Fig. 1

Reversible coating of nanoparticles surface with PEG

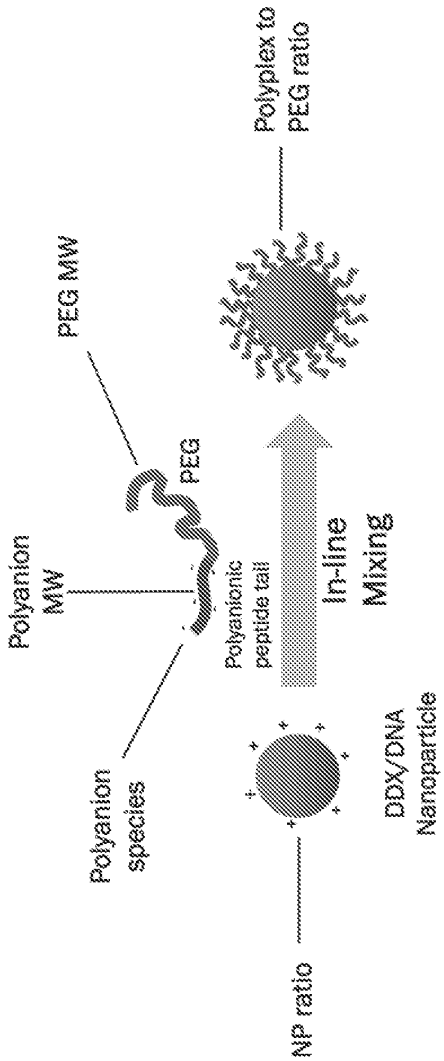


Fig. 2

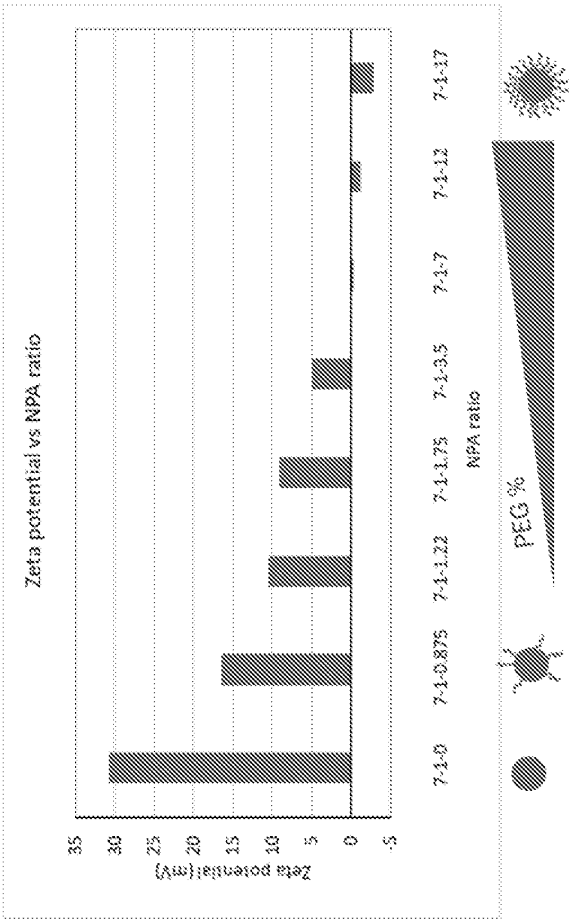
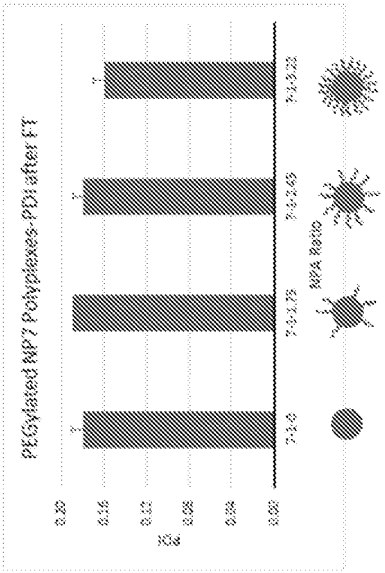

Zeta potential vs NPA ratio
Zeta potential(mV)
35
30
25
20
15
10
5
0
-5
7-1-0
7-1-0.875
7-1-1.22
7-1-1.75
7-1-3.5
7-1-7
7-1-12
7-1-17
NPA ratio
PEG %

Fig. 3


PEGylated NP7 Polyplexes-PDI after FT
PDI
0.20
0.15
0.10
0.05
0.00
7:1:0
7:1:1.75
7:1:2.45
7:1:3.22
NRA Ratio

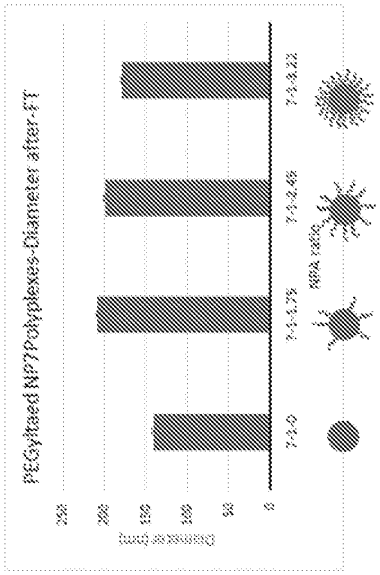

PEGylated NP7Polyplexes-Diameter after FT
Diameter (nm)
250
200
150
100
50
0
7:1:0
7:1:1.75
7:1:2.45
7:1:3.22
NRA ratio

Fig. 4

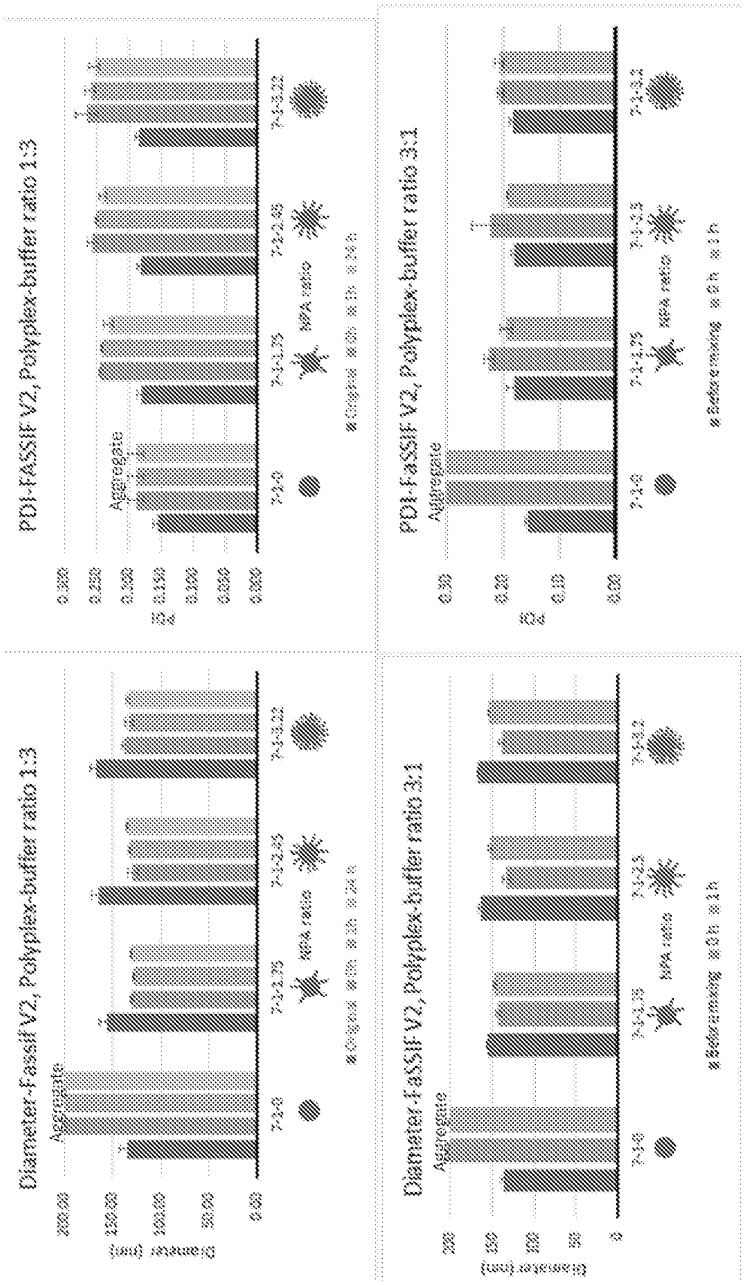
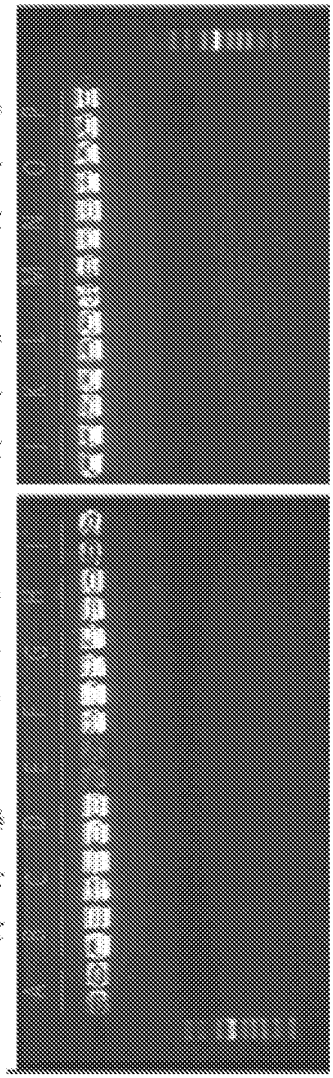
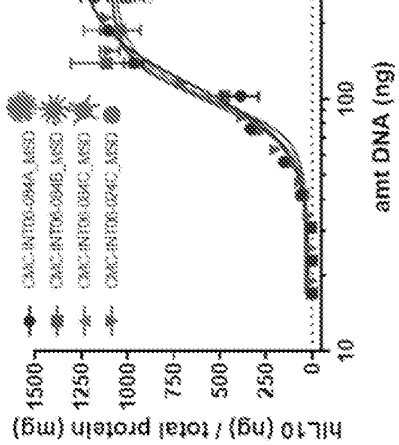

