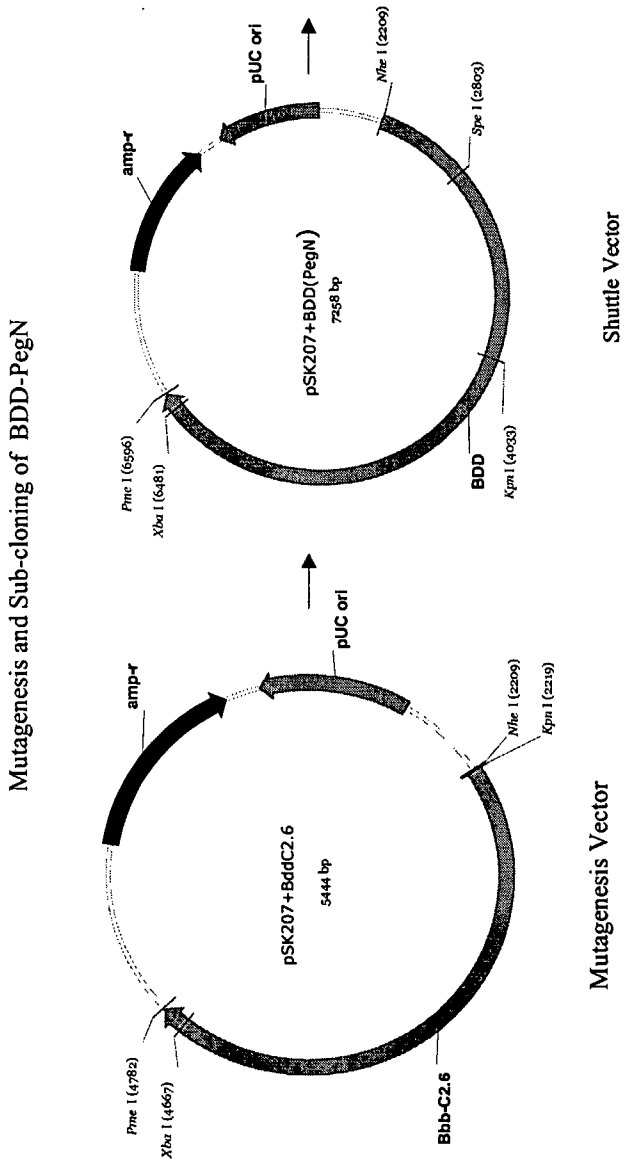

Mutagenesis and Sub-cloning of BDD-PegN
amp-r
pUC ori
pSK207+BddC2.6
5444 bp
Pme I (4782)
Xba I (4667)
Nhe I (2209)
Kpn I (2219)
Bbb-C2.6
Mutagenesis Vector
amp-r
pUC ori
pSK207+BDD(PegN)
7258 bp
Pme I (6596)
Xba I (6481)
Nhe I (2209)
Spe I (2803)
BDD
Kpn I (4033)
Shuttle Vector

FIG. 1a

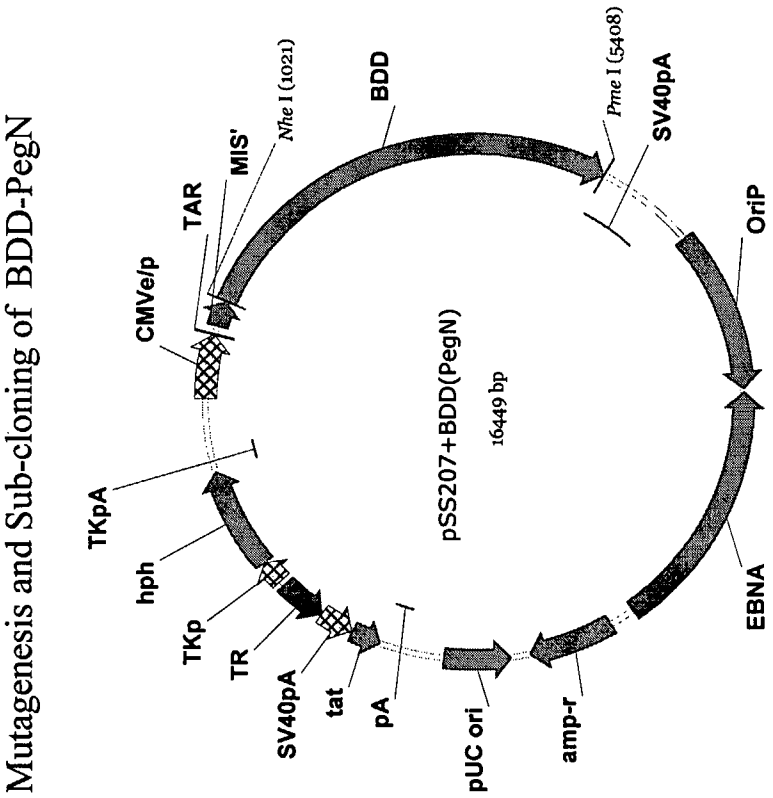

Mutagenesis and Sub-cloning of BDD-PegN
pSS207+BDD(PegN)
16449 bp
CMVe/p
TAR
MIS'
Nhe I (1021)
BDD
Pme I (5408)
SV40pA
OriP
EBNA
amp-r
pUC ori
pA
tat
SV40pA
TR
TKp
hph
TKpA
Expression Vector

FIG. 1b

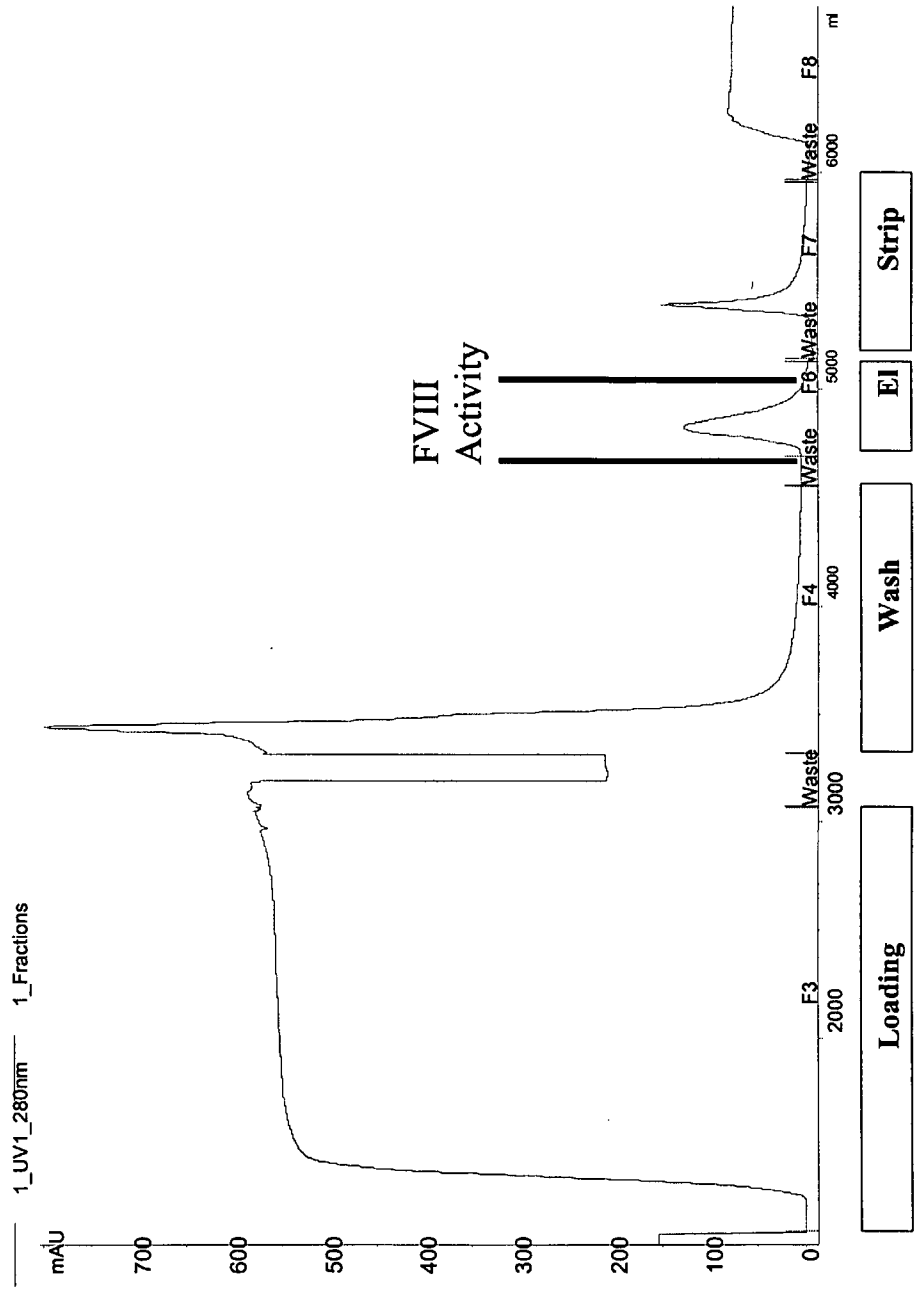

1_UV1_280nm
1_Fractions
mAU
700
600
500
400
300
200
100
0
2000
3000
4000
5000
6000
ml
F3
F4
F6
F7
F8
Waste
Waste
Waste
Waste
Waste
FVIII
Activity
Loading
Wash
El
Strip

FIG. 2

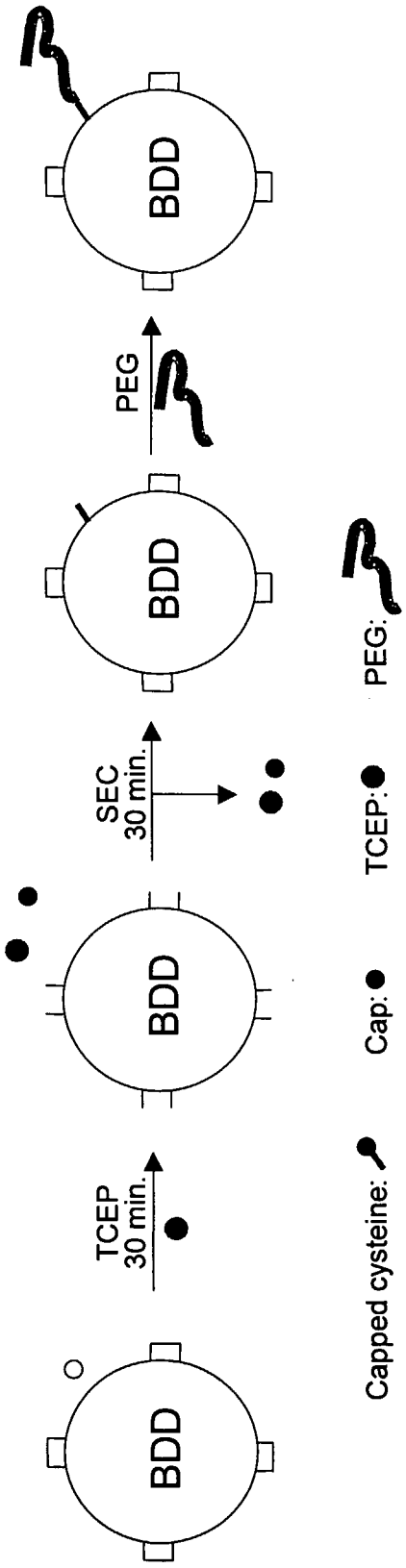

BDD
PEG
BDD
SEC
30 min.
BDD
TCEP
30 min.
BDD
PEG:
TCEP:
Cap:
Capped cysteine:

FIG. 3

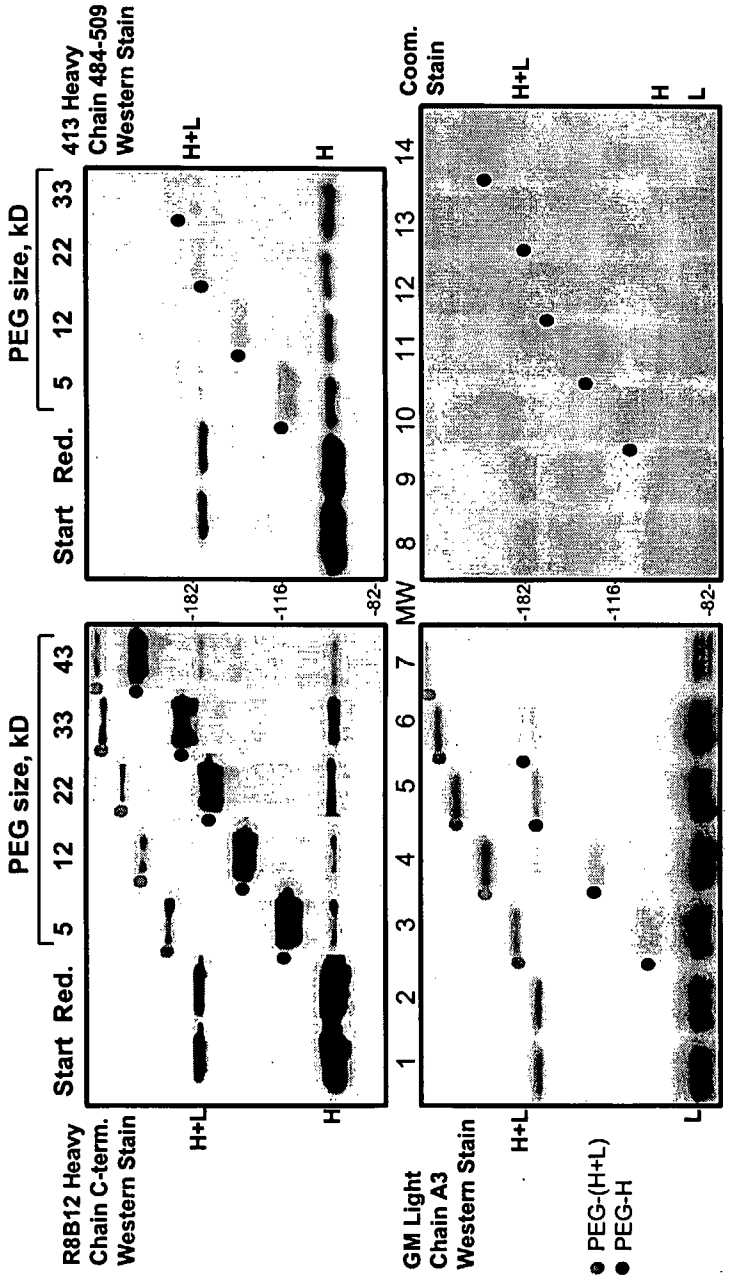

PEG size, kD
Start Red. 5 12 22 33 43
1 2 3 4 5 6 7
MW
8 9 10 11 12 13 14
R8B12 Heavy Chain C-term. Western Stain
413 Heavy Chain 484-509 Western Stain
GM Light Chain A3 Western Stain
Coom. Stain
H+L
H
L
-182
-116
-82
PEG-(H+L)
PEG-H

