sulfoximine (MSX) selection marker. The truncated vWF variants fused with the IgG1 Fc domain are defined as follows: (a) Pro-D'D3-Fc (D1/D2/D'/

D3-Fc) represents a construct that contains vWF propeptide domain (D1D2, amino acids +1-741 of the wild-type vWF molecule, GenBank NM_000552) and domains D'D3 (amino acids 742-1247 of the wild-type vWF molecule,), fused at its C-terminus with an immunoglobulin Fc domain (e.g., UniProtKB 01857, comprising amino acids 104-330) The dual expression vector of BDD-FVIII and Pro-D'D3-Fc is named HX11. (b) D'D3-Fc (D'/D3-Fc) represents a construct that contains wild-type vWF domains D'D3, fused at its C-terminus with an immunoglobulin Fc domain; in contrast to construct (a), the propeptide domain (D1D2) has been deleted. The dual expression vector of BDD-FVIII and D'D3-Fc is named HX12. (c) Pro-D'D3mut-Fc (D1/D2/D'/D3 + mutation-Fc) represents a construct similar to (a), except having alanine substitution mutations at C1077 and C1120 of the mature, wild-type vWF molecule (counting starts from the vWF signal sequence). The dual expression vector of BDD-FVIII and Pro-D'D3mut-Fc is named HX13. (d) D'D3mut-Fc (D'/D3 + mutation) represents a construct similar to (b) except having alanine mutations at C1077 and C1120 of the mature, wild-type vWF molecule. The dual expression vector of BDD-FVIII and Pro-D'D3mut-Fc is named HX14.

2.2. Cell-Line Generation

Four cell lines of HX11, HX12, HX13 and HX14 were generated for evaluating which construct is the best for high level expression of human FVIII. CHO K1 cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC 85051005) and the stable cell-line were generated by conventional recombinant expression vector transfection, cell clone screening and clone selections. Once the expected clone was confirmed, the clones were expanded and scaled up for FVIII expression comparison.

2.3. Recombinant FVIII Expression

To compare the selected FVIII expression cell lines of HX11, HX12, HX13 and HX14, fed-batch culture using shake-flasks was performed with standard technology and cell density and FVIII activity were checked regularly to monitor cell growth and FVIII expression. Production of recombinant FVIII from vector HX14 was carried out in 200L pilot-scale production campaigns in a fed-batch process using chemically-defined cell culture media. The resulting purified rhFVIII from such a bioreactor campaign is named NX01 BDD-FVIII. All of the analytical characterization and quality control testing of the drug product batches were performed according to Chinese Food and Drug control regulations.

2.4. One-Stage Clotting Assay

FVIII activity was measured by conventional one-stage clotting assay on an STA Compact MAX or a Diagnostica Stago STart 4 hemostasis analyzer using activated partial thromboplastin time reagent (STAGO US, Parsippany, NJ) and FVIII-deficient plasma (George King, Overland Park, KS). All FVIII samples were pre-diluted to approximately 1.0 IU before experiments. The clotting times were recorded and converted to IU using a calibration curve.

2.5. Purification of Recombinant FVIII and vWF Variants

For purification of recombinantly-expressed FVIII, the culture supernatant was first processed with high-speed centrifugation (3000g for 30 min at 4°C) and filtration (0.45μ); the supernatant was further diluted with an equal volume of buffer (40mM Tris-HCl, pH 7.0, 150 mM NaCl). Using the AKTA Pure chromatography system, the processed supernatant was loaded onto a HiTrap MabSelect Prism A column, and the column was then washed with 10 column volumes (CV) of wash buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl). The elution buffer (20 mM Tris-HCl, pH 7.0, 0.3 M CaCl2) was applied to the protein A column to separate the FVIII from bound vWF variant-Fc fusions. The purified BDD-FVIII was then buffer-exchanged into 0.3% (w/v) sucrose, 2.2% (w/v) glycine, 20 mM histidine, 220 mM NaCl, 25 mM CaCl2 and 0.008% Tween-80, pH 6.9, for further protein characterization. Bound vWF variant-Fc was further eluted with 200 mM HAc, pH 2.8, from a HiTrap MabSelect Prism A column and buffer-exchanged into a 20 mM Tris-HCl, pH 7.0, 150 mM NaCl solution.