PDI-FASSIF V2, Polyplex-buffer ratio 1:3
PDI-FaSSIF V2, Polyplex-buffer ratio 3:1
Diameter-Fassif V2, Polyplex-buffer ratio 1:3
Diameter-FaSSIF V2, Polyplex-buffer ratio 3:1
Aggregate
MPA ratio
Diameter (nm)
PDI

Fig. 5



Lane	N:P:A ratio	Buffer	Mixing volume (FaSSIF uL+PP uL)
A	7:1:0	FaSSIFV1	75+50
B	7:1:1.75	FaSSIFV1	75+50
C	7:1:2.45	FaSSIFV1	75+50
D	7:1:3.22	FaSSIFV1	75+50
E	7:1:0	FaSSIFV2	75+50
F	7:1:1.75	FaSSIFV2	75+50
G	7:1:2.45	FaSSIFV2	75+50
H	7:1:3.22	FaSSIFV2	75+50
I	7:1:0	FaSSIFV1	50+100
J	7:1:1.75	FaSSIFV1	50+100
K	7:1:2.45	FaSSIFV1	50+100
L	7:1:3.22	FaSSIFV1	50+100
M	7:1:0	FaSSIFV2	50+100
N	7:1:1.75	FaSSIFV2	50+100
O	7:1:2.45	FaSSIFV2	50+100
P	7:1:3.22	FaSSIFV2	50+100

Fig. 6



	Top	HillSlope	EC50	R squared
CMC-INT06-084A_MSD	1269	3.7	112.6	0.98
CMC-INT06-084B_MSD	1064	3.9	99.7	0.95
CMC-INT06-084C_MSD	1074	4.6	98.4	0.96
CMC-INT06-024C_MSD	1085	4.8	96.9	0.98

Rows 1,2 &3 are PEGylated NP7 polyplexes

Row 4 is NP7 non-PEGylated polyplex

Fig. 7

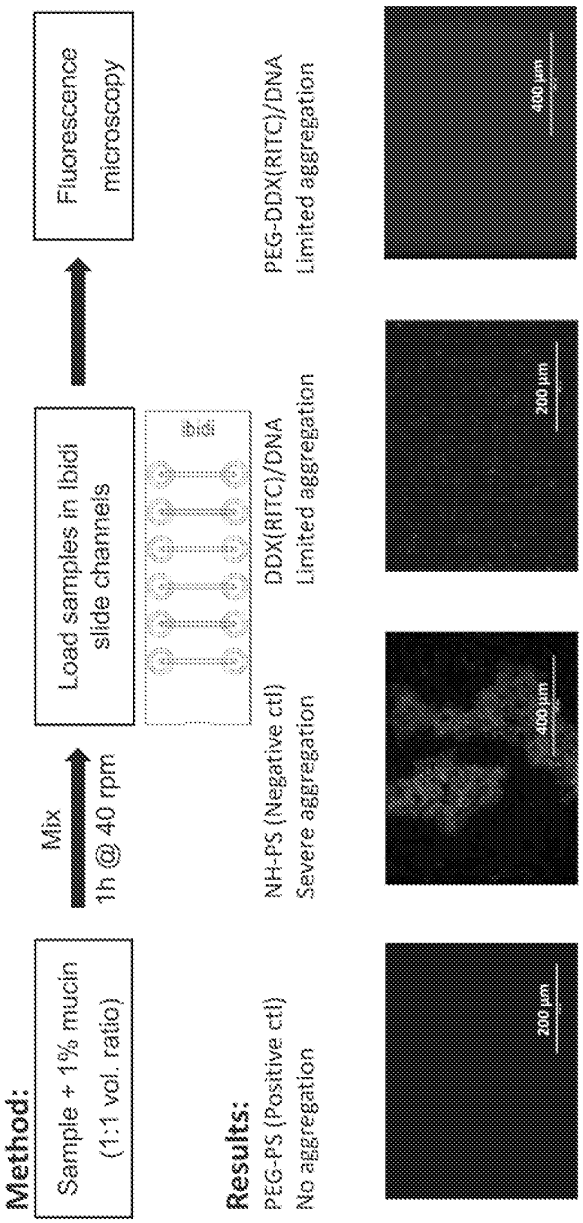
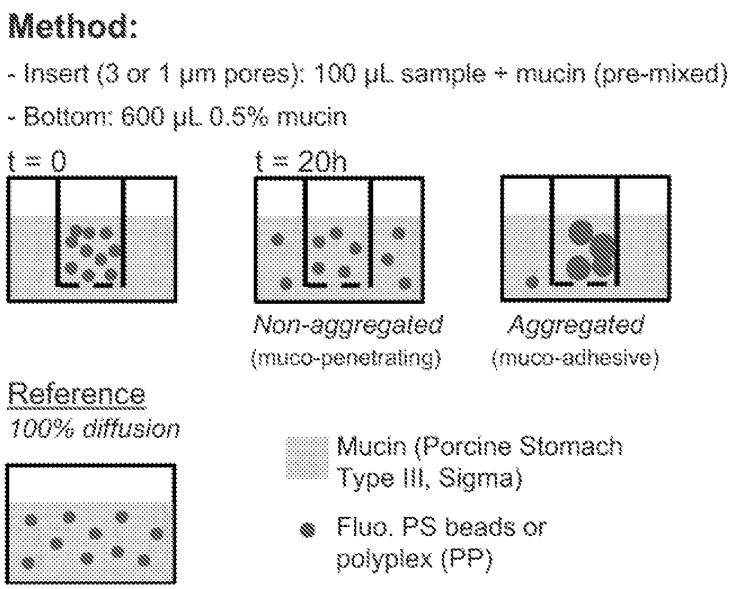

Method:
Sample + 1% mucin (1:1 vol. ratio)
Mix
1h @ 40 rpm
Load samples in Ibidi slide channels
Fluorescence microscopy
Results:
PEG-PS (Positive ctl)
No aggregation
NH-PS (Negative ctl)
Severe aggregation
DDX(RITC)/DNA
Limited aggregation
PEG-DDX(RITC)/DNA
Limited aggregation
200 µm
400 µm
200 µm
400 µm

Fig. 8


Method:
- Insert (3 or 1 µm pores): 100 µL sample + mucin (pre-mixed)
- Bottom: 600 µL 0.5% mucin
t = 0
t = 20h
Non-aggregated
(muco-penetrating)
Aggregated
(muco-adhesive)
Reference
100% diffusion
Mucin (Porcine Stomach Type III, Sigma)
Fluo. PS beads or polyplex (PP)

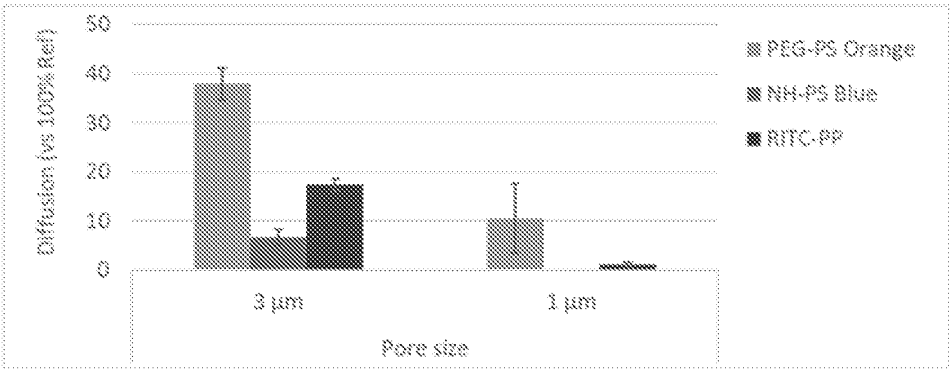

Diffusion (vs 100% Ref)
50
40
30
20
10
0
3 µm
1 µm
Pore size
PEG-PS Orange
NH-PS Blue
RITC-PP