FIG. 4

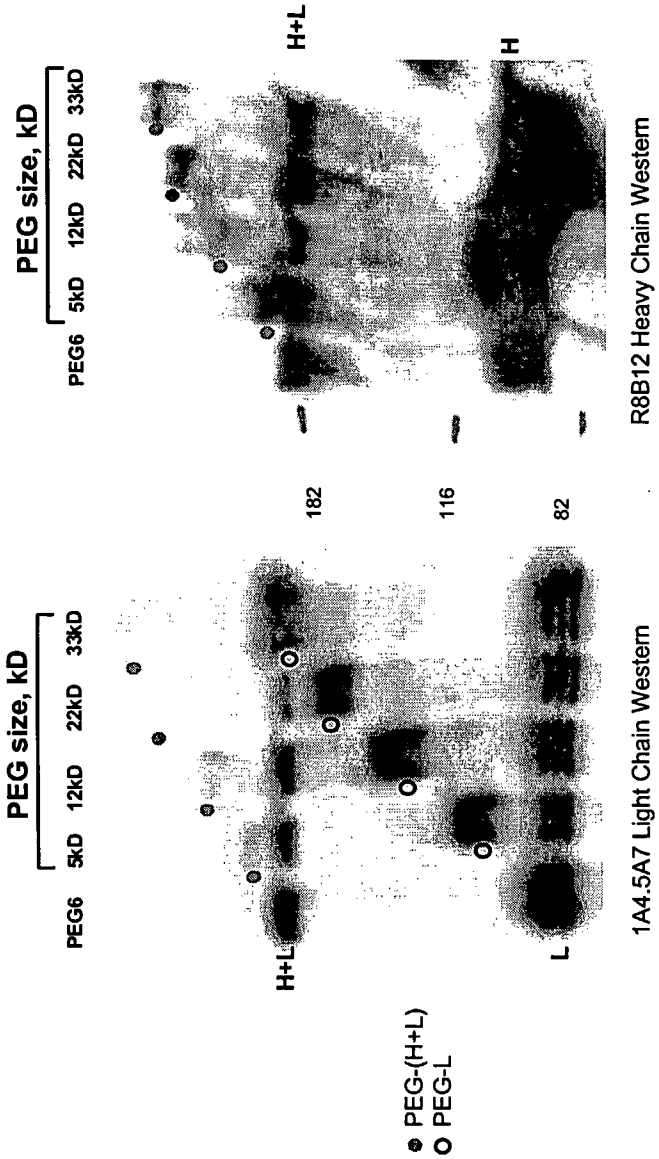

PEG size, kD
PEG6 5kD 12kD 22kD 33kD
H+L
182
116
82
H
L
R8B12 Heavy Chain Western
1A4.5A7 Light Chain Western
● PEG-(H+L)
○ PEG-L

FIG. 5

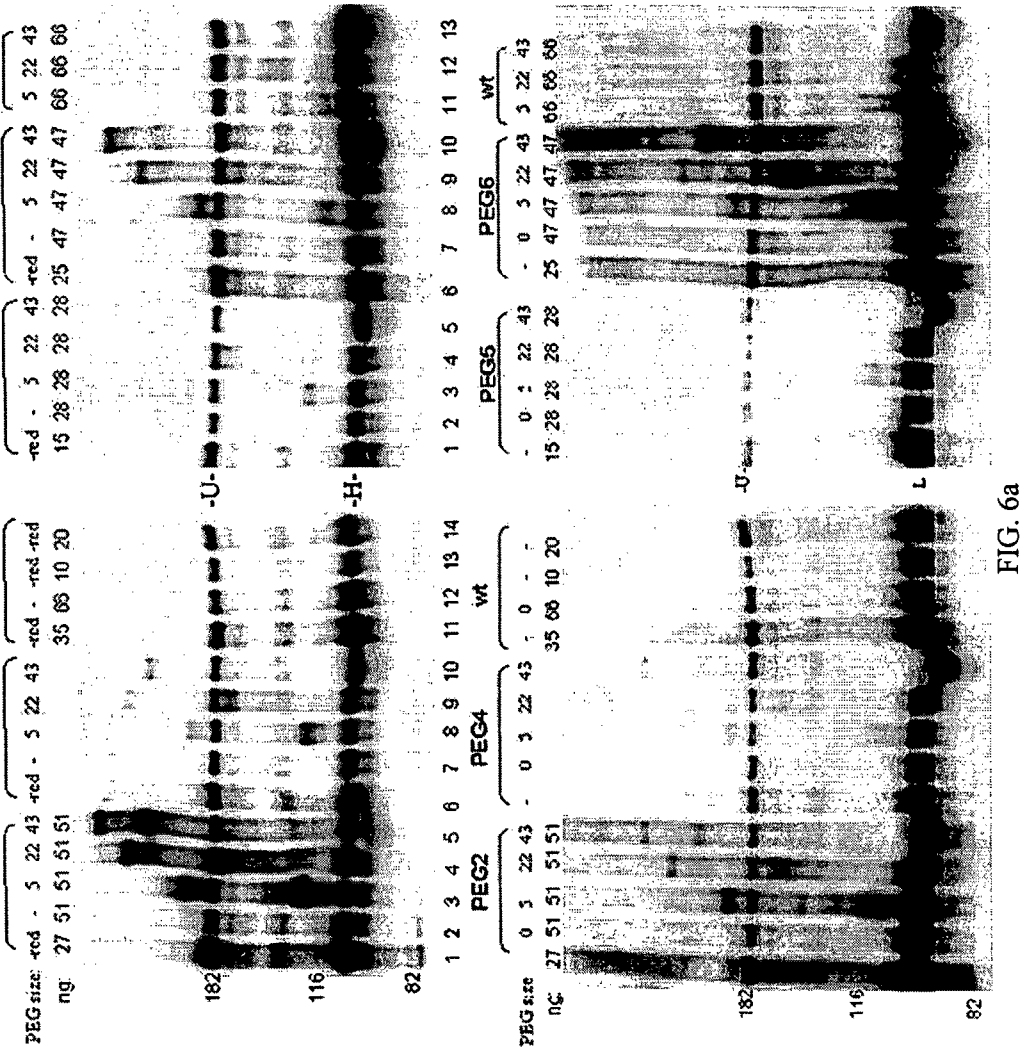

PEG2
PEG4
BDD
PEG5
PEG6
BDD
PEG size: -red - 5 22 43 -red - 5 22 43 -red -red -red -red - 5 22 43 -red - 5 22 43 5 22 43
ng: 27 51 51 51 51 35 66 10 20 15 28 28 28 28 25 47 47 47 47 66 66 66
1 2 3 4 5 6 7 8 9 10 11 12 13 14
182
116
82
-U-
-H-
PEG2
PEG4
wt
PEG5
PEG6
wt
PEG size 0 5 22 43 - 0 3 22 43 - 0 - - - - 0 1 22 43 - 0 3 22 43 3 22 43
ng 27 51 51 51 51 35 66 10 20 15 28 28 28 - 0 3 22 47 47 47 66 66 66
1 2 3 4 5 6 7 8 9 10 11 12 13
182
116
82
-U-
-L-

FIG. 6a

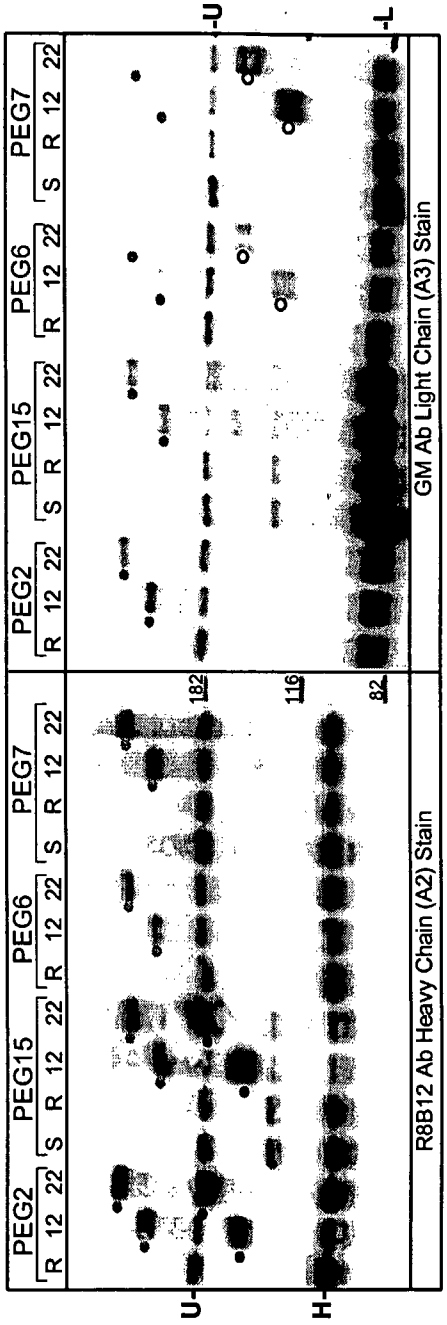

PEG2
PEG15
PEG6
PEG7
R 12 22
S R 12 22
182
116
82
U
H
L
R8B12 Ab Heavy Chain (A2) Stain
GM Ab Light Chain (A3) Stain
PEG-(H+L)
PEG-H
PEG-L

FIG. 6b

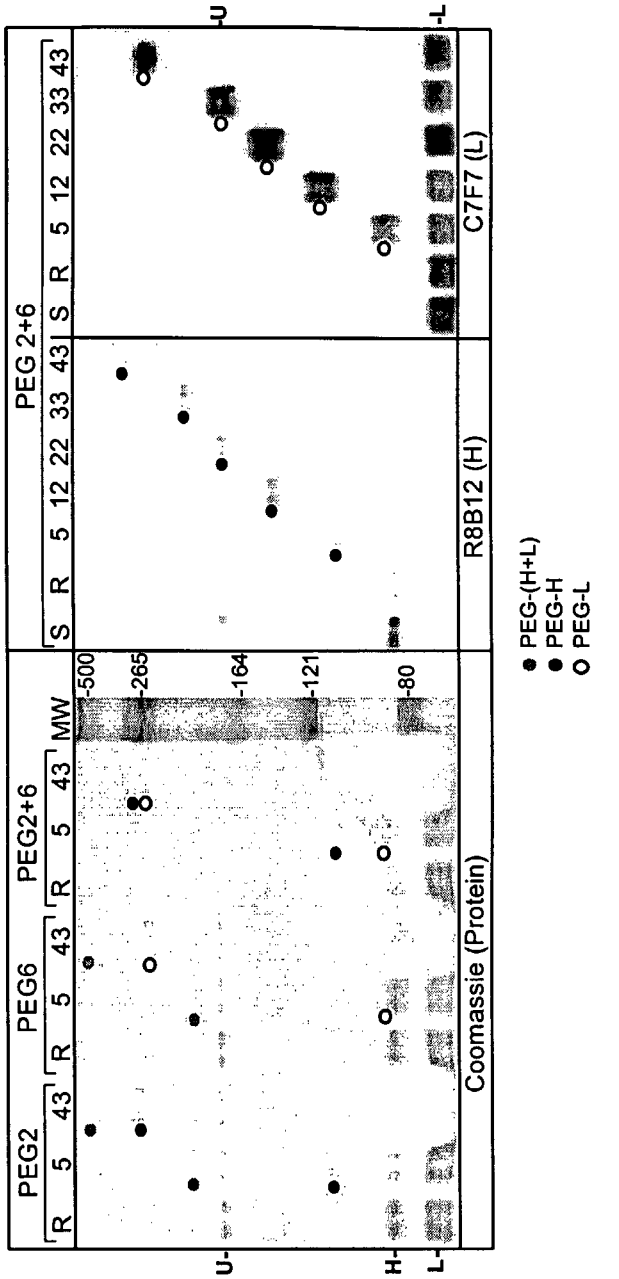

PEG2
PEG6
PEG2+6
R 5 43 R 5 43 R 5 43
MW
-500
-265
-164
-121
-80
U-
H-
L-
Coomassie (Protein)
PEG 2+6
S R 5 12 22 33 43 S R 5 12 22 33 43
-U
-L
R8B12 (H)
C7F7 (L)
PEG-(H+L)
PEG-H
PEG-L

FIG. 6c

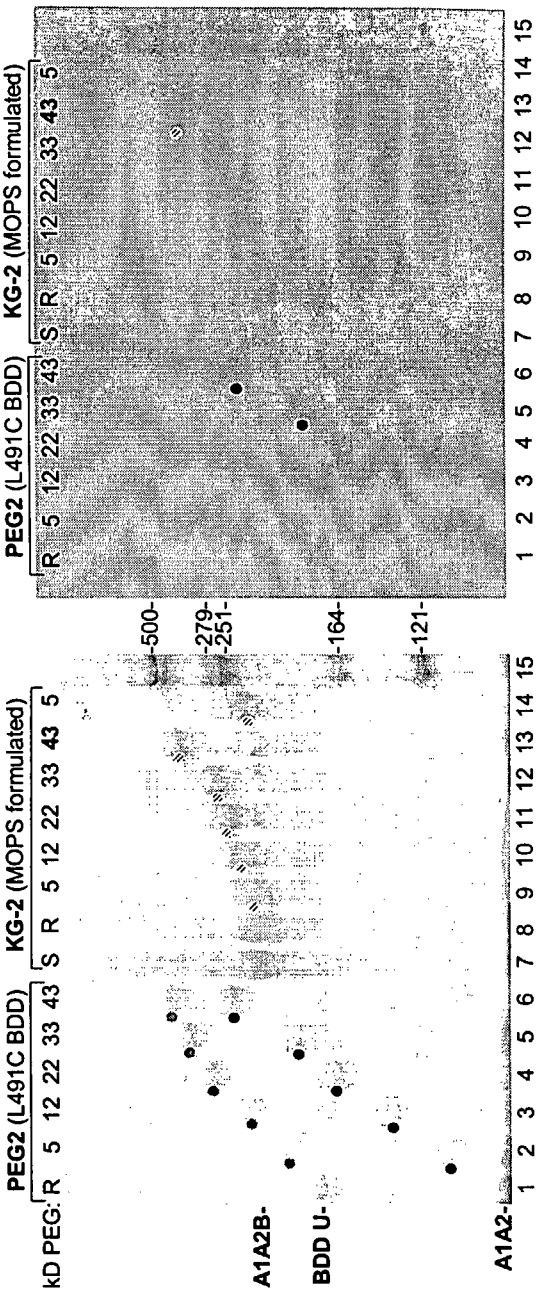

PEG2 (L491C BDD)
KG-2 (MOPS formulated)
R 5 12 22 33 43 S R 5 12 22 33 43 5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
-500-
-279-
-251-
-164-
-121-
kD PEG:
A1A2B-
BDD U-
A1A2-
PEG-(H+L)
PEG-H
PEG-A1A2B