For manufacturing, several steps were involved in recombinant FVIII purification process including depth filtration; viral inactivation; protein an affinity chromatography; anion exchange chromatography; hydrophobic interaction chromatography; viral filtration and ultrafiltration/ diafiltration. The FVIII intermediate and final products thus obtained were visualized directly by SDS-PAGE electrophoresis on 10% SDS-PAGE gels; corresponding Western blots were likewise used to visualize proteins via specific antibody binding and HRP-mediated detection. For vWF variant-Fc fusions, non-reducing 4% SDS-PAGE gels were used to visualize truncated vWF fragments (i.e., dimer, multimer), then stained with 0.25% Coomassie Brilliant Blue.

2.6. Thrombin Digestion

Thrombin cleavage of purified rFVIII protein was assessed by SDS-PAGE. One Hundred microliter volumes containing approximately 25 nM FVIII protein and 1 nM bovine α-thrombin in assay buffer (20mM HEPES, 150mM NaCl, 5 mM CaCl2) was incubated for 15 minutes at 37°C. As a negative control, an aliquot was collected before adding thrombin (time=0). The reaction was quenched by adding 2xSDS PAGE nonreducing loading dye after digestion and boiling at 95°C for 2 minutes. Cleavage products along with control sample were resolved (10 ug per well) by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then stained with Coomassie Blue. Xyntha® was used as the positive control.

2.7. In Vitro Enhancement of FVIII Recovery

To test if co-expression of the truncated vWF-Fc fusion protein could increase recombinant FVIII expression, four purified vWF-Fc variants (Pro-D'D3-Fc, Pro-D'D3muts-Fc, D'D3mut-Fc and D'D3-Fc proteins), plasma vWF, and a negative control protein (HSA) were included in the assay. In brief, BDD-FVIII expression only cell line was seeded in a 48-well plate at 0.8 x 106 cells/well in CD-CHO medium and grown for 48 hrs. Two micrograms/well of plasma vWF and recombinant vWF-Fc fragments, including Pro-D'D3-Fc, Pro-D'D3mut-Fc, D'D3mut-Fc and D'D3-Fc, were then added

into their respective wells. Wells with no addition of vWF-Fc or with the addition of human serum albumin (HSA) were included as controls. Supernatant samples were collected at 0, 2, 4, and 8 hours after addition of all testing proteins and FVIII activity was tested with the one-stage clotting assay described in Materials and Methods.

2.8. Affinity Determination of Truncated vWF Variant and Full-Length vWF with FVIII

Full-length vWF and the vWF variant fragment (D'D3mut-Fc) were characterized for their FVIII binding affinity. All binding experiments were performed using the Octet Red 96e (Sartorius) and PBST based kinetic buffer at 25°C. Full-length vWF and the vWF variant (5 μg/mL) were immobilized onto a Protein A probe. Von Willebrand factor-coated biosensors were washed with kinetic buffer before association and dissociation of varying concentrations of FVIII (0-100 nM). Xyntha®, a commercial rhFVIII product, was included in the study as control. The binding data were analyzed by Prism (GraphPad) and nonlinearly regressed to the "Global Fit" mode to determine kinetic constants.

2.9. Factor Xa Generation and APC Inhibition Assays

The COATEST SP4 FVIII chromogenic kit (Diapharma, West Chester, OH) was used to evaluate if NX01 BDD-FVIII is able to generate FXa comparable to commercial FVIII product. According to modified COATEST SP4 FVIII protocol, 25 μl of various concentration of FVIII samples (in triplicates) including the national FVIII standard (China, NIFDC) were added to the 96-well microplate and incubated for 4 min; 50 μl of pre-warmed factor reagent containing Factor IXa, Factor X and phospholipid was then added to the well and incubated for 5 min, followed by addition of 25 μl/well of CaCl2 (0.025 mol/L) and incubated at 37ºC for 5 min. At the end of incubation, 50 μl of chromogenic substrates (S-2765 and I-2581) were added and incubated for 5 min; 25 μl of 20% acetic acid was then added to stop the reaction. The OD absorbance at 405 nm were read using BioTek 800 TS (Agilent BioTek). For APC inhibition assay, the one stage aPTT assay was used to examine if NX01 BDD-FVIII has a similar sensitivity to activated Protein C (APC) compared with commercial FVIII product (Xyntha®) to generate thrombin. In brief, 50 μl of FVIII samples (in triplicate), 50 μl of aPTT reagent and 50 μl of FVIII deficiency plasma were incubated for 240 seconds, and then various concentrations of APC (60-150 mM) were added into above solutions. The aPTT time were measured using STA Compact Max (STAGO) to determine APC-mediated FVIII inhibition.