Fig. 9

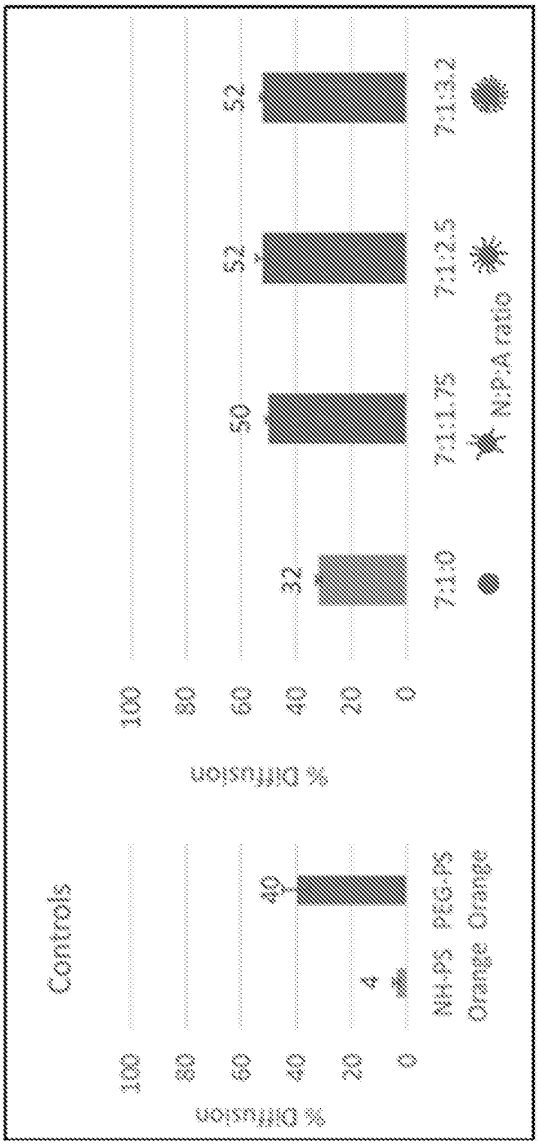

Controls
% Diffusion
100
80
60
40
20
0
4
40
NH-PS
PEG-PS
Orange
Orange
% Diffusion
100
80
60
40
20
0
32
50
52
52
7:1:0
7:1:1.75
7:1:2.5
7:1:3.2
N:P:A ratio

Fig. 10

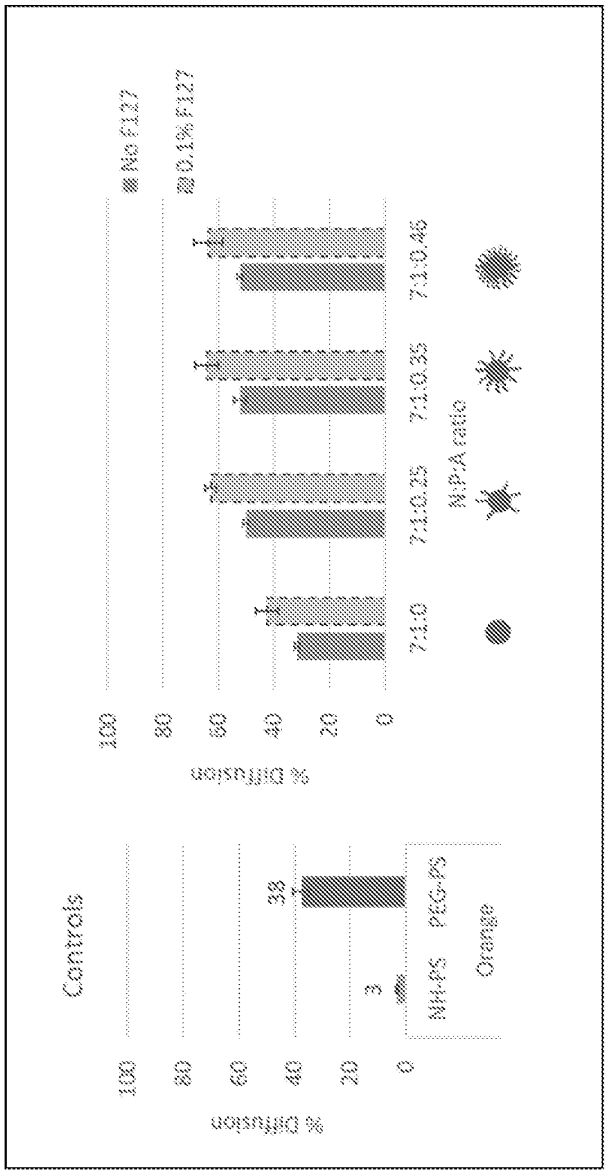

Controls
% Diffusion
100
80
60
40
20
0
38
3
NH-PS
PEG-PS
Orange
No F127
0.1% F127
7:1:0
7:1:0.25
7:1:0.35
7:1:0.46
N:P:A ratio

Fig. 11

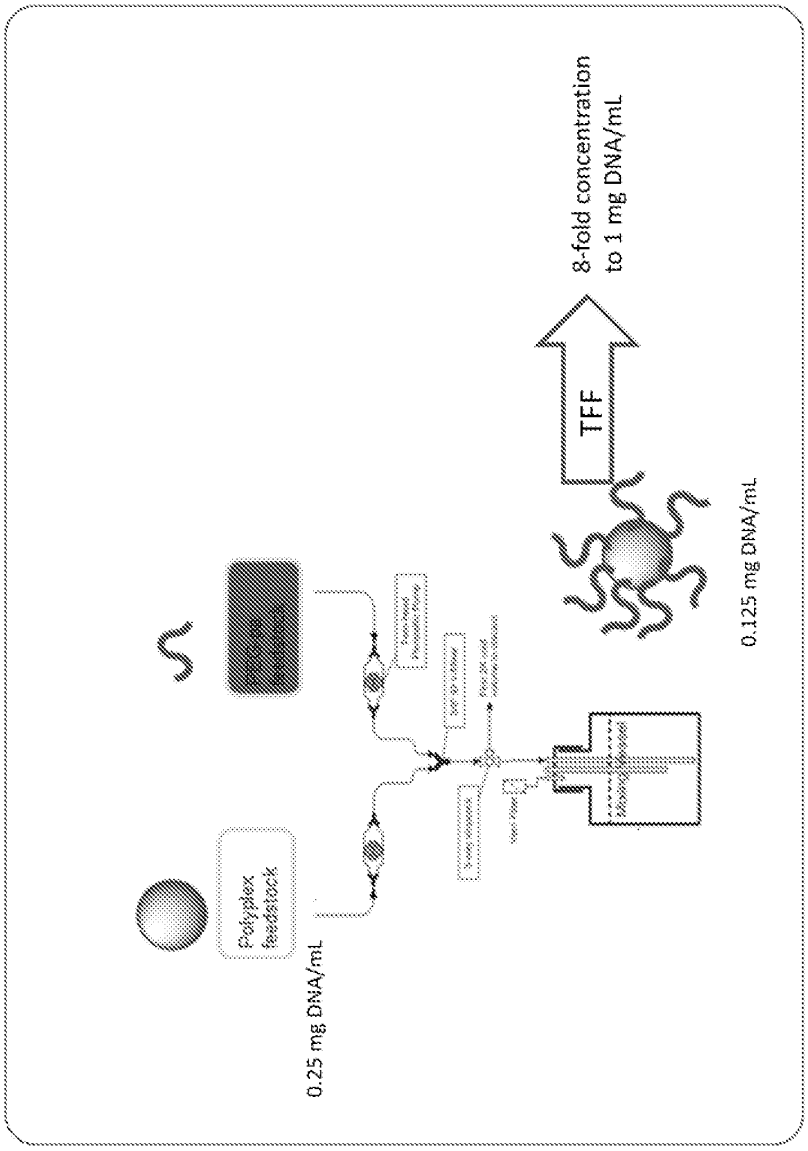

Polyplex feedstock
0.25 mg DNA/mL
TFF
8-fold concentration
to 1 mg DNA/mL
0.125 mg DNA/mL

Fig. 12

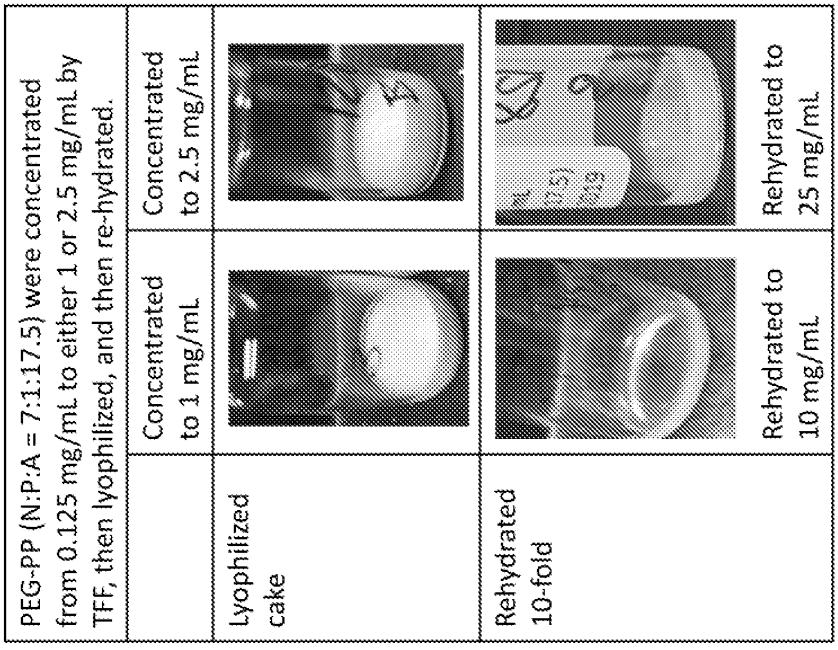

PEG-PP (N:P:A = 7:1:17.5) were concentrated from 0.125 mg/mL to either 1 or 2.5 mg/mL by TFF, then lyophilized, and then re-hydrated.
Concentrated to 1 mg/mL
Concentrated to 2.5 mg/mL
Lyophilized cake
Rehydrated 10-fold
Rehydrated to 10 mg/mL
Rehydrated to 25 mg/mL
Lyophilized PEG-PP (N:P:A=7:1:17.5)
Rehydration and Concentration
Size - 0h
Size - 48h at 4°C
PDI - 0h
PDI - 48h at 4°C
Particle size and PDI of re-hydrated PEG-PP, and stability up to 48 hours.