FIG. 6d

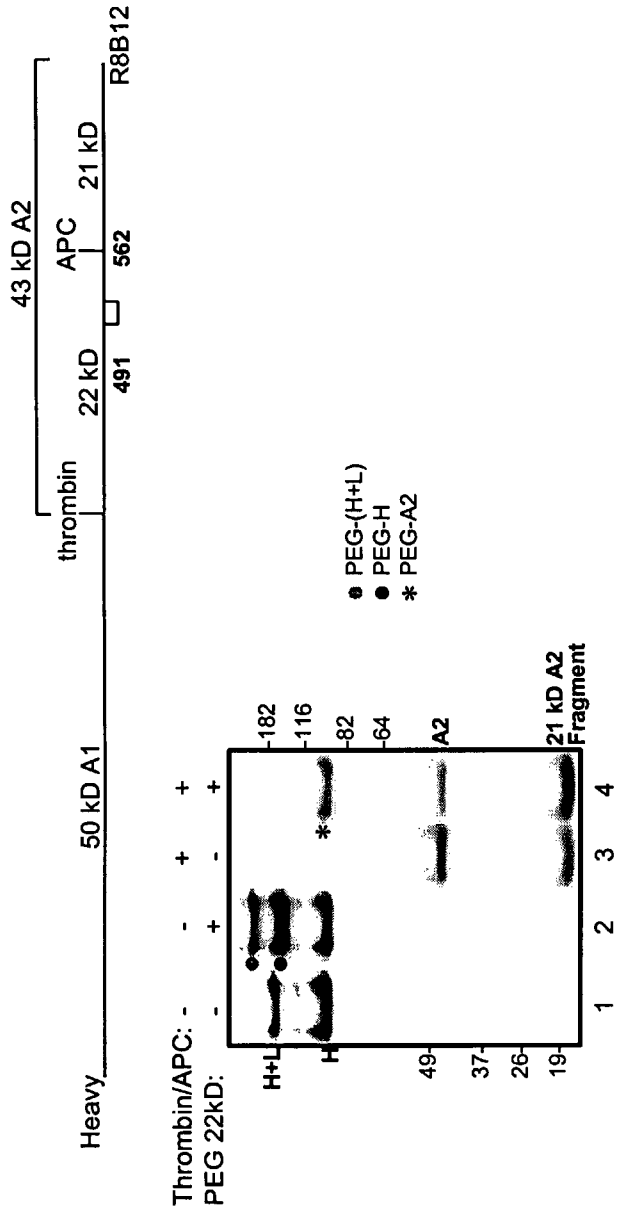

43 kD A2
APC
21 kD
R8B12
thrombin
22 kD
491
562
50 kD A1
Heavy
PEG-(H+L)
PEG-H
PEG-A2
Thrombin/APC: - - + + - + + +
PEG 22kD: - + - + - - - +
182
116
82
64
49
37
26
19
H+L
H
A2
21 kD A2 Fragment
1 2 3 4

FIG. 7

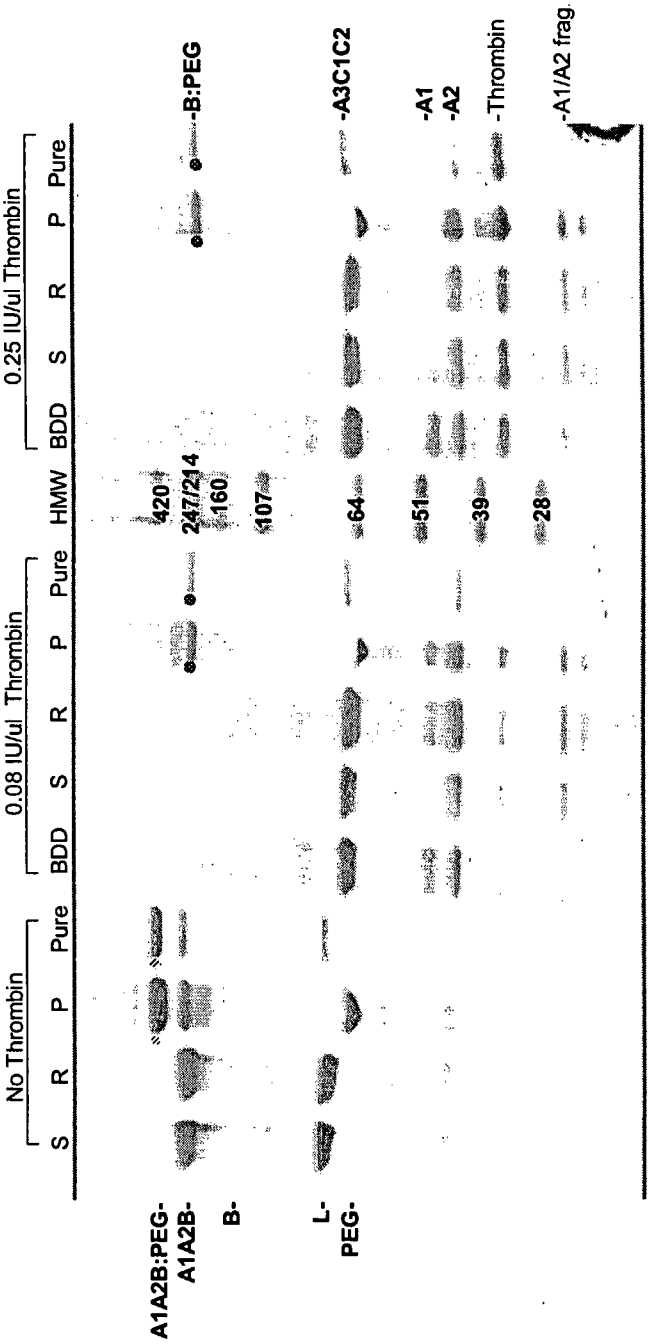

No Thrombin
0.08 IU/ul Thrombin
0.25 IU/ul Thrombin
S
R
P
Pure
BDD
S
R
P
Pure
BDD
S
R
P
Pure
HMW
A1A2B:PEG-A1A2B-
B-
L-PEG-
-B:PEG
-A3C1C2
-A1
-A2
-Thrombin
-A1/A2 frag
420
247/214
160
107
64
51
39
28
PEG-B
PEG-A1A2B

FIG. 8

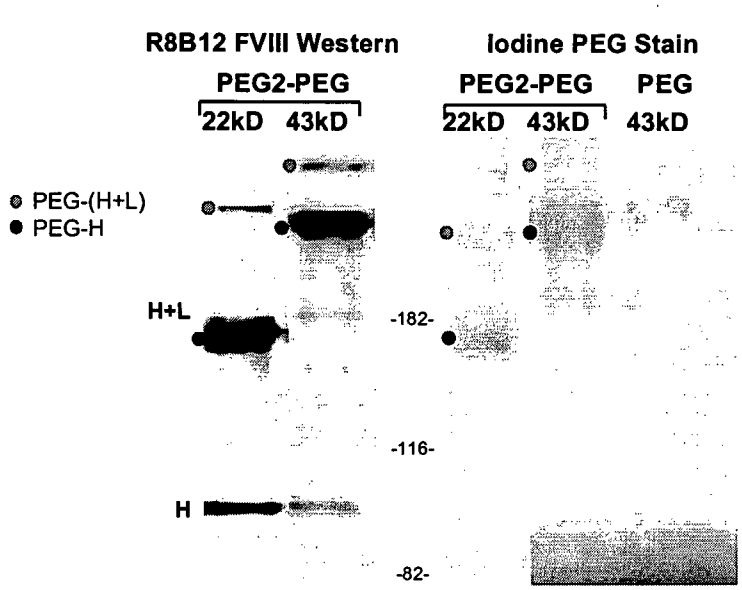

R8B12 FVIII Western
Iodine PEG Stain
PEG2-PEG
22kD 43kD
PEG2-PEG
22kD 43kD
PEG
43kD
PEG-(H+L)
PEG-H
H+L
H
-182-
-116-
-82-

FIG. 9

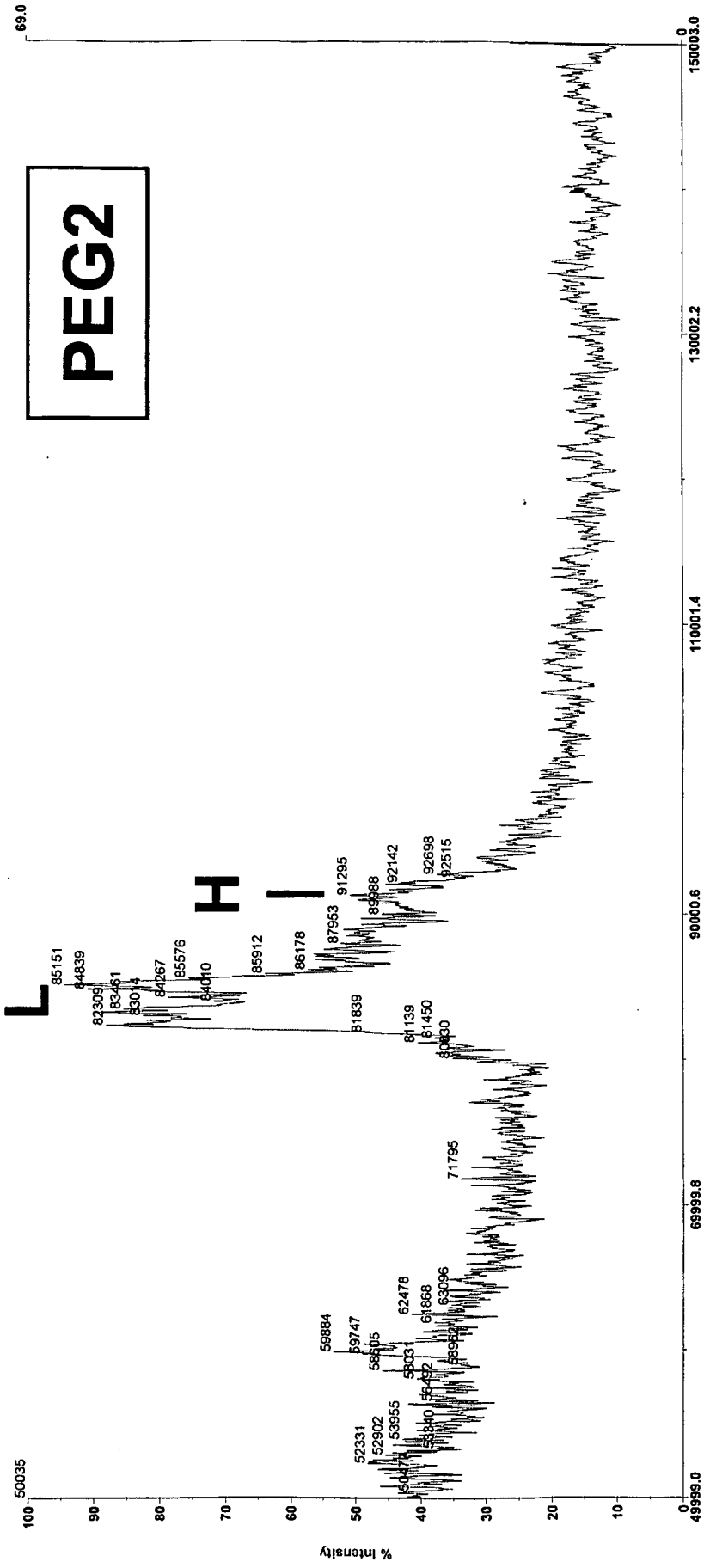
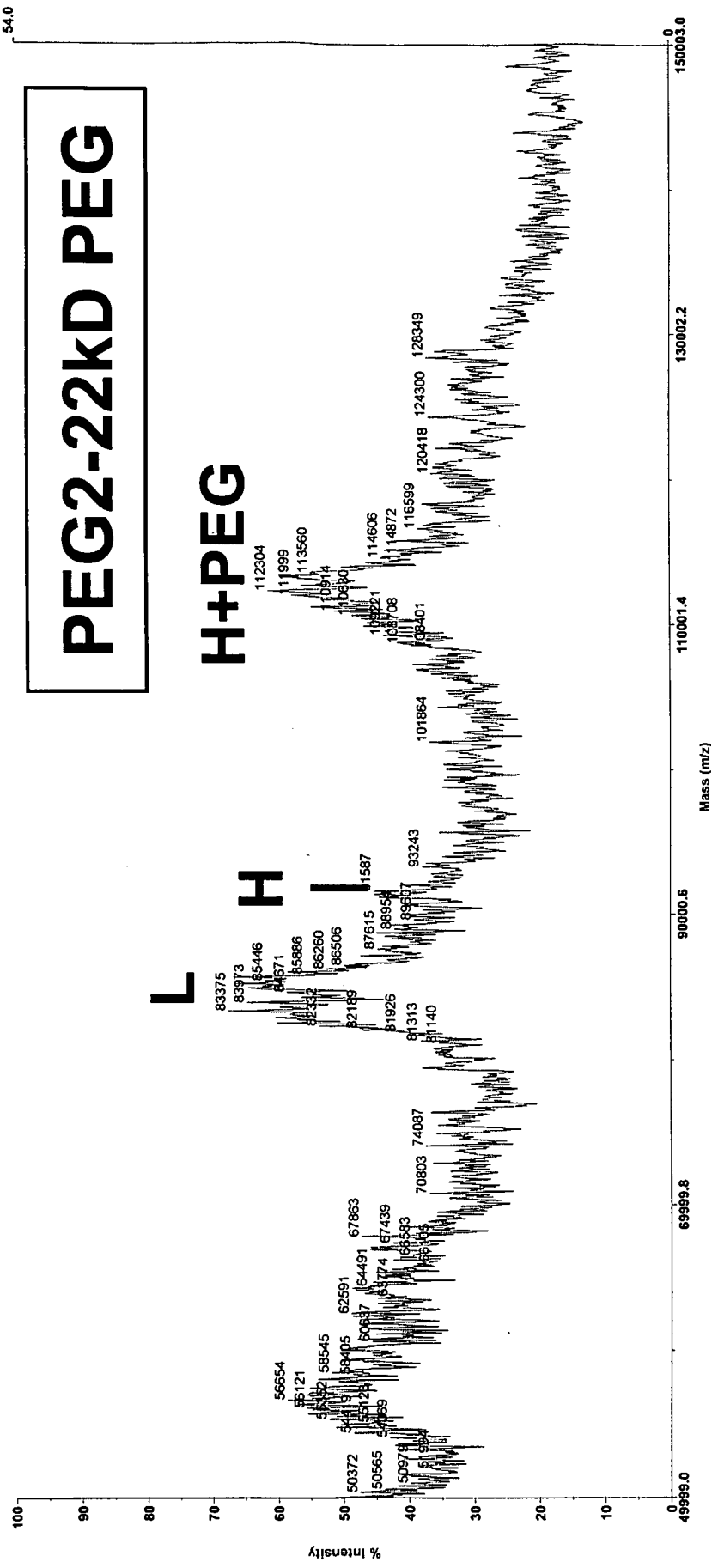