2.10. Post-Translational Modifications

Samples of NX01 BDD-FVIII were treated with guanidine hydrochloride and dithiothreitol at the final concentration 6 M and 20 mM respectively for 30 min at 56°C. Protein alkylating was then performed using 50 mM iodoacetamide (final concentration) at room temperature for 45 min. After buffer exchange of 50 mM Tris-HCl, pH 7.8, enzyme digestion using trypsin (Promega, Madison) was carried at 37°C for 15 hours. Digested protein samples were analyzed using a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, MA, USA) coupled to an UHPLC Vanquish system (Thermo Fisher Scientific, MA, USA).

3. Results and Discussion

3.1. Expressed vWF-Fc Variants Bind to and Protect FVIII

Full-length vWF is a large, multimeric glycoprotein that is critical for hemostasis and thrombus formation. It also functions as a carrier molecule for FVIII and protects its fast clearance in plasma. Research showed that co-expressed full-length vWF is able to increase recombinant FVIII accumulation in cell culture, however the multimeric vWF molecules may become problematic for FVIII downstream processing [9,11]. We have generated several recombinant variants of vWF that contain different versions of the D'D3 domains for FVIII binding and also further modified them at their C-termini with a human IgG1 domain (Fc) to facilitate downstream manipulation and dimer formation (D'D3mut-Fc) for FVIII interaction. Two of the variants were expressed with the vWF propeptide domain (ProD'D3-Fc and ProD'D3mut-Fc) that is believed to be important for proper folding of vWF and subsequent binding to FVIII, while two variants were expressed without the propeptide (D'D3-Fc and D'D3mut-Fc) [12,13]. We prepared these vWF variants to determine a) if the propeptide was critical for expression of vWF and efficient binding to FVIII since cleavage of the propeptide by furin could be problematic when these molecules are over-expressed and b) if reducing the extent of vWF multimerization by insertion of mutations considered important for multimerization could promote improved recovery of FVIII-vWF complexes during the expression and purification [14-16]. Perhaps continuous binding by vWF mitigate some of the issues associated with chaperone binding in the Golgi and endoplasmic reticulum and thus promote improved secretion.

SDS-PAGE was used to analyze the expression and multimerization of four vWF variants compared with plasma full-length vWF (Figure 1)

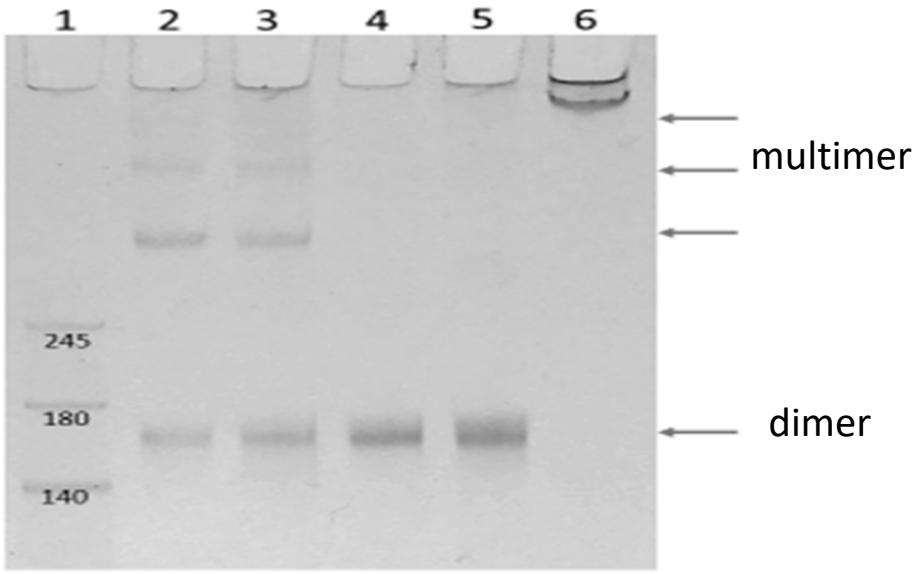

Figure 1: Expression of Truncated von Willebrand's Factor-Fc Variants. Equal Volumes of Supernatants from Small-Scale Cultures Expressing ProD'D3-Fc (Lane 2), D'D3-Fc (Lane 3) and ProD'D3mut-Fc (Lane 4) and D'D3mut-Fc (Lane 5) were Loaded Onto a Non-Reducing PAGE Gel and Stained with Coomassie Blue. Plasma-Derived vWF was Included as a Comparator (Lane 6); Molecular Weight Markers are Shown in Lane 1