Fig. 13

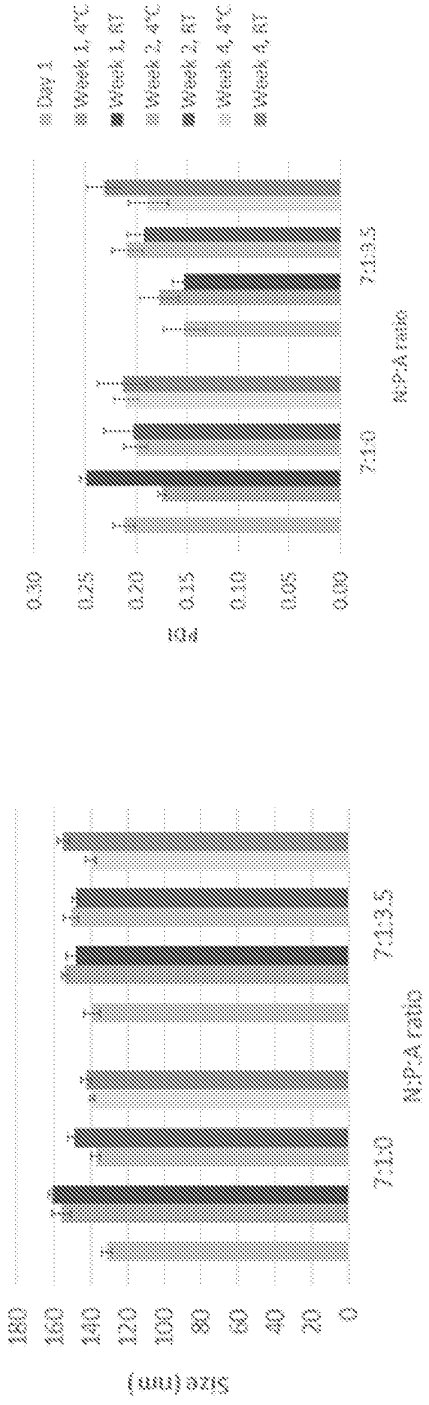
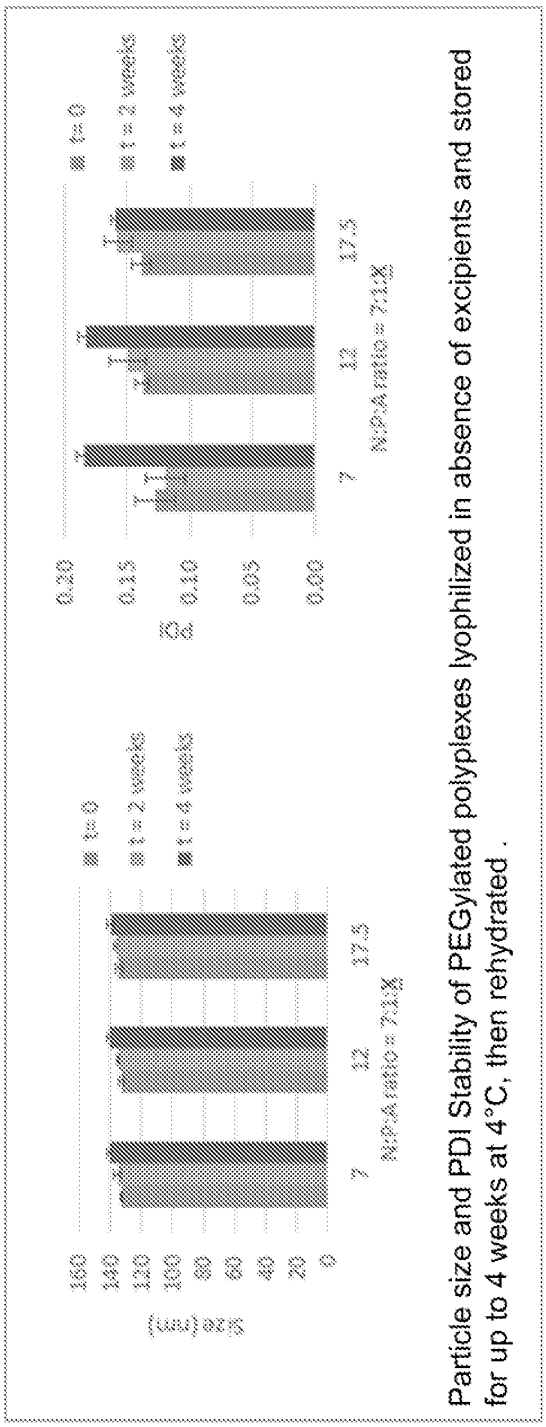

Day 1
Week 1, 4°C
Week 1, RT
Week 2, 4°C
Week 2, RT
Week 4, 4°C
Week 4, RT
PDI
0.30
0.25
0.20
0.15
0.10
0.05
0.00
7:1:0
7:1:3.5
N:P:A ratio
Size (nm)
180
160
140
120
100
80
60
40
20
0
7:1:0
7:1:3.5
N:P:A ratio

Fig. 14



Particle size and PDI Stability of PEGylated polyplexes lyophilized in absence of excipients and stored for up to 4 weeks at 4°C, then rehydrated .

Fig. 15

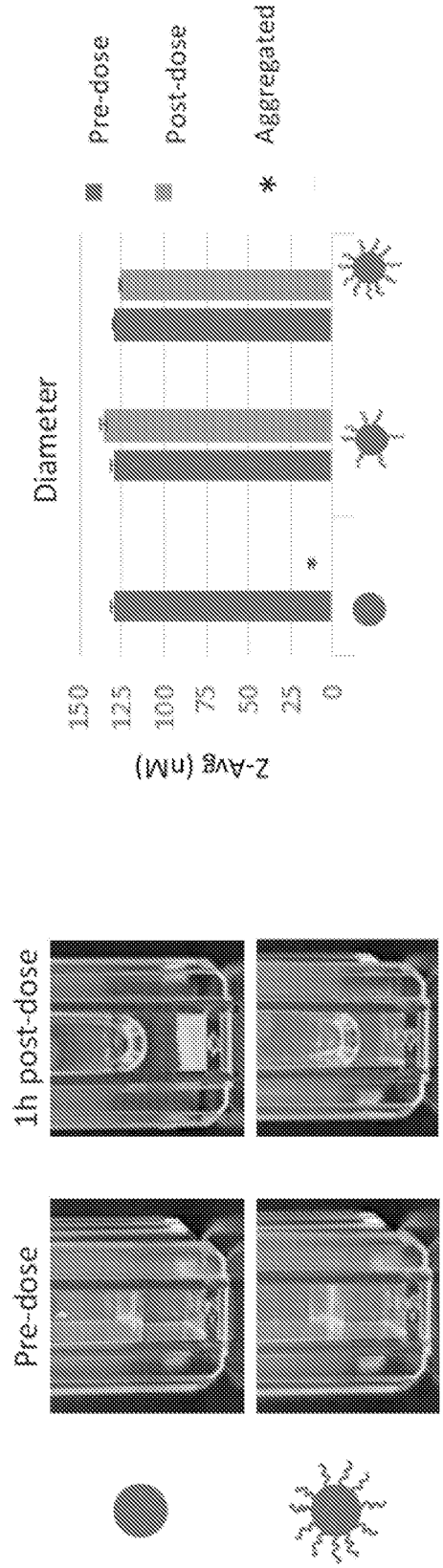

Pre-dose
Post-dose
Aggregated
Diameter
Z-Avg (nM)
150
125
100
75
50
25
0
1h post-dose
Pre-dose

Fig. 16

Formulations	NPA
Non-PEG DDX	NP7, NP15 and NP20 (combined since they were equivalent)
PEG-DDX (low)	7:1:3.5
PEG-DDX (high)	7:1:17.5

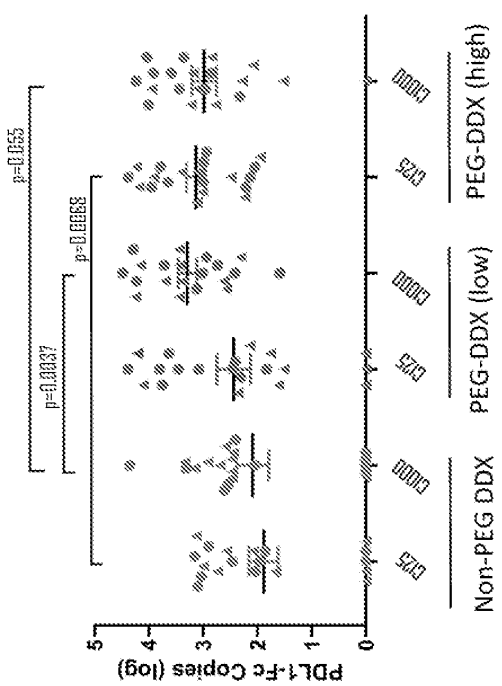


Fig. 17

Group	Polyplex	Excipient	NP ratio	PEG ratio
Gr. 1	Naive	N/A	N/A	N/A
Gr. 2	CMC-INT01-001	9% sucrose	NP15	N/A
Gr. 3	CMC-INT03-004	5% trehalose	NP20	N/A
Gr. 4	INT06-099A	5% trehalose	NP7	N/A
Gr. 5	INT06-099B	5% trehalose	NP7	17.5
Gr. 6	INT06-099C	5% trehalose	NP7	17.5
Gr. 7	INT06-098E	5% trehalose	NP7	3.5