PEG2
L
H
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
50035
49999.0
69999.8
90000.6
110001.4
130002.2
150003.0
69.0
85151
84839
82309
83461
83014
84267
85576
84010
85912
86178
87953
91295
92142
92698
92515
81839
81139
81450
71795
59884
59747
62478
61868
63096
52331
52902
53955

FIG. 10a


PEG2-22kD PEG
H+PEG
L
H
% Intensity
Mass (m/z)
54.0
0
150003.0
130002.2
110001.4
90000.6
69999.8
49999.0
100
90
80
70
60
50
40
30
20
10
0
128349
124300
120418
116599
114872
114606
113560
112304
111999
110914
101864
93243
88958
87615
86506
86260
85886
85446
84671
83973
83375
82189
81926
81313
81140
74087
70803
67863
67439
64491
62591
60687
58545
56654
55121
50565
50372

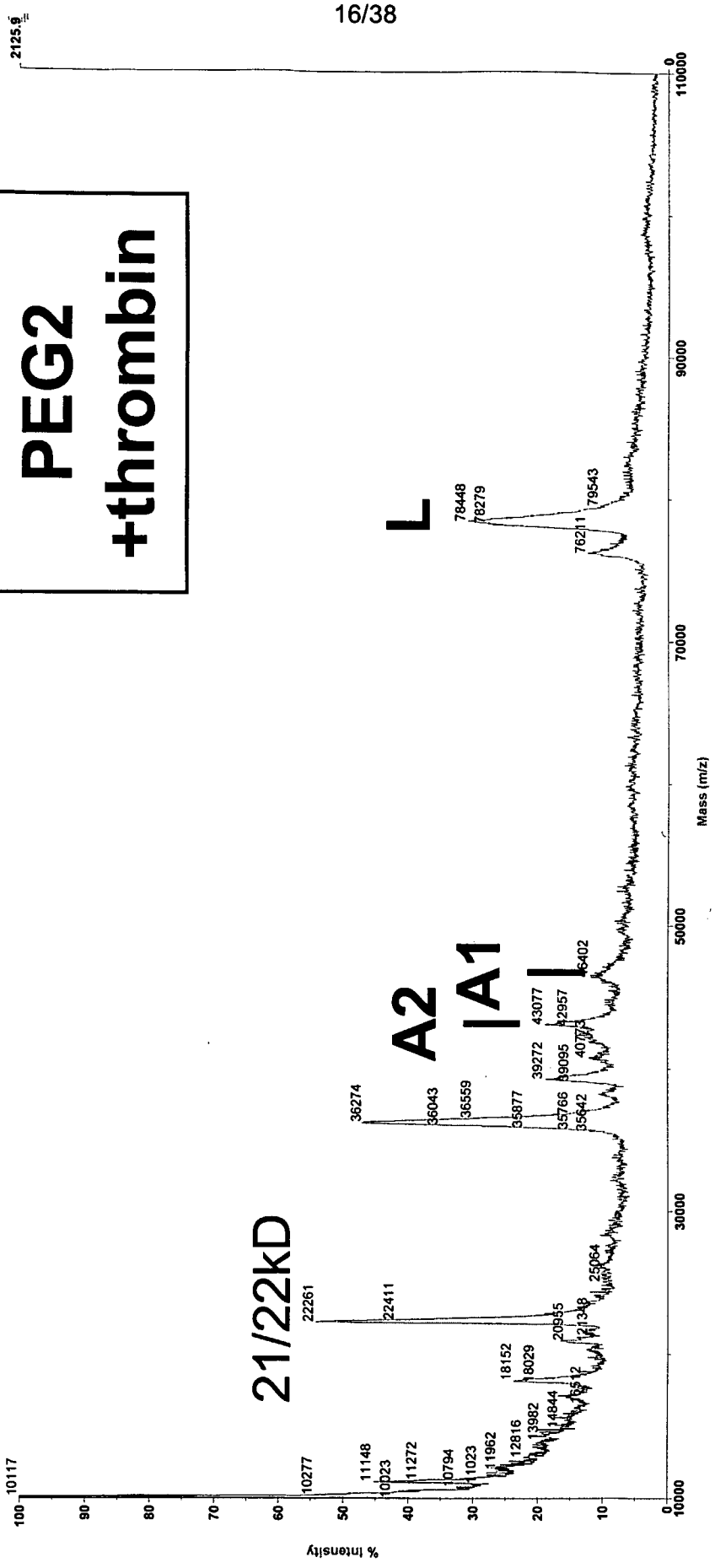


FIG. 10b

FIG. 11a

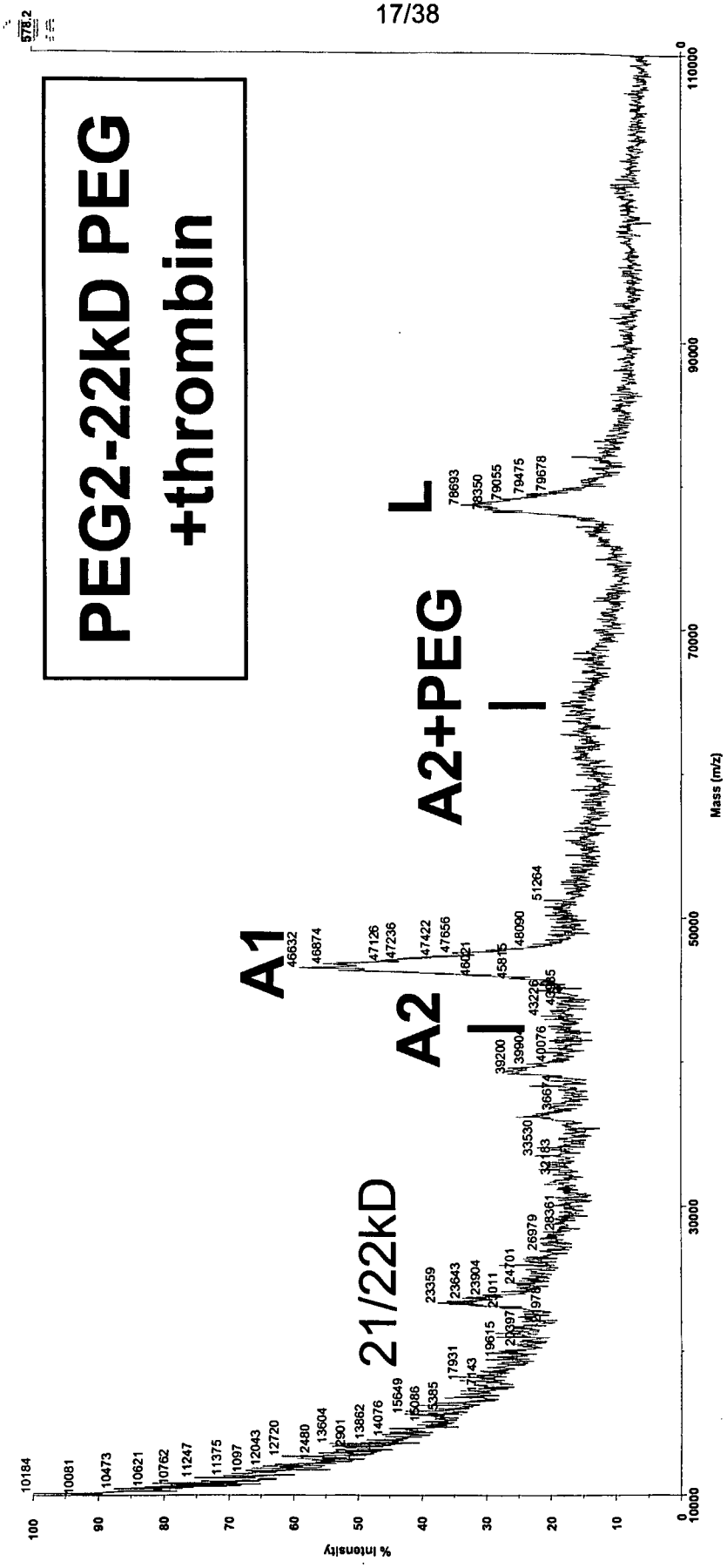

PEG2-22kD PEG
+thrombin
A1
A2
A2+PEG
L
21/22kD
% Intensity
Mass (m/z)

FIG. 11b

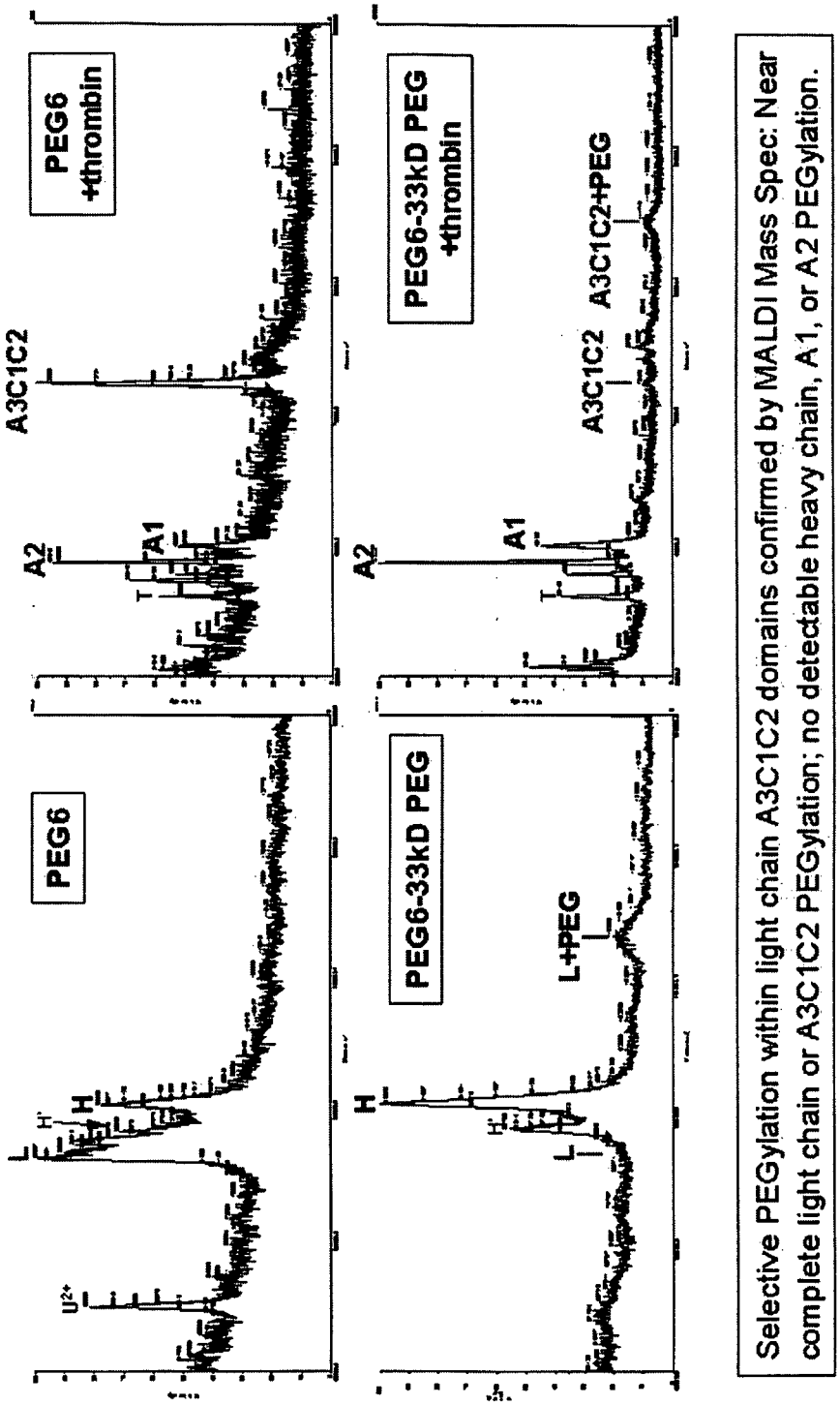

PEG6
+thrombin
A3C1C2
A2
A1
PEG6
H
U2+
PEG6-33kD PEG
+thrombin
A3C1C2+PEG
A3C1C2
A1
A2
PEG6-33kD PEG
L+PEG
H
Selective PEGylation within light chain A3C1C2 domains confirmed by MALDI Mass Spec: Near complete light chain or A3C1C2 PEGylation; no detectable heavy chain, A1, or A2 PEGylation.