While full-length, plasma-derived (pd) vWF virtually remains near its well (lane 6), the recombinant variants show considerably reduced multimerization. Both versions of wild-type D'D3 fusions, with and without the propeptide, show various degrees of multimers (lanes 2 and 3); the variants containing mutations at C1077A and C1120A, however, only show the dimer form (lanes 4 and 5), and further indicated that the ProD'D3-Fc and ProD'D3mut-Fc variants were efficiently cleaved by furin in these cells. Considering their high and efficient expression and binding ability, it seems that vWF propeptide may not be required for FVIII binding, as generally believed [11]. In order to assess if any of these versions bind and protect FVIII during expression in cell culture, we added equimolar amounts of each recombinant vWF variant, as well as pd-vWF, to cells expressing recombinant FVIII and tested for FVIII activity at different times.

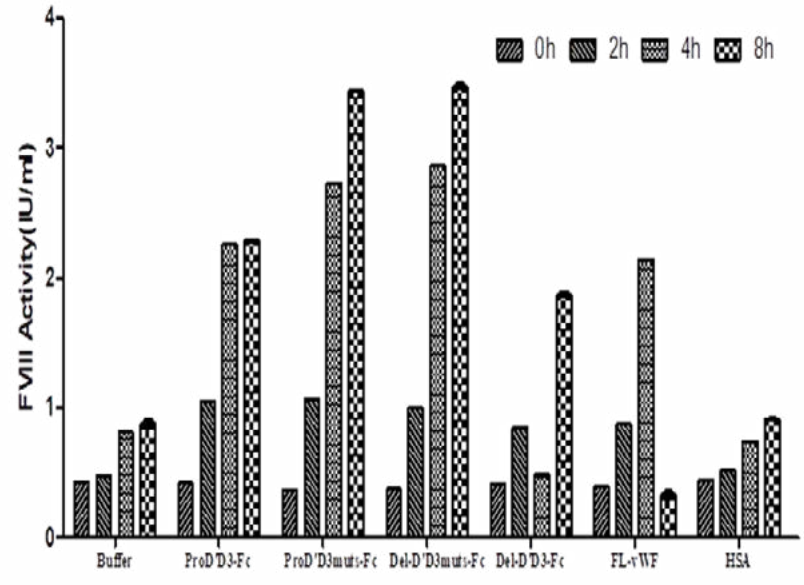

Figure 2: vWF Variants Bind to and Protect BDD-FVIII. Equimolar Amounts of Purified, Truncated vWF Variants Were Added to Cell Cultures Expressing BDD-FVIII. Samples of Supernatants Were Tested for FVIII Activity at 0, 2, 4 and 8 hrs Post-Addition. Both Buffer, Human Serum Albumin (HSA) and Full-Length vWF Were Added as Controls and Comparators. The Legend is Shown in the Upper Right-Hand Corner

The results (Figure 2) suggest that not only do the vWF variants provide increased protection of FVIII activity over time, but that, unexpectedly, the mutated versions of the truncated variants were even better than the wild-type versions in promoting protection. As a result of these analyses, we selected D'D3mut-Fc for further characterization and utility in FVIII expression in culture.

3.2. High-Level Expression and Improved Purification of Recombinant FVIII

Four expression cell lines (HX11, HX12, HX13 and HX14) were evaluated for their FVIII expression productivity. HX11, HX12, and HX13 gave higher expression level than a FVIII gene alone (data not shown), but HX14 gave an unprecedented high level of FVIII expression. At the 200L production scale, the FVIII expression level of HX14 reached >2000IU/ml (23.38 IU/106 cells/24 hours) at 12 days of fed-batch culture. The mechanism by which vWF fragments promote a high level of recombinant FVIII accumulation is unknown, but we speculate that it occurs by protection of FVIII from post-secretion degradation and reduction of expressed FVIII binding to negative charged cell membrane, as addition of exogenous vWF added to FVIII-producing cells in culture increases and maintains FVIII levels in culture supernatants (Fig 1.).