INT06-099B polyplex prepared at c125
INT06-099C polyplex was prepared at c125, underwent TFF and was diluted to c125

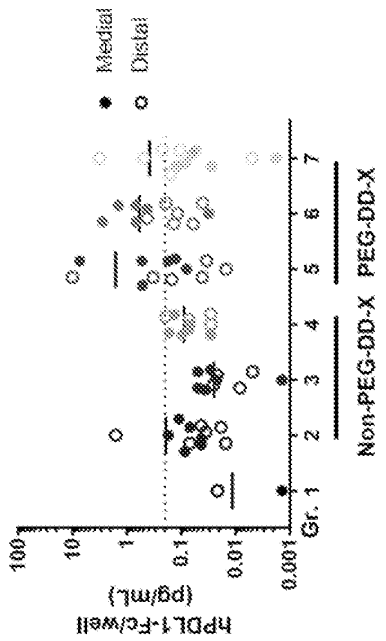
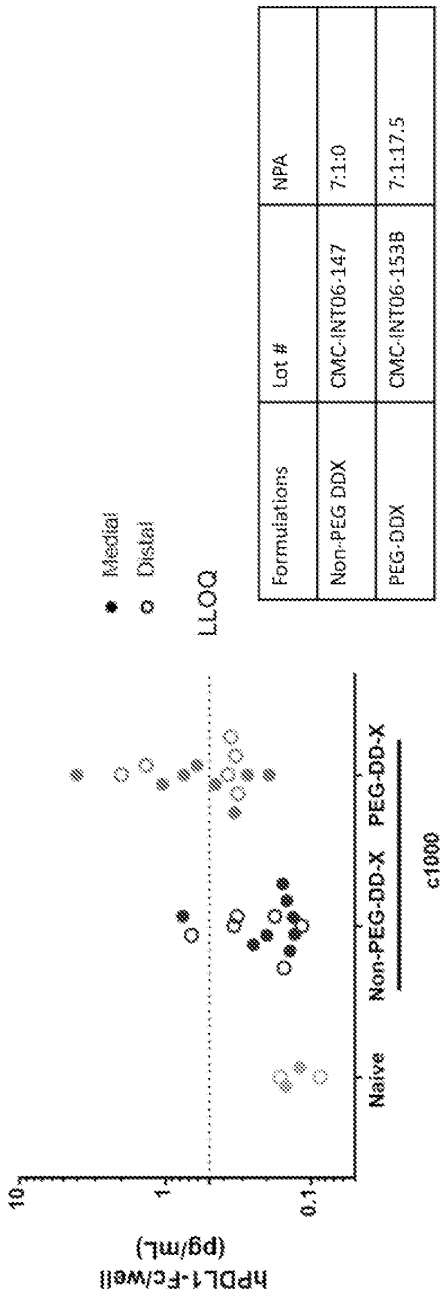


Fig. 18



Formulations	Lot #	NPA
Non-PEG DDX	CMC-INT06-147	7:1:0
PEG-DDX	CMC-INT06-153B	7:1:17.5

Fig. 19

Phys – Chem	Non-PEGylated RXG(28%R,10%G)/DNA NP20, 1 mg DNA/mL 5% Tre (CMC-INT02-033 C)		PEGylated PEG-RXG(25%R,10%G)/DNA NPA 10:1:5, 1 mg DNA/mL 1% man (CMC-INT02-039)	
	Frozen	Lyophilized	Frozen	Lyophilized
Supercoil (%)	92.5	81.3	94.6	94.1
Appearance	Opalescent			
Size (nm)	108	114	111	113
PDI	0.14	0.13	0.14	0.14
ZP (mV)	29.1	30.0	2.5	2.3
pH	6.12		6.30	6.15

Fig. 20

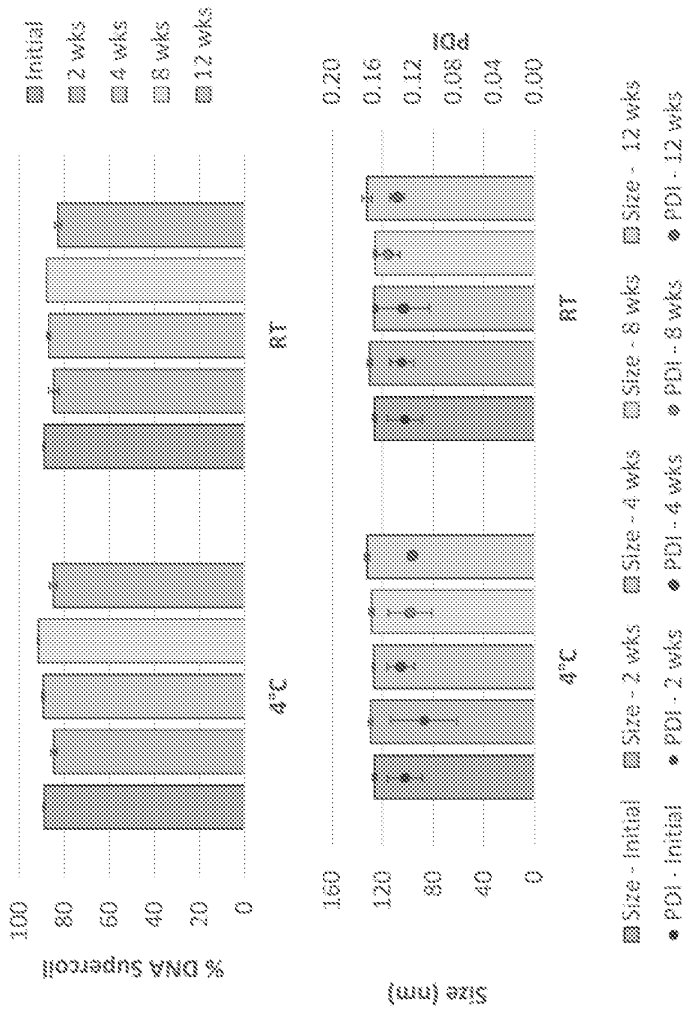
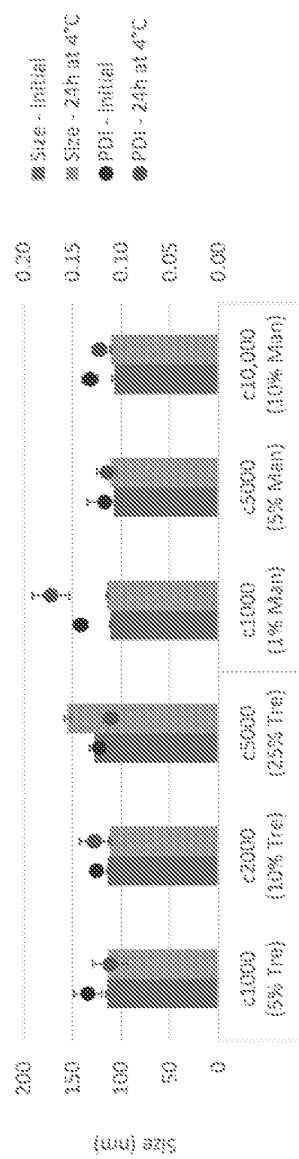

% DNA Supercoil
100
80
60
40
20
0
4°C
RT
Initial
2 wks
4 wks
8 wks
12 wks
Size (nm)
160
120
80
40
0
PDI
0.20
0.16
0.12
0.08
0.04
0.00
Size - Initial
Size - 2 wks
Size - 4 wks
Size - 8 wks
Size - 12 wks
PDI - Initial
PDI - 2 wks
PDI - 4 wks
PDI - 8 wks
PDI - 12 wks

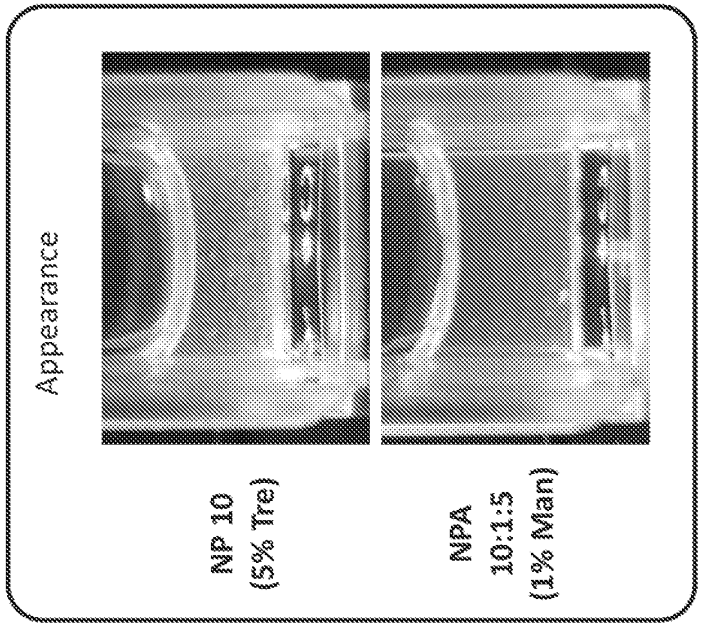
Fig. 21



Lot #	NP or NPA ratio	Max conc [1] (µg of DNA/mL)	Excipient content	
			Initial	Final
Non-PEGylated (CMC-INT02-033 c)	20	2000	5% tre	10% Tre
PEGylated (CMC-INT02-039)	10:1:5	10,000	1% man	5% Man

[1] Size, PDI, Zeta potential stable for at least 24h at 4°C.
[2] c5000 possible, but unknown effect of 25% trehalose on potency (highly hypertonic!).
[3] c10000 possible, but 10% mannitol seems to affect potency (NCL-INT02-168).