FIG. 12

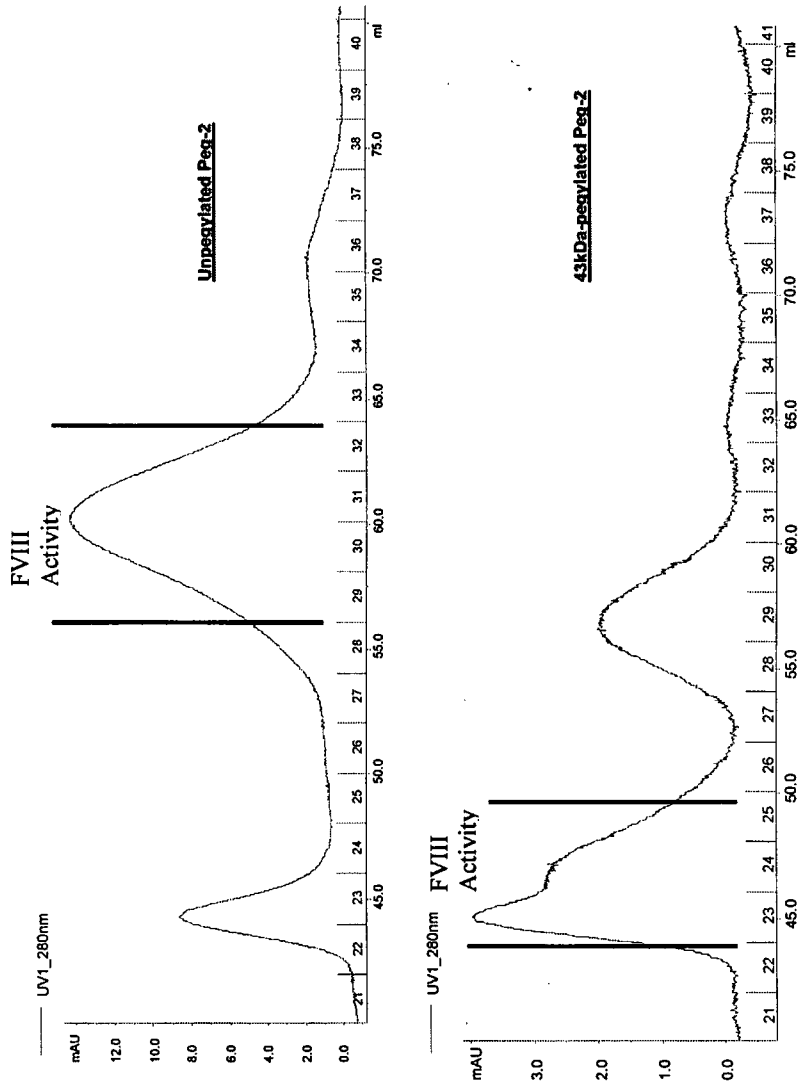

Unpegylated Peg-2
FVIII Activity
UV1_280nm
mAU
ml
43kDa-pegylated Peg-2
FVIII Activity
UV1_280nm
mAU
ml

FIG. 13

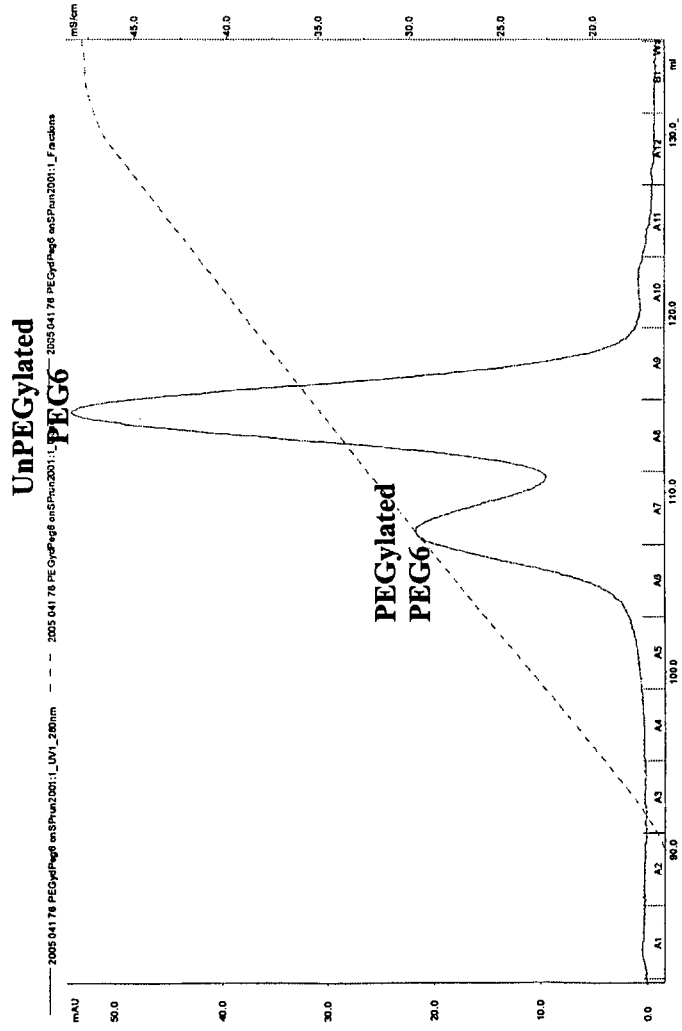


FIG. 14

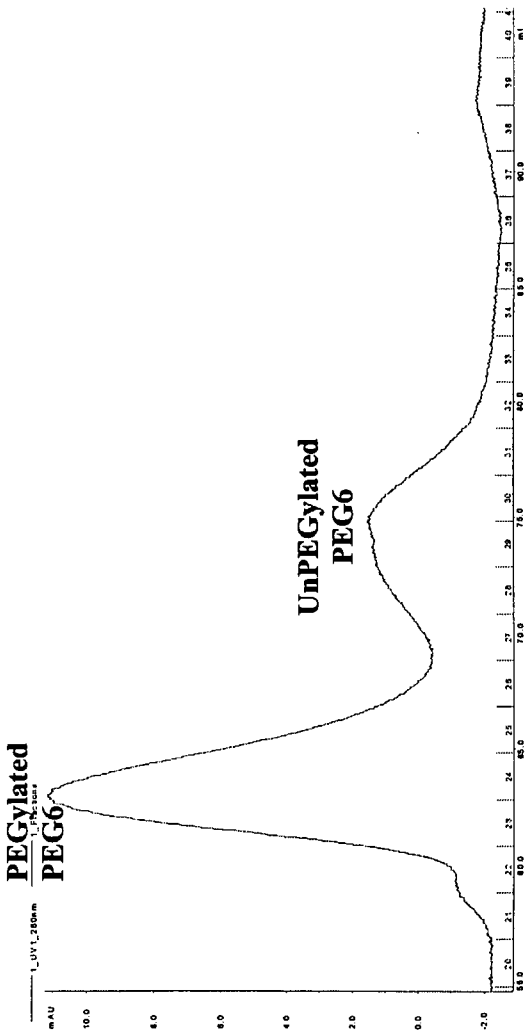


FIG. 15

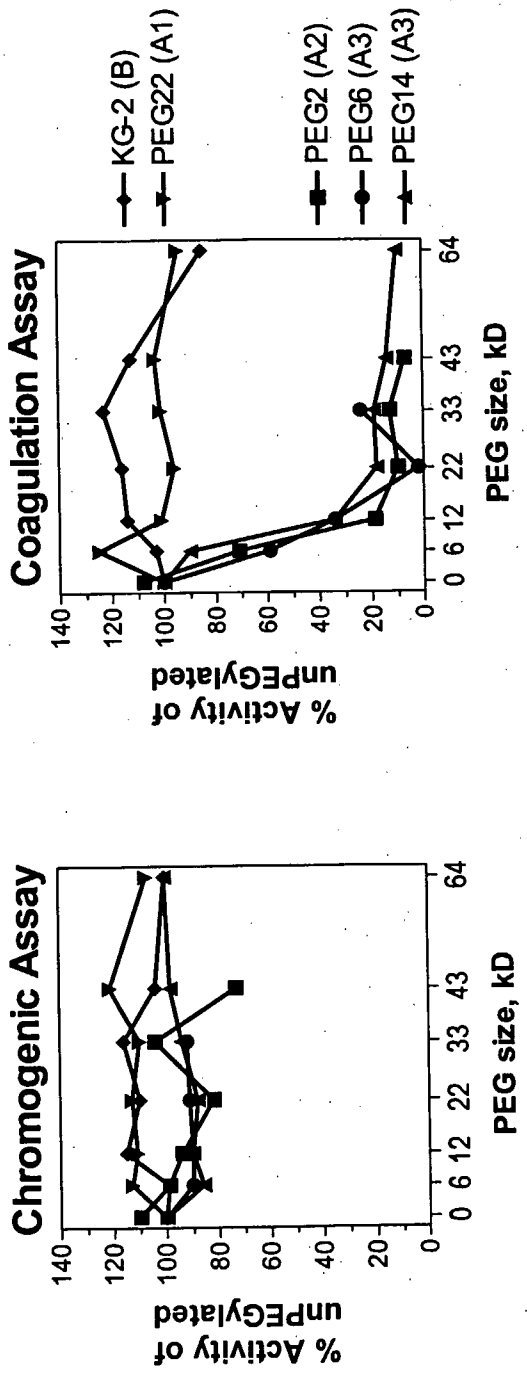

Coagulation Assay
KG-2 (B)
PEG22 (A1)
PEG2 (A2)
PEG6 (A3)
PEG14 (A3)
% Activity of unPEGylated
PEG size, kD
0 6 12 22 33 43 64
0 20 40 60 80 100 120 140
Chromogenic Assay
% Activity of unPEGylated
PEG size, kD
0 6 12 22 33 43 64
0 20 40 60 80 100 120 140

FIG. 16

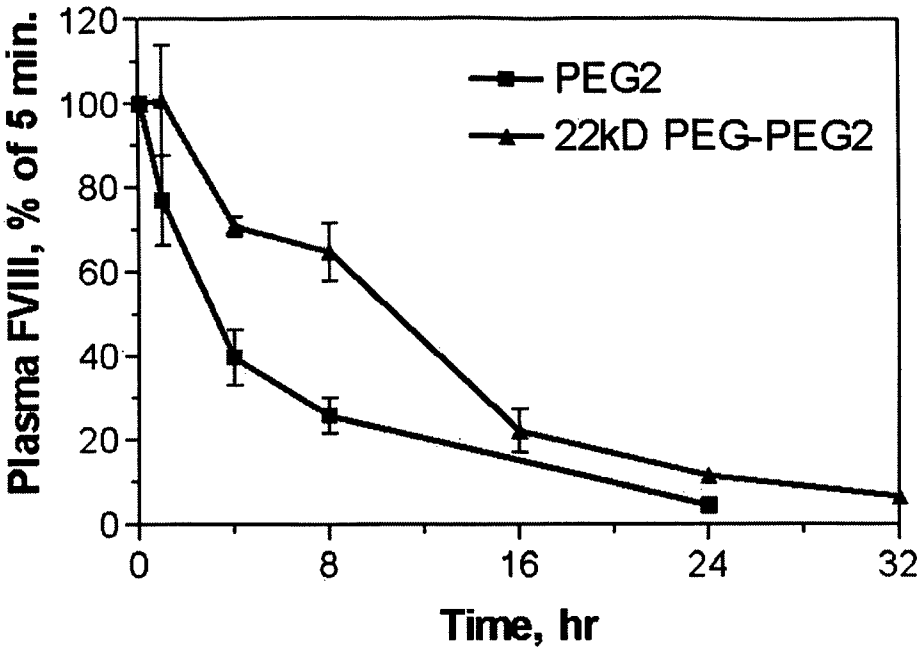

Plasma FVIII, % of 5 min.
120
100
80
60
40
20
0
0
8
16
24
32
Time, hr
PEG2
22kD PEG-PEG2

FIG. 17

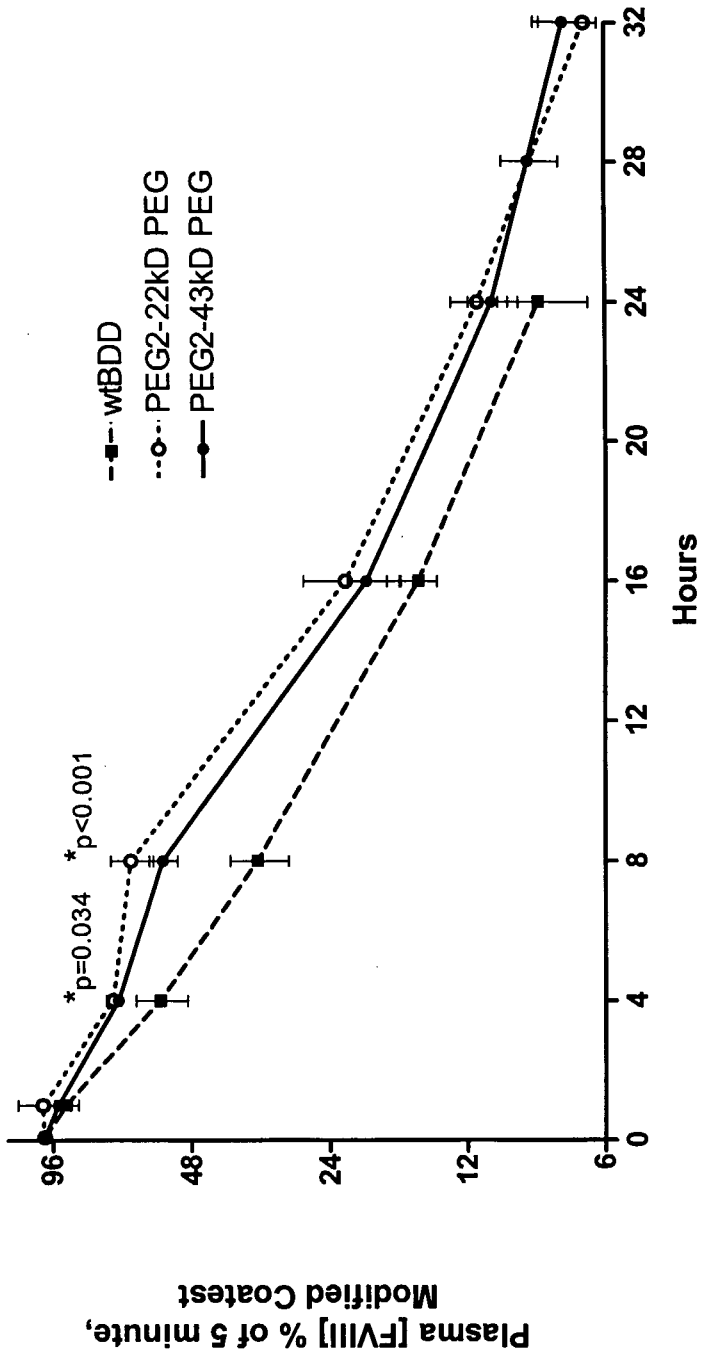

wtBDD
PEG2-22kD PEG
PEG2-43kD PEG
*p=0.034
*p<0.001
Hours
0
4
8
12
16
20
24
28
32
6
12
24
48
96
Plasma [FVIII] % of 5 minute, Modified Coatest