The efficiency of purification provided by Fc fragment fused at the C-termini of vWF variants proved to be highly useful and economically-sound compared with other conventional methods using FVIII monoclonal antibody affinity chromatography, such as FVIIISelect, anion-exchange (AEX) and Hydrophobic (HIC) resins. We were able to prepare NX01 BDD-FVIII to over 90% purity with high yield by a single chromatography column step over Protein A (Figure 3A), the specific activity (5000-10000IU/mg) of the final FVIII product is comparable to Xyntha, a commercial FVIII product. In addition, this process has been extended to high-yield expression of recombinant porcine BDD-FVIII (Figure 3B) and to a new long-acting human BDD-FVIII in development (Figure 3C). Relatively straightforward workflow using Protein A, AEX and HIC chromatography for recombinant FVIII purification and high purity of the final protein product with minimal host cell protein contaminants (Table 1) and demonstrated that this simplified downstream process could dramatically reduce the overall cost of FVIII production.

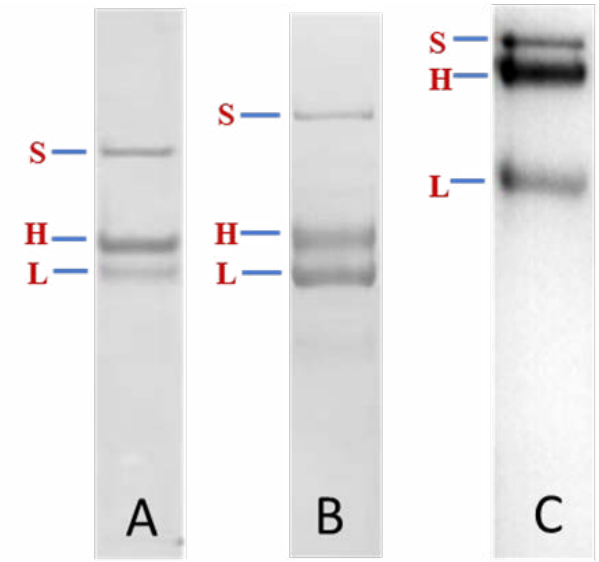

Figure 3: Purified FVIII Variants Co-Expressed with D'D3mut-Fc vWF. Supernatants were Taken from Cell Cultures Co-Expressing Different BDD-FVIIIs and D'D3mut-Fc and Purified by Protein an Affinity Resins as Described in Methods. Purified Proteins Were Electrophoresed on 10% SDS-PAGE Gels and Stained with Coomassie Blue. The Heavy (H) and Light (L) Chains of the FVIII Molecule, as well as its Single-Chain Form (S), are Indicated. (A) Human BDD-FVIII. (B) Porcine BDD-VIII with a Human-Derived B-Domain. (C) Long-Acting Human BDD-FVIII (in Development). Gel Lanes Represented are from Separate Gel Runs

Purification Table					
Steps	**FVIII Recovery Rate（%）**	**FVIII Purity by SEC（%）**	**Specific Activity（IU/mg）**	**HCPb（ppmc）**	**vWF D'D3mut-Fc（ppm）**
Protein A	73.4	92.9	5912	2279	4575
Protein A FTa	94.6	93.5	5108	1614	1523
Anion Exchange	81.5	96.3	8281	155	607
Hydrophobic	63	97.6	7769	18	282

Table 1: Purification Table of Recombinant Human FVIII (200L Production scale)

3.3. Analysis of Recombinant FVIII by SDS-PAGE

Thrombin-catalyzed factor VIII activation, at Arg372 (A1-A2 junction), Arg740 (A2-B junction), and Arg1689 (near NH2 terminus of A3), is an essential positive feedback mechanism regulating intrinsic blood coagulation. To determine if the robust expressed FVIII maintains the correct structure for thrombin cleavage, thrombin cleavage assays were performed and the results were examined using SDS-PAGE. As expected, rhFVIII is cleaved by thrombin at the above-mentioned amino acids and generates three major polypeptides (Fig. 4, lane 3) corresponding to the following segments of the A1 and A2 and A3-C1-C2 domains. There were compared with the results of analysis of Xyntha® (a CHO-derived rhBDD FVIII protein, Fig. 4, lane 2) by the same method.