Fig. 22


Appearance
NP 10
(5% Tre)
NPA
10:1:5
(1% Man)

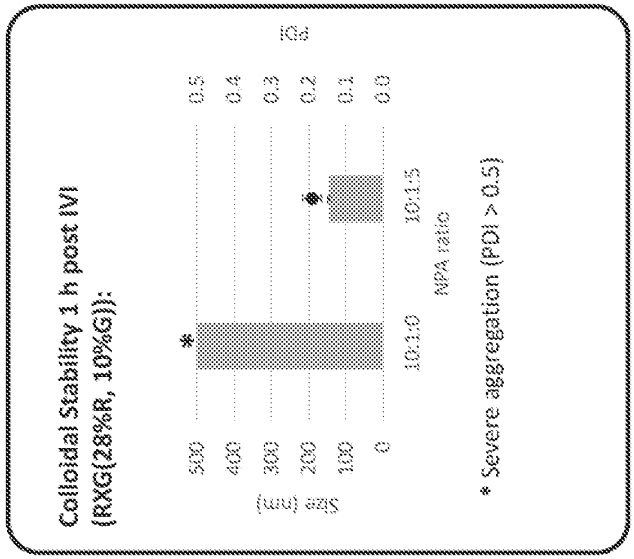

Colloidal Stability 1 h post IVI
(RXG(28%R, 10%G)):
Size (nm)
PDI
500
400
300
200
100
0
0.5
0.4
0.3
0.2
0.1
0.0
*
*
10:1:0
10:1:5
NPA ratio
* Severe aggregation (PDI > 0.5)

Fig. 23

Test	Value	Non-PEGylated RXG(25%R.10%G)/ DNA, 5% Trehalose NPA 20:1:0 (CMC-INT02-033 C)						PEGylated PEG-RXG(25%R.10%G)/ DNA, 1% mannitol NPA 10:1:5 (CMC-INT02-039)					
		Unfiltered	0.2um PES	0.2um PVDF	0.2um wwPTFE	0.2um Nylon	Unfiltered	0.2um PES	0.2um PVDF	0.2um wwPTFE	0.2um Nylon
Size (nm)	Average	117.6	111.7	107.1	110.9	109.3	114.2	107.3	102.5	105.1	105.7
	% Diff from unfiltered	n/a	n/a	3%	7%	3%	5%	n/a	6%	10%	8%	7%
PDI	Average	0.114	0.132	0.114	0.092	0.114	0.120	0.134	0.098	0.099	0.108	0.090
	% Diff from unfiltered	n/a	n/a	7%	25%	7%	3%	n/a	27%	26%	19%	33%
Zeta Potential (mV)	Average	36.4	36.2	34.4	33.7	33.5	34.3	3.5	3.1	3.6	3.4	3.3
	% Diff from unfiltered	n/a	n/a	5%	7%	8%	6%	n/a	11%	4%	1%	4%
Conductivity (mS/cm)	Average	1.180	1.173	1.177	1.153	1.133	1.143	1.113	1.057	1.133	1.113	1.093
	% Diff from unfiltered	n/a	n/a	0%	2%	4%	3%	n/a	5%	2%	0%	2%
pH	Average	6.03	6.19	6.04	6.05	6.04	6.05	6.23	6.27	6.27	6.26	6.19
	% Diff from unfiltered	n/a	n/a	1%	1%	1%	1%	n/a	1%	1%	0%	1%
N+ (mM)	Average	43.3	42.9	43.2	42.4	42.3	43.2	27.7	28.0	27.5	26.8	27.2
	% Diff from unfiltered	n/a	n/a	0%	2%	2%	0%	n/a	1%	1%	3%	2%
Total DNA (ug/mL)	Average	814.0	818.0	691.0	553.0	659.0	661.0	1095.0	1022.0	991.0	1040.0	996.0
	% Diff from unfiltered	n/a	n/a	15%	32%	19%	19%	n/a	7%	9%	5%	9%

Fig. 24

Filter	Non-PEGylated RXG(25%R,10%G)/DNA 5% Trehalose NP20:1:0, 1 mg/mL		PEGylated PEG-RXG(25%R,10%G)/DNA 1% mannitol NPA 10:1:5, 1 mg/mL	
	Qty Filterable (g/cm²)	Phys-Chem*	Qty Filterable (g/cm²)	Phys-Chem*
25mm (2.8 cm²) 0.8/0.2 µm pores (dual membranes)	1.375	[DNA] loss: 19%	> 5.35 (no clogging with 15 mL)	[DNA] loss < 10%

From: Millipore Application Note – Filter Sizing Methods

Fig. 25

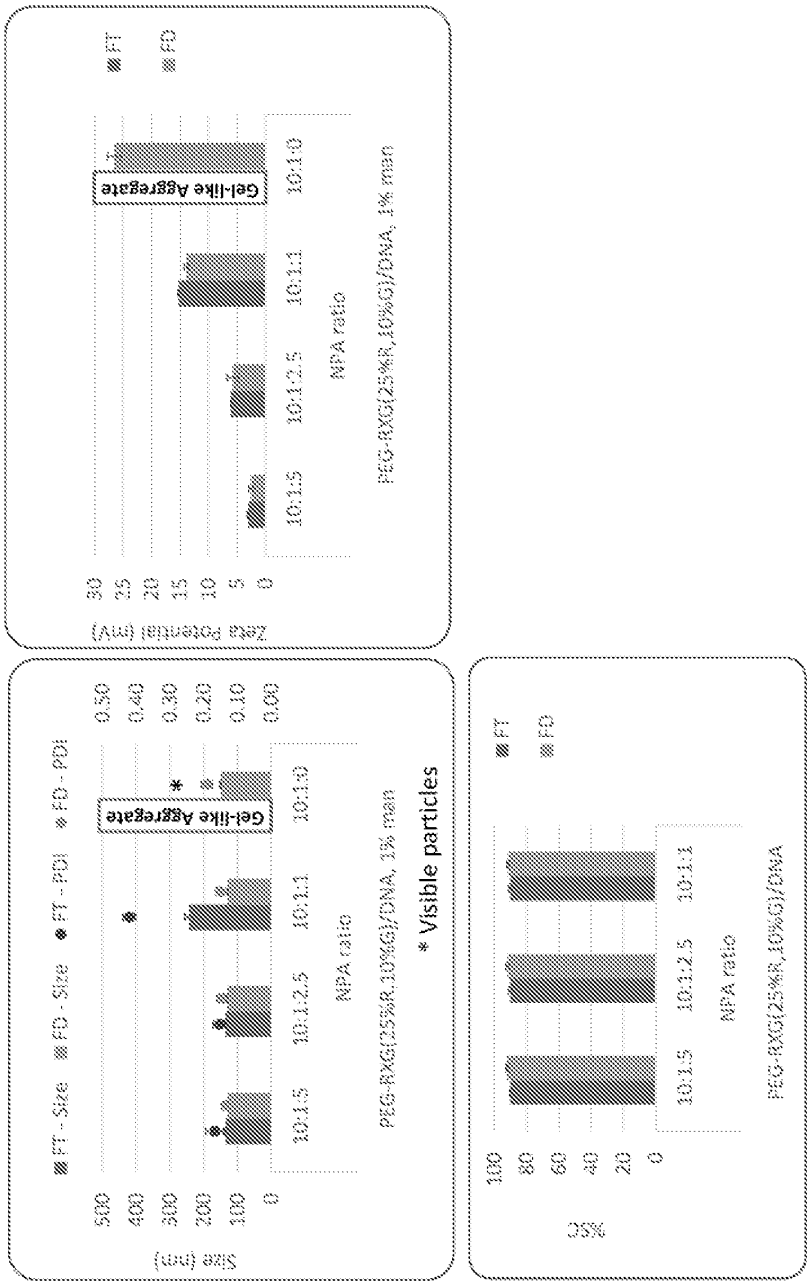

Zeta Potential (mV)
30
25
20
15
10
5
0
FT
FD
Gel-like Aggregate
10:1:5
10:1:2.5
10:1:1
10:1:0
NPA ratio
PEG-RXG(25%R,10%G)/DNA, 1% man
Size (nm)
500
400
300
200
100
0
0.50
0.40
0.30
0.20
0.10
0.00
FT - Size
FD - Size
FT - PDI
FD - PDI
* Visible particles
%SC
100
80
60
40
20
0