FIG. 18

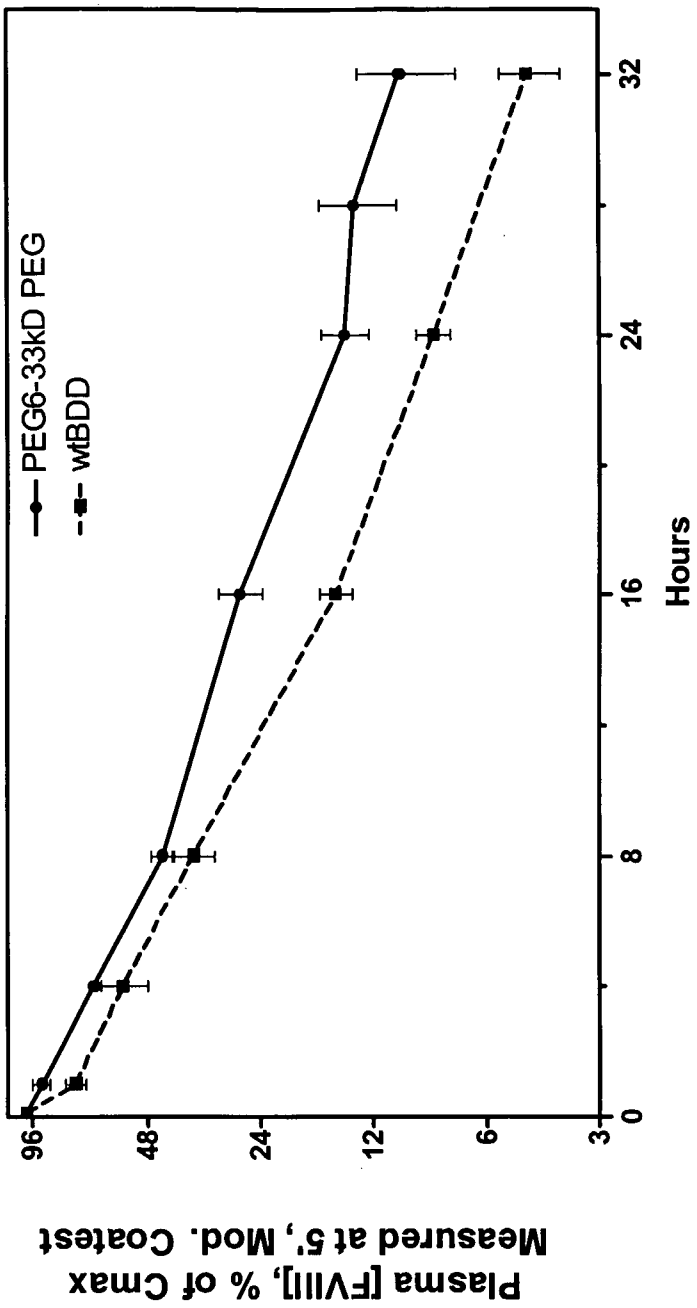

PEG6-33kD PEG
wtBDD
Hours
0
8
16
24
32
3
6
12
24
48
96
Plasma [FVIII], % of Cmax
Measured at 5', Mod. Coatest

FIG. 19

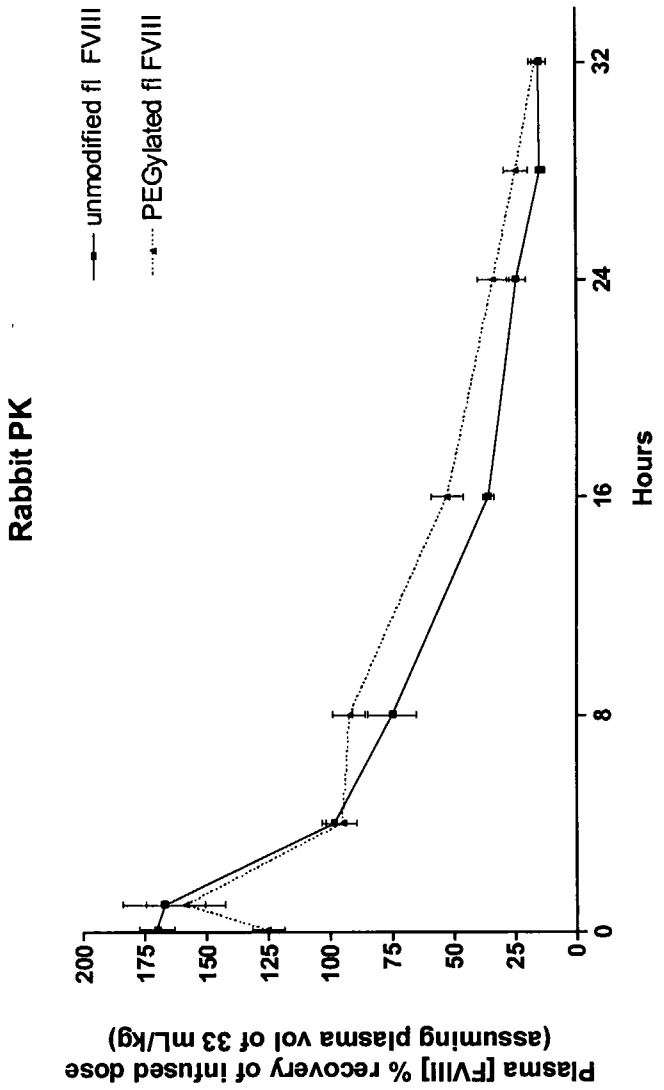

Rabbit PK
unmodified fl FVIII
PEGylated fl FVIII
Plasma [FVIII] % recovery of infused dose
(assuming plasma vol of 33 mL/kg)
200
175
150
125
100
75
50
25
0
0
8
16
24
32
Hours

FIG. 20

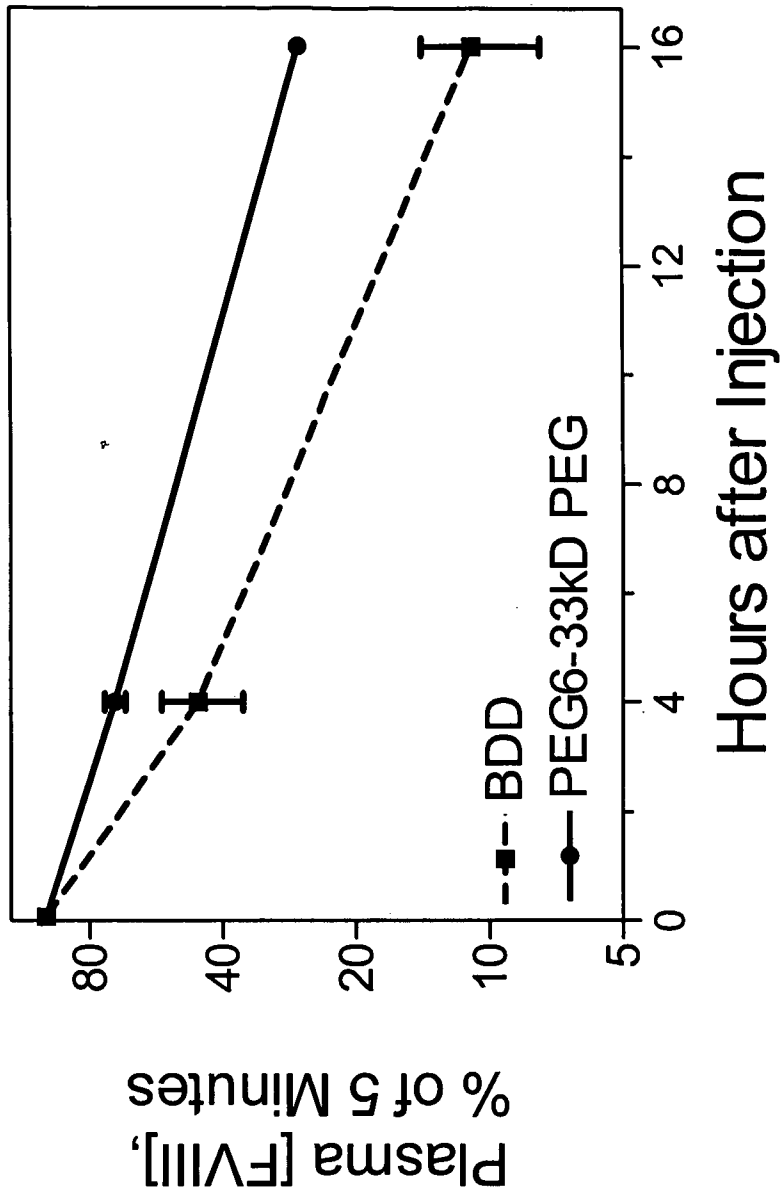

Plasma [FVIII], % of 5 Minutes
80
40
20
10
5
0
4
8
12
16
Hours after Injection
BDD
PEG6-33kD PEG

FIG. 21

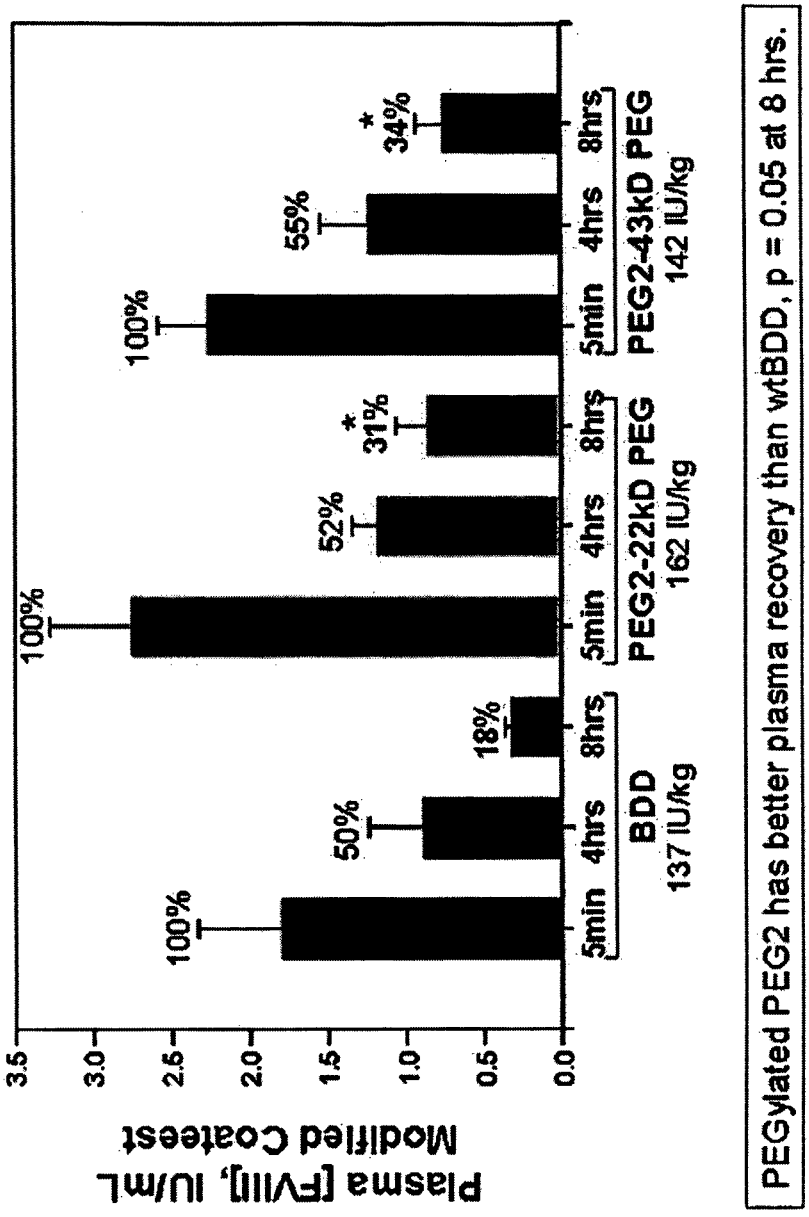

Plasma [FVIII], IU/mL
Modified Coatest
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100%
50%
18%
100%
52%
*
31%
100%
55%
*
34%
5min
4hrs
8hrs
5min
4hrs
8hrs
5min
4hrs
8hrs
BDD
137 IU/kg
PEG2-22kD PEG
162 IU/kg
PEG2-43kD PEG
142 IU/kg
PEGylated PEG2 has better plasma recovery than wtBDD, p = 0.05 at 8 hrs.

FIG. 22.