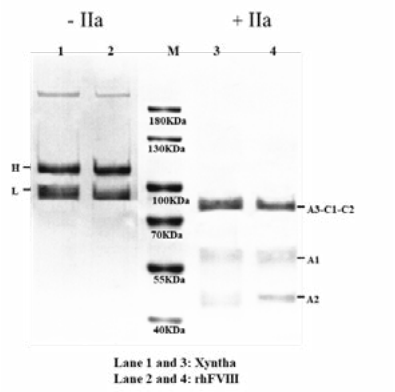

Figure 4: Cleavage Products of FVIII Activation by Thrombin. Recombinant hFVIII and +/-Thrombin Treatment Was Resolved by SDS-PAGE and Visualized by Coomassie Blue Staining. A Molecular Weight Ladder (M) Was Used to Determine the Relative Mobility of the FVIII Cleavage Fragments. Lane 1 and 2 were Xyntha, a Commercial Recombinant FVIII Product, and rhFVIII Samples Respectively. Lane 3 and 4 Were Xyntha and rhFVIII Samples Treated with Thrombin, Respectively. Heavy chain (H), Light Chain (L), A3-C1-C2, A1 and A2 Polypeptides are Labeled

3.4. Binding of D'D3mut-Fc to FVIII

In light of the ability of D'D3mut-Fc to promote high expression of NX01 BDD-FVIII, we wished to determine the apparent binding constants of full-length vWF and D'D3mut-Fc to NX01 BDD-FVIII as well as to a commercial BDD-FVIII product, Xyntha®. This was important particularly to determine the ability of full-length, plasma-derived vWF to bind to NX01 BDD-FVIII since pd-vWF is the chaperone for recombinant FVIII administered intravenously to patients. Our results indicate that the 'on-rate' of pd-vWF for NX01 BDD-FVIII is slightly more than for Xyntha® (k_{on} = 2.61 x 10^6 vs. 1.55 x 10^6 1/Ms) while its 'off-rate' is virtually identical for the two FVIII molecules (k_{diss} = 7.01 x 10^{-3} vs. 7.04 x 10^{-3} 1/s), yielding binding affinities that were similar but favored higher binding to NX01 BDD-FVIII than to Xyntha® (Kd of 2.68 nM vs. 4.56 nM). The truncated and mutated variant, D'D3mut-Fc, also bound NX01 BDD-FVIII slightly better than it did to Xyntha® (Kd of 9.62 nM vs. 15 nM). D'D3mut-Fc bound NX01 BDD-FVIII and Xyntha® with nearly ten-fold lower affinity than full-length vWF, consistent with results reported for efanesoctocog [17]. These results suggest that D'D3mut-Fc is in molar excess over FVIII and saturating with respect to KD, thus overcoming any reduction in its apparent affinity for NX01 BDD-FVIII compared to full-length vWF.

3.5. Factor Xa Generation and APC-Mediated Thrombin Inhibition

The cofactor activities of NX01 BDD-FVIII and Xyntha in an intrinsic FXase activity assay were compared by a chromogenic assay as described in Materials and Methods. In this assay, purified NX01 BDD-FVIII was evaluated for its relative potency to commercial rhFVIII (Xyntha®) at various concentrations. NX01 BDD-FVIII, Xyntha, as well as a national FVIII standard, had similar activities in this assay indicating that the NX01 BDD-FVIII activity in generating FXa is fully functional. (Figure 5A).

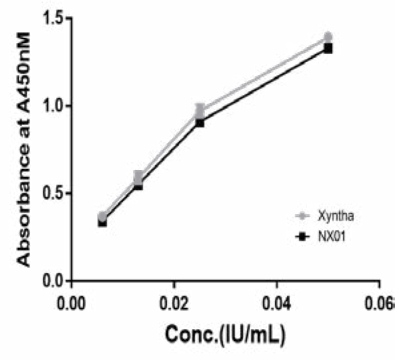

Figure 5A: FVIII-Mediated Interactions with Co-Factors. (A) FXa Generation Was Performed with FVIII Concentrations Diluted from 1.0 IU/ mL Stocks of NX01 BDD-FVIII and Xyntha®. There is no Significant Difference Between NX01 BDD-FVIII and Xyntha® at Each Concentration. P < 0.05

The inhibition of FVIIIa mediated by the protein C system provides a highly efficient and specific regulation of blood coagulation. To study if NX01 BDD-FVIII has similar sensitivity to activated Protein C compared to commercial rhFVIII, its activity in response to increasing doses of APC was studied in a one-stage aPTT assay. The results indicated that the clotting times are prolonged as APC concentrations in the assay are increased as it is likely that FVIII-mediated thrombin generation is abrogated due to APC cleavage. The APC inhibitory effects of NX01 BDD-FVIII, commercial rhFVIII (Xyntha®) and a plasma-derived FVIII standard (obtained from National Institutes for Food and Drug Control) are all virtually identical (Figure 5B).