Fig. 26

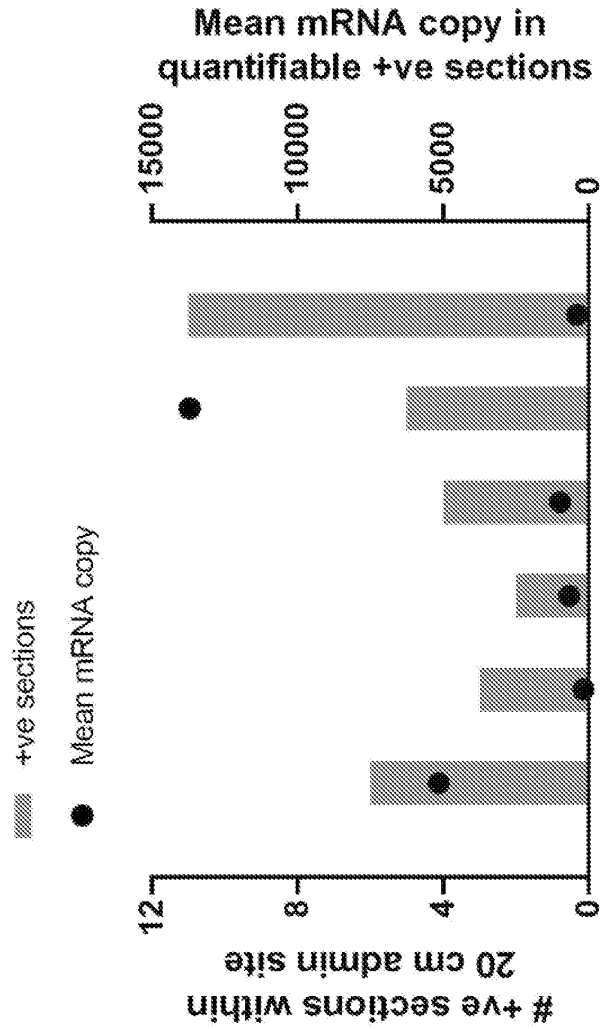

Mean mRNA copy in quantifiable +ve sections
15000
10000
5000
0
12
8
4
0
+ve sections within 20 cm admin site
+ve sections
Mean mRNA copy

Fig. 27

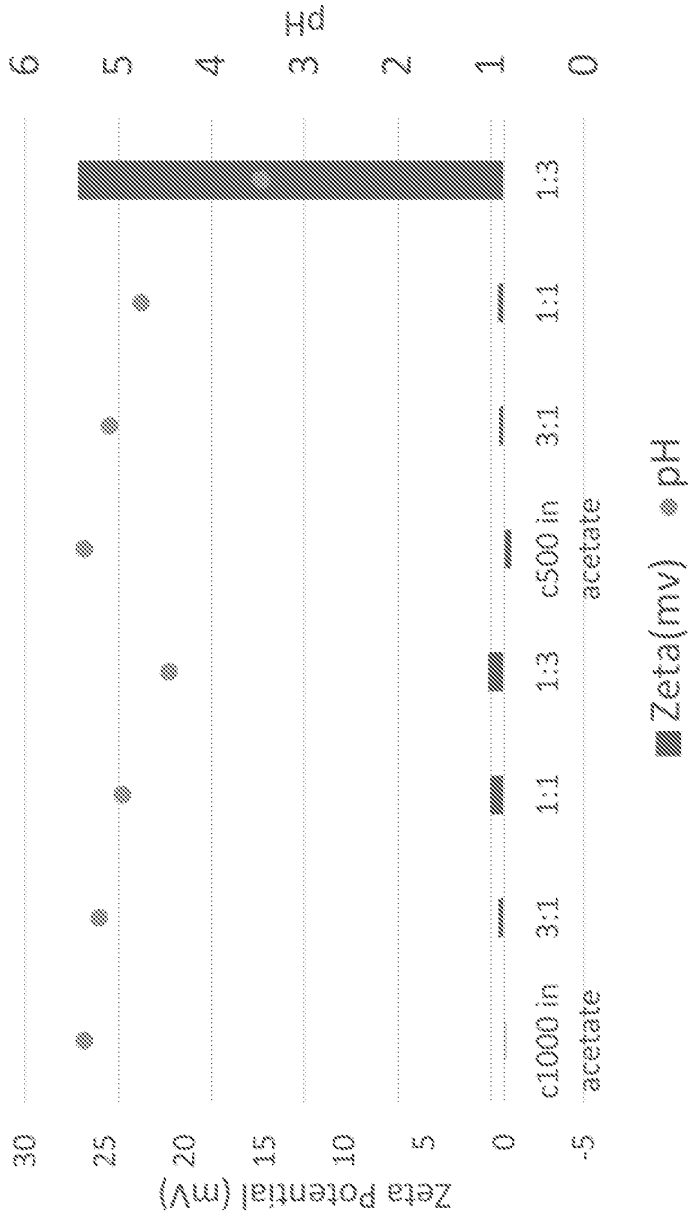

pH
6
5
4
3
2
1
0
Zeta Potential (mV)
30
25
20
15
10
5
0
-5
c1000 in acetate
3:1
1:1
1:3
c500 in acetate
3:1
1:1
1:3
Zeta(mV)
pH

Fig. 28

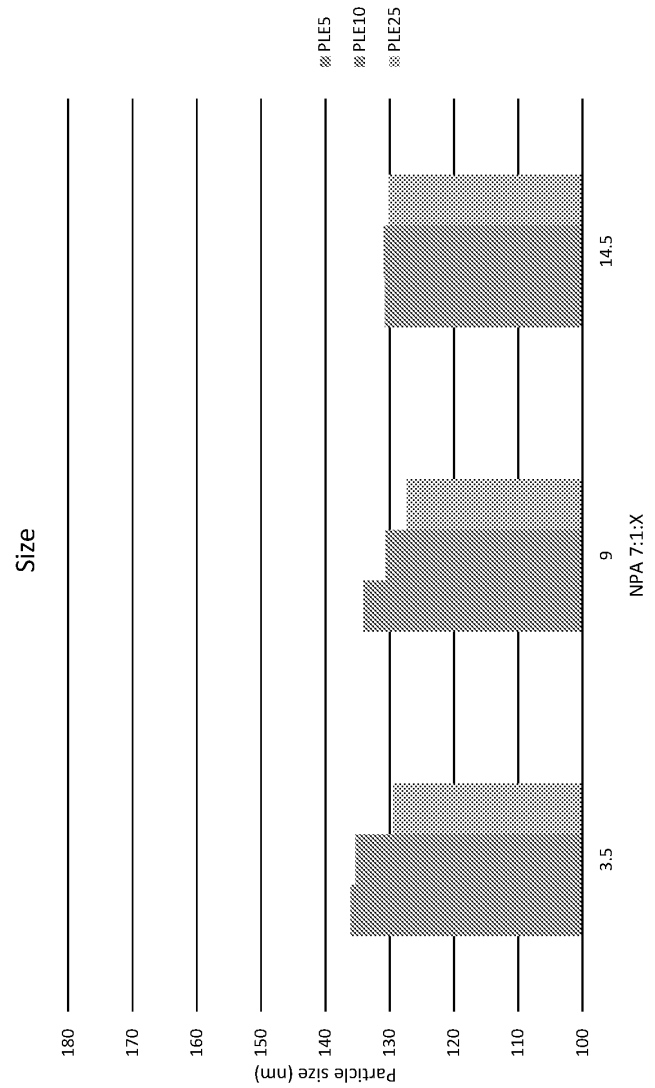

Size
PLE5
PLE10
PLE25
Particle size (nm)
180
170
160
150
140
130
120
110
100
3.5
9
14.5
NPA 7:1:X