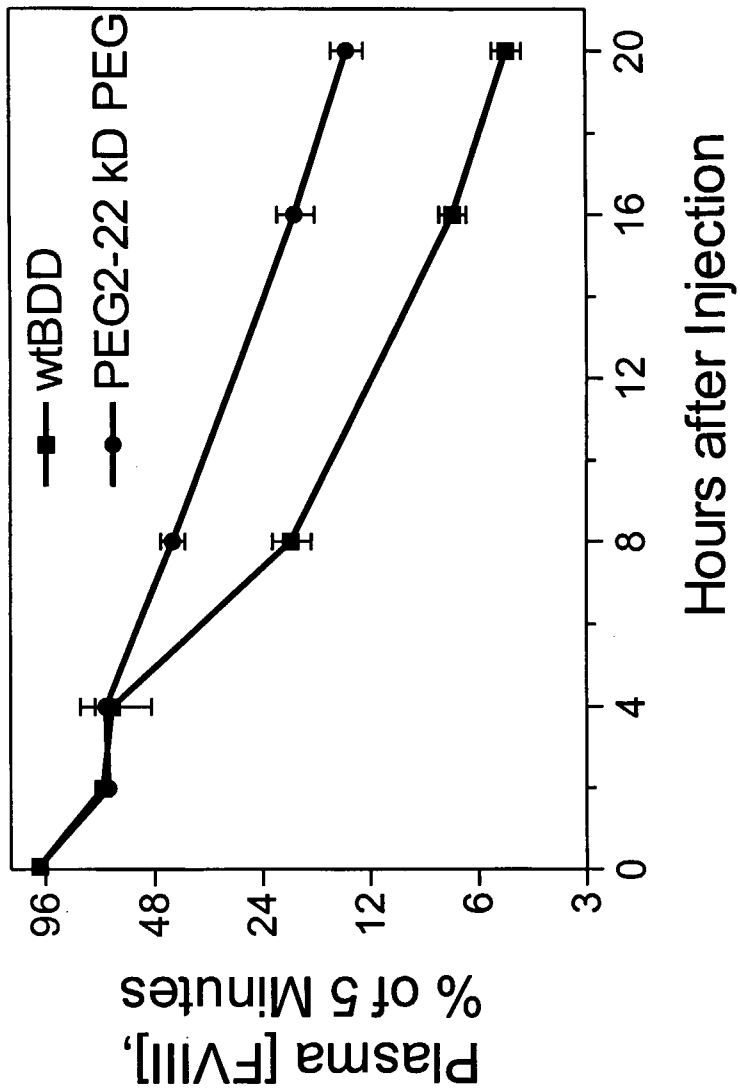

wtBDD
PEG2-22 kD PEG
Plasma [FVIII], % of 5 Minutes
96
48
24
12
6
3
0
4
8
12
16
20
Hours after Injection

FIG. 23

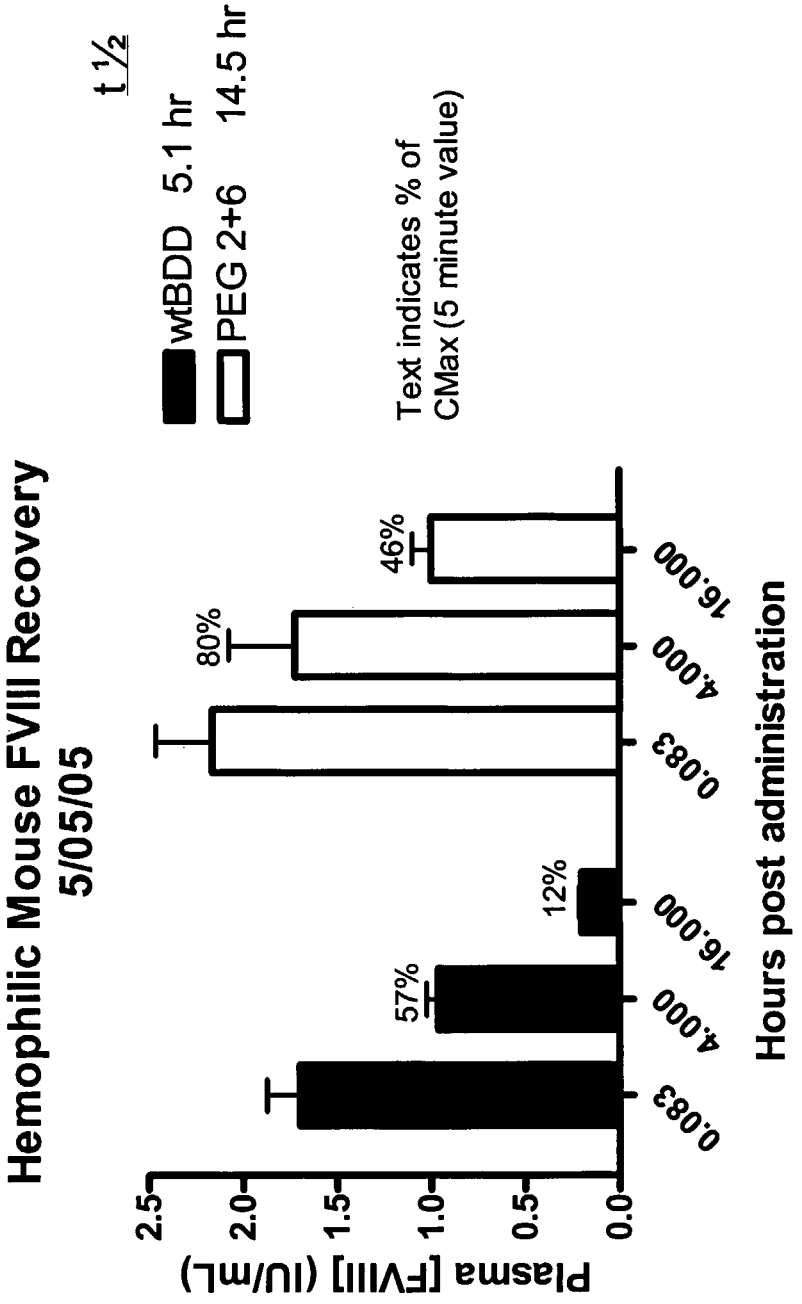

Hemophilic Mouse FVIII Recovery
5/05/05
t ½
wtBDD 5.1 hr
PEG 2+6 14.5 hr
Text indicates % of
CMax (5 minute value)
Plasma [FVIII] (IU/mL)
2.5
2.0
1.5
1.0
0.5
0.0
57%
12%
80%
46%
0.083
4.000
16.000
0.083
4.000
16.000
Hours post administration

FIG. 24

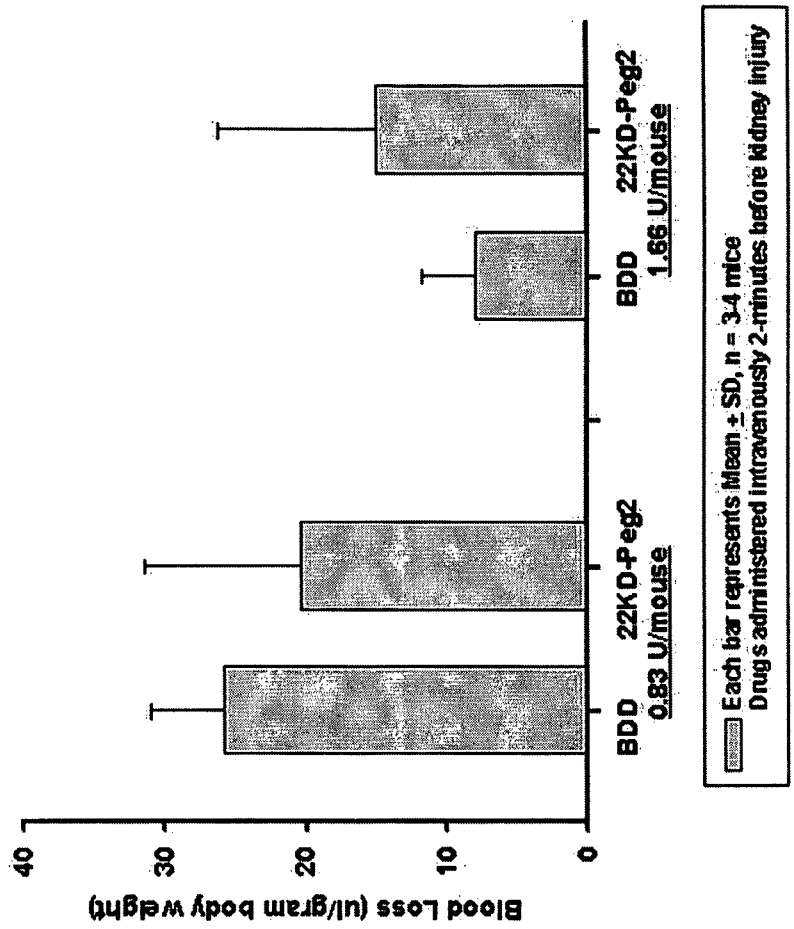

Blood Loss (ul/gram body weight)
0
10
20
30
40
BDD
22KD-Peg2
0.83 U/mouse
BDD
22KD-Peg2
1.66 U/mouse
Each bar represents Mean ± SD, n = 3-4 mice
Drugs administered intravenously 2-minutes before kidney injury

FIG. 25.

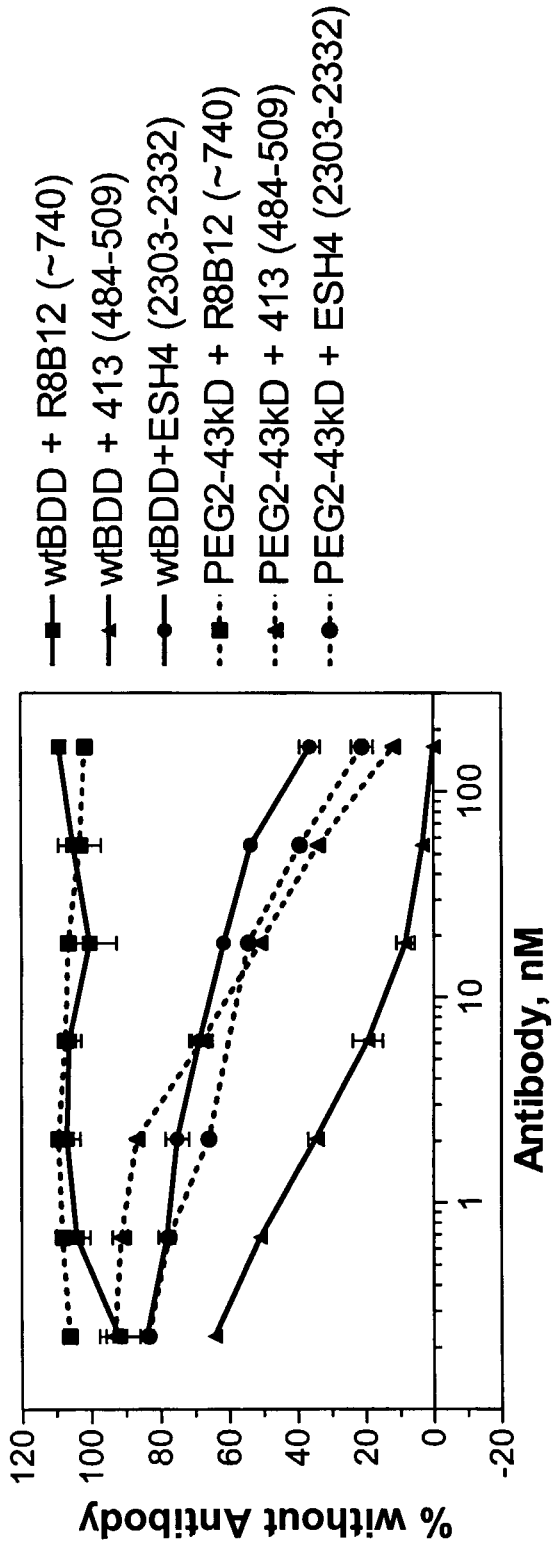

wtBDD + R8B12 (~740)
wtBDD + 413 (484-509)
wtBDD+ESH4 (2303-2332)
PEG2-43kD + R8B12 (~740)
PEG2-43kD + 413 (484-509)
PEG2-43kD + ESH4 (2303-2332)
% without Antibody
120
100
80
60
40
20
0
-20
1
10
100
Antibody, nM

FIG. 26

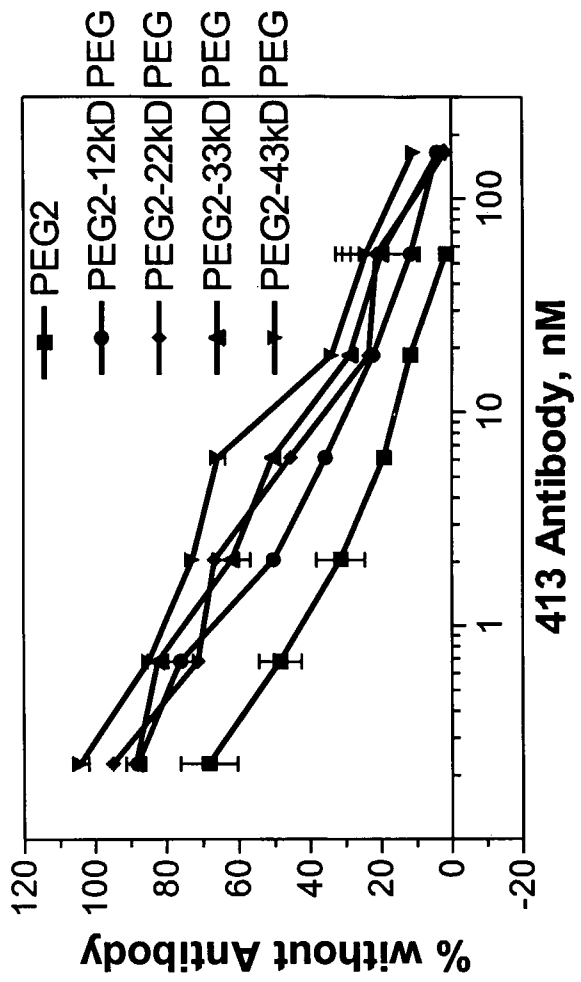

PEG2
PEG2-12kD PEG
PEG2-22kD PEG
PEG2-33kD PEG
PEG2-43kD PEG
% without Antibody
120
100
80
60
40
20
0
-20
1
10
100
413 Antibody, nM