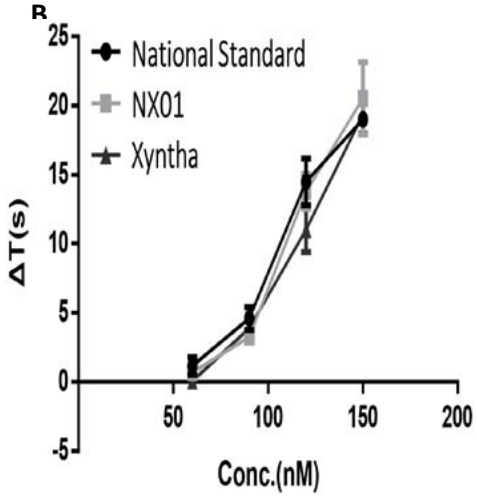

Figure 5B: (B) APC-Mediated FVIII Inhibition of Thrombin Generation Was Performed by Determining the Clotting Time to Evaluate the Thrombin Generation in the Present of Increased Concentration of APC in Human FVIII Deficient Plasma Where Equal Amounts of NX01 BDD-FVIII and Xyntha®. ΔT(s) is the Clotting Time Difference Between FVIII Only and FVIII+APC

3.6. Glycan Analysis and Tyrosine Sulfation

The glycosylation and tyrosine sulfation of recombinant FVIII has been recognized as important to FVIII's binding to vWF and its stability in vivo. In order to assess if overexpression of NX01 BDD-FVIII by co-expression with D'D3mut-Fc (as its chaperone) resulted in any significant changes to the glycosylation and sialylation of the four major glycans on the protein, we used mass spectroscopy to determine sialylated, unglycosylated, high mannose-modified and other glycans at asparagine positions N41, N239, N916 (=N1810 for FL-FVIII) and N1224 (=N2118 for FL-FVIII) (Table 2) on three lots of NX01 BDD-FVIII. While it is difficult to compare glycosylation/sialylation results between FVIII molecules expressed even in similar cell lines, it is clear that, as expected, positions N239 and N1224 contained predominantly high-mannose compared to the other two sites with 1224 being almost exclusively high-mannose modified [18]. This result was consistent between NX01 BDD-FVIII compared with commercial Xyntha® when both were analyzed concurrently. Overall, the most significant features of post-translational modifications related to N-linked glycosylation are very similar to Xyntha as well as to other BDD-FVIII molecules expressed in CHO cells (Table 3).

Peptide sequence	Glycosylation Site	N-linked glycosylation classification	NX01 BDD-FVIII (%) (average of 3 lots)		Xyntha (%)
			Mean	SD	
SFPFNTSVVK	N41	High-mannose	12.17	2.09	0.00
		Sialic acid	54.64	2.61	98.29
		Unglycosylated	0.17	0.09	0.79
		Other Glycans	33.01	2.01	0.92
VNGYVNRSLP	N239	High-mannose	35.19	0.74	50.78
		Sialic acid	21.37	2.60	25.23
		Unglycosylated	4.11	0.38	4.94
		Other Glycans	39.03	2.62	18.74
NFVKPNETK	N916(1810)	High-mannose	Not detected	NA	Not detected
		Sialic acid	30.92	2.30	58.14
		Unglycosylated	43.41	1.92	34.00
		Other Glycans	21.8	0.26	5.21

GNSTGTLMV	N1224(2118)	High-mannose	97.43	0.10	94.40
		Sialic acid	Not detected		Not detected
		Unglycosylated	2.32	0.16	5.60
		Other Glycans	0.19	0.18	0.00

Table 2: Primary N-Glycan Site Glycosylation/Sialyation of FVIII. NX01 BDD-FVIII Were Prepared for Mass Spectroscopy to Evaluate the Kinds of Modifications at the Four Primary N-Glycan Sites on BDD-FVIII, Namely, N41, N239, N916 and N2118. NX01 BDD-FVIII Protein from Three Different Bioreactor Runs Were Tested and Their Mean Values and SD are Shown; Commercial-Grade Xyntha® was Used as a Comparator