FIG. 27

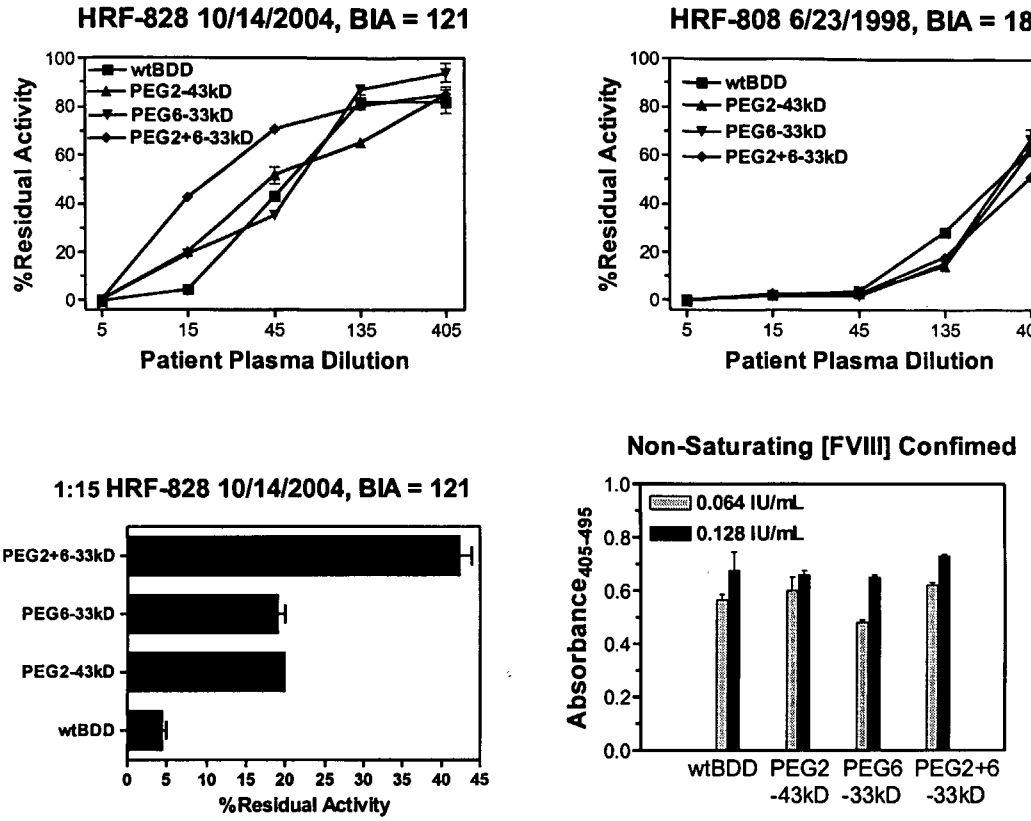

HRF-828 10/14/2004, BIA = 121
%Residual Activity
Patient Plasma Dilution
wtBDD
PEG2-43kD
PEG6-33kD
PEG2+6-33kD
HRF-808 6/23/1998, BIA = 188
1:15 HRF-828 10/14/2004, BIA = 121
%Residual Activity
Non-Saturating [FVIII] Confimed
Absorbance405-495
0.064 IU/mL
0.128 IU/mL
wtBDD
PEG2 -43kD
PEG6 -33kD
PEG2+6 -33kD

FIG. 28

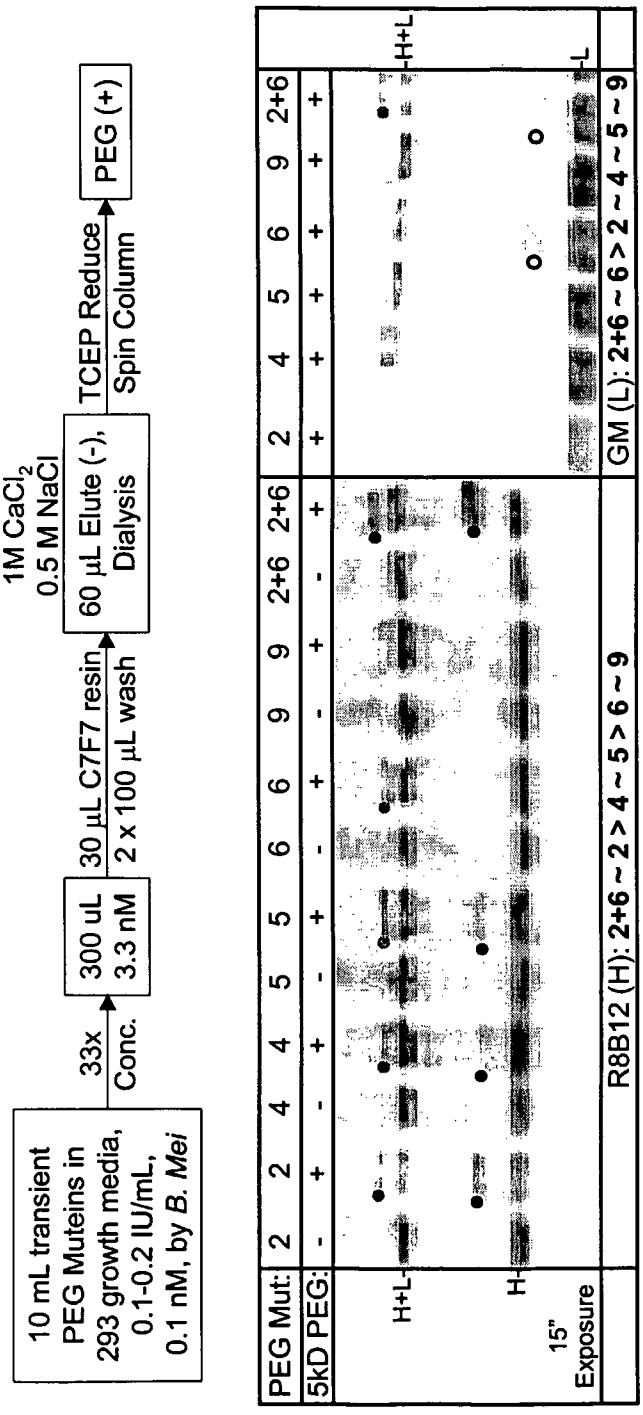

10 mL transient PEG Muteins in 293 growth media, 0.1-0.2 IU/mL, 0.1 nM, by B. Mei
33x Conc.
300 uL 3.3 nM
30 µL C7F7 resin
2 x 100 µL wash
60 µL Elute (-), Dialysis
1M CaCl2 0.5 M NaCl
TCEP Reduce Spin Column
PEG (+)
PEG Mut: 2 2 4 4 5 5 6 6 9 9 2+6 2+6 2 4 5 6 9 2+6
5kD PEG: - + - + - + - + - + - + + + + + + +
H+L
H
15" Exposure
R8B12 (H): 2+6 ~ 2 > 4 ~ 5 > 6 ~ 9
GM (L): 2+6 ~ 6 > 2 ~ 4 ~ 5 ~ 9
PEG-(H+L)
PEG-H
PEG-L

FIG. 29

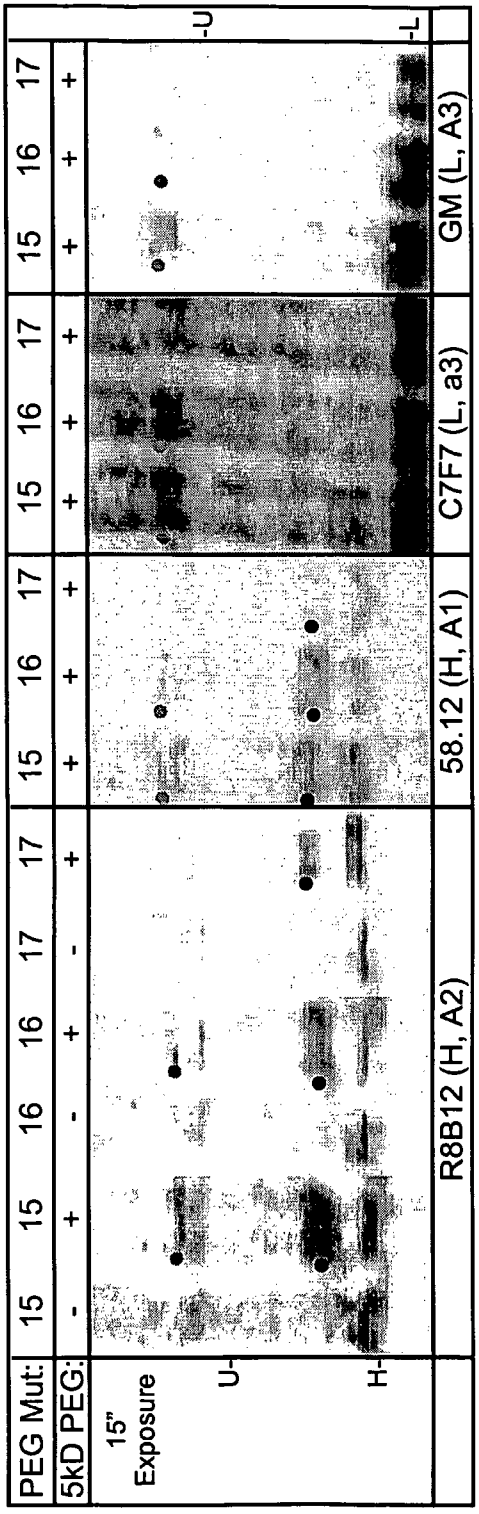

PEG Mut:
15 15 16 16 17 17
5kD PEG:
- + - + - +
15" Exposure
U-
H-
R8B12 (H, A2)
15 16 17
+ + +
58.12 (H, A1)
15 16 17
+ + +
C7F7 (L, a3)
15 16 17
+ + +
GM (L, A3)
-U
-L
PEG-(H+L)
PEG-H

FIG. 30

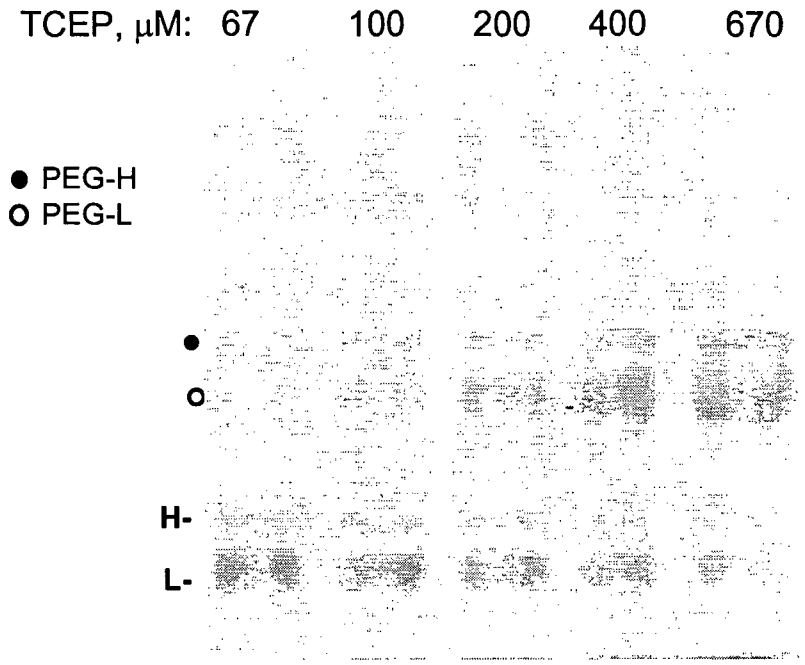


FIG. 31

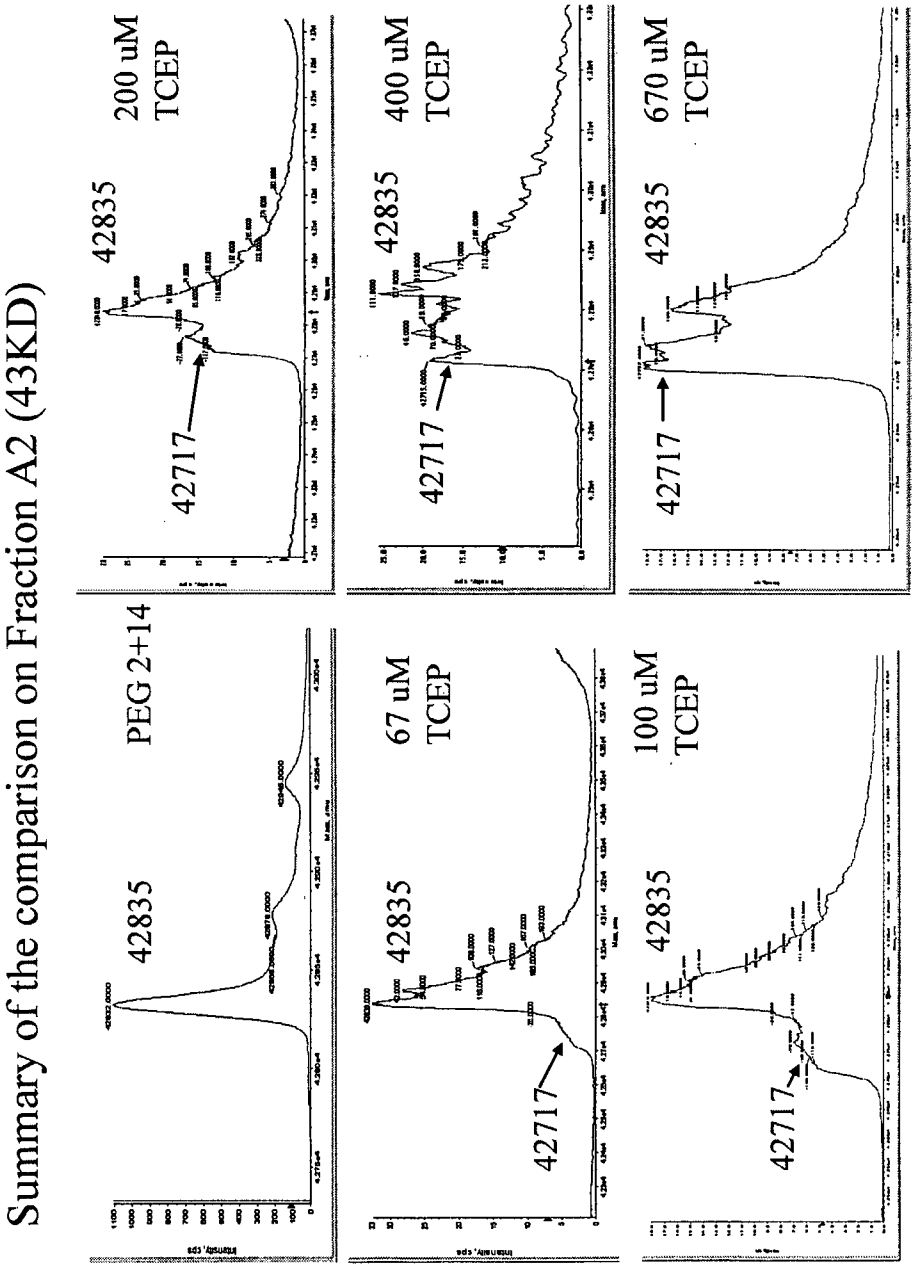

Summary of the comparison on Fraction A2 (43KD)
42835
PEG 2+14
42717
42835
67 uM
TCEP
42717
42835
100 uM
TCEP
42717
42835
200 uM
TCEP
42717
42835
400 uM
TCEP
42717
42835
670 uM
TCEP
42717

FIG. 32