Glycan Type	Attribute	NX01 BDD-FVIII (average of 3 lots)		Xyntha (commercial)
		Mean	SD	
High-mannose N-glycans	Overall proportion (%)	35.1	0.70	34.5
	Average mannose residues	7.62	0.11	7.42
Complex and hybrid N-glycans	Overall proportion (%)	64.89	0.70	65.5
	Antennae capping rate (%)	69.2	1.80	92.5
	Sialic acid (%)	68.9	1.87	92.5
	Fucose (%)	0	0.00	0
	Hexose (%)	0.27	0.12	0
	Ratio sialylated N-glycans	0.48	0.00	0.7
	Bi - Tri -Tetra antennary (%)	88-10-2	2.08 - 1.73 - 0.58	85-10-5
	Average number of antennae	2.15	0.02	2.2

Table 3: Summary of Predominant FVIII Glycan Populations. Breakdown of High-Mannose and Complex Glycan Structures on NX01 BDD-FVIII (Mean of Three Different Lots) and on Commercial-Grade Xyntha® that was used as an Internal Comparator. See also Canis et al. (2108) for Comparison with Other Commercial rhFVIII Products

The degree of sialylation on specific asparagines of NX01 BDD-FVIII varied to different extents from Xyntha® and other BDD-FVIIIs but it is difficult to a priori assess the impact of these differences on interaction with factor IX or factor X in a tenase complex since neither clotting time (by aPTT) nor FXa generation were impacted by using NX01 BDD-FVIII compared to Xyntha®. Likewise, the animal PK/PD studies and, more importantly, the preliminary clinical studies in hemophilia A human subjects did not see impact by the variation of glycosylation and sialylation of recombinant FVIII produced with this technology(unpublished). This further corroborates the findings above suggesting that the differences between NX01 BDD-FVIII and Xyntha® are not particularly meaningful and cannot be used in isolation to assess how FVIIIs will perform in the clinic.

In addition to glycan analysis, we also examined three lots of NX01 BDD-FVIII for the sulfation of tyrosines Y346, Y770 (=Y1664 for FL-FVIII) and Y786 (=Y1680 for FL-FVIII) since these three positions were considered preferentially required for FVIII procoagulant activity and stability in plasma [19]. While Y346 and Y770 are considered important for the intrinsic activity of FVIII, position Y786 is considered critical for binding of FVIII to vWF, its chaperone and binding partner in plasma. Our data (Table 4) indicate that Y786 is > 90% sulfated and, in this study, was considerably higher than another commercial BDD-FVIII product used as comparator. Therefore, the percentage of FVIII binding with plasma vWF might be higher in NX01 BDD-FVIII, which may translate to a better stability and less immunogenicity for NX01 BDD-FVIII [20].

Sequence	Modification	Site	NX01 BDD-FVIII		Xyntha (%) (commercial)
			Mean	SD	
EDFDIYDEDENQSPR	Sulfation	Y786 (1680)	91.5	0.76	79.48
MKNNEEAEDYDDDLTDSEMDVVR	Sulfation	Y346	71	3.51	81.70
TTLQSDQEEIDYDDTISVEMK	Sulfation	Y770(1664)	70.4	2.30	67.16

Table 4: Sulfation of Select Tyrosines on FVIII. Mass Spectroscopy was Used to Identify the Percentages of Sulfated Tyrosines at Three Selected Sites, Y346, Y770 and Y786, on NX01 BDD-FVIII (From Three Different Lots) and on Commercial-Grade Xyntha®. Y786 (or Y1680 on FL-FVIII) is Considered a Primary Determinant of vWF Binding

These combined data further demonstrate that despite the massive over-expression of FVIII mediated by co-expression with vWF fragment, its glycosylation, sialylation and sulfation appear to be generally well-supported by post-translational cell machinery.

4. Conclusion

In summary, we have developed a versatile platform for overexpressing recombinant human FVIII and other assorted variants from both humans and pigs; this strategy will likely also provide an opportunity for expressing large quantities

of long-acting FVIII proteins that are in development. The methods described in this publication provides an opportunity to potentially create one or more generic versions of assorted FVIII proteins with novel attributes that can be made available to needy patients at considerably reduced cost.

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Conflict of Interests

Xu Liu, Zhijun Wang, Qianhui Li, and Chester Q. Li are employees of Beijing Neoletix Biological Technology Co. Ltd. Lijun Xu, Qingzhang Meng and Min Shen are employees of Nanjing Probiotic Biotech Co., Ltd. Chester Qi Li has patents issued to Beijing Neoletix Biological Technology Co. Ltd.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Contribution of each author

Authors 1-6: Data Curation, Resources, Investigation and Methodology. Author 7: Conceptualization, Writing-Original Draft and Supervision

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