Fig. 29

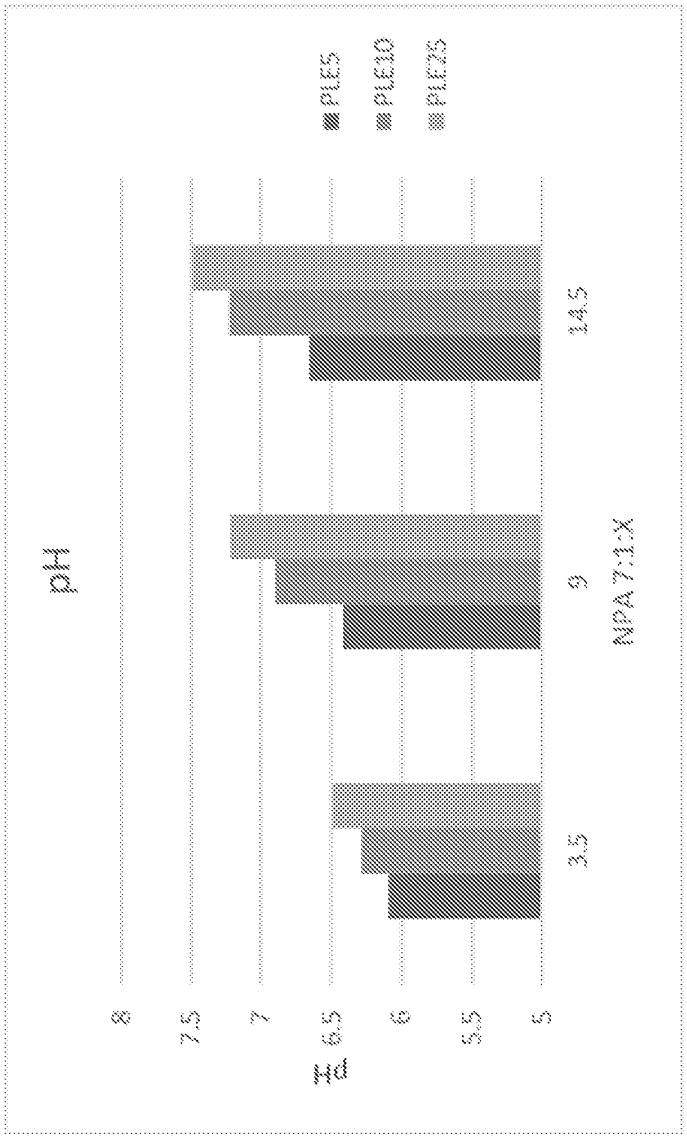
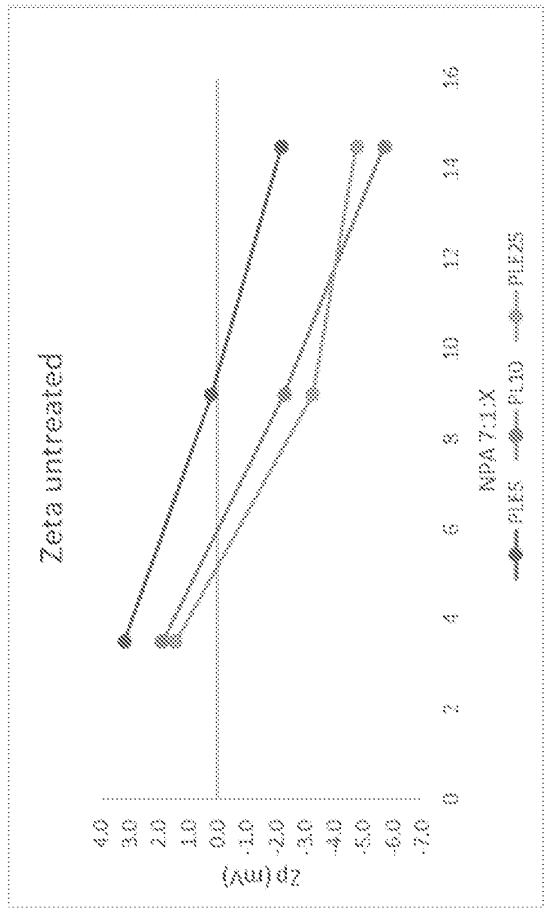


Fig. 30


Zeta untreated
zp (mV)
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
-7.0
0
2
4
6
8
10
12
14
16
NPA 7:1:X
PLE5
PLE10
PLE25

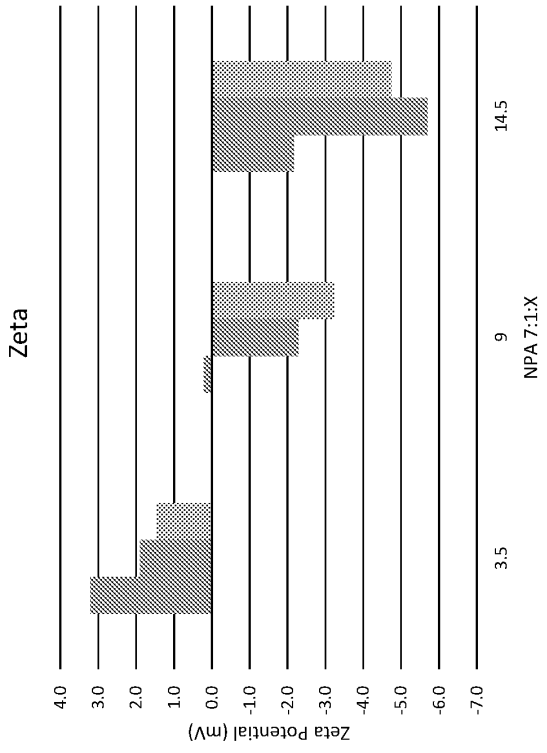

Zeta
Zeta Potential (mV)
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
-7.0
3.5
9
14.5
NPA 7:1:X
PLE5
PLE10
PLE25

Fig. 31

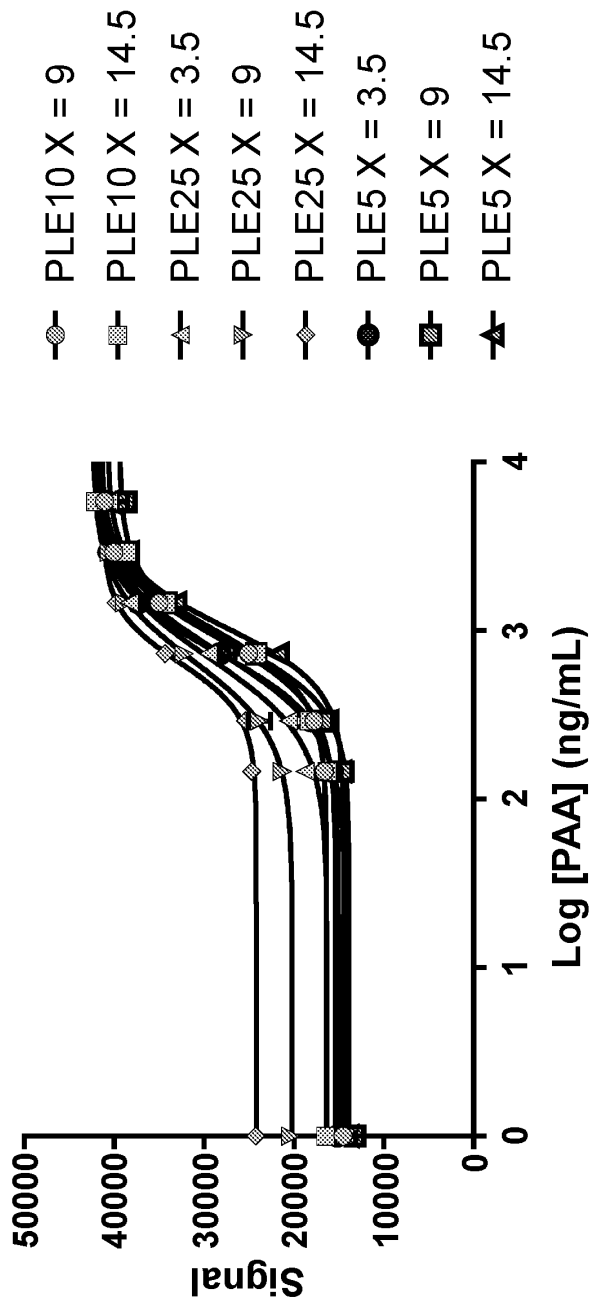

All N:P:A = 7:1:X
PLE10 X = 3.5
PLE10 X = 9
PLE10 X = 14.5
PLE25 X = 3.5
PLE25 X = 9
PLE25 X = 14.5
PLE5 X = 3.5
PLE5 X = 9
PLE5 X = 14.5
Signal
50000
40000
30000
20000
10000
0
0
1
2
3
4
Log [PAA] (ng/mL)

Fig. 32

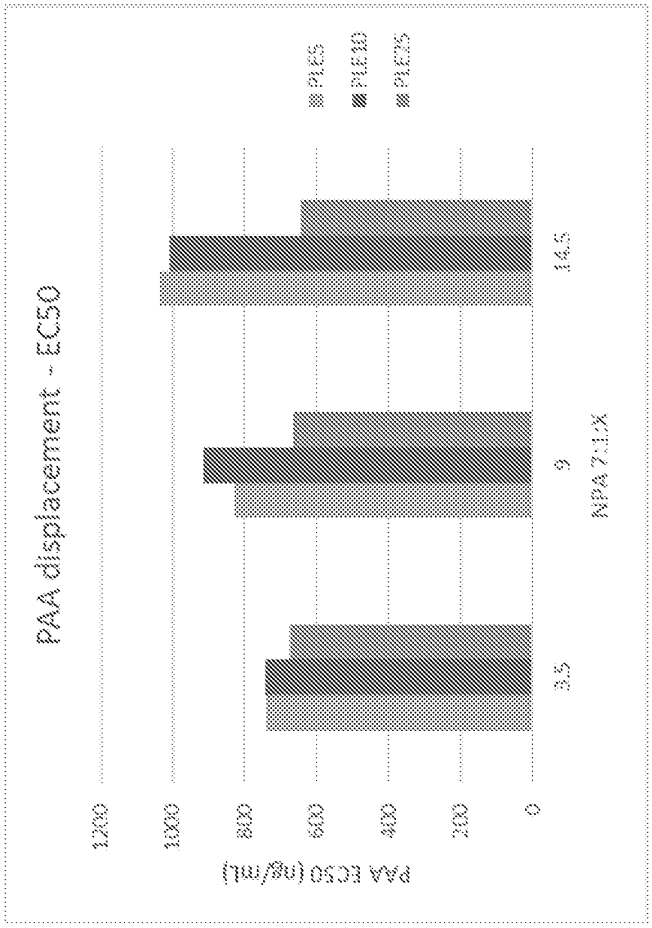

PAA displacement - EC50
PAA EC50 (ng/mL)
1200
1000
800
600
400
200
0
3.5
9
14.5
NPA 7:1:X
PLES
PLE3D
PLE2S

Fig. 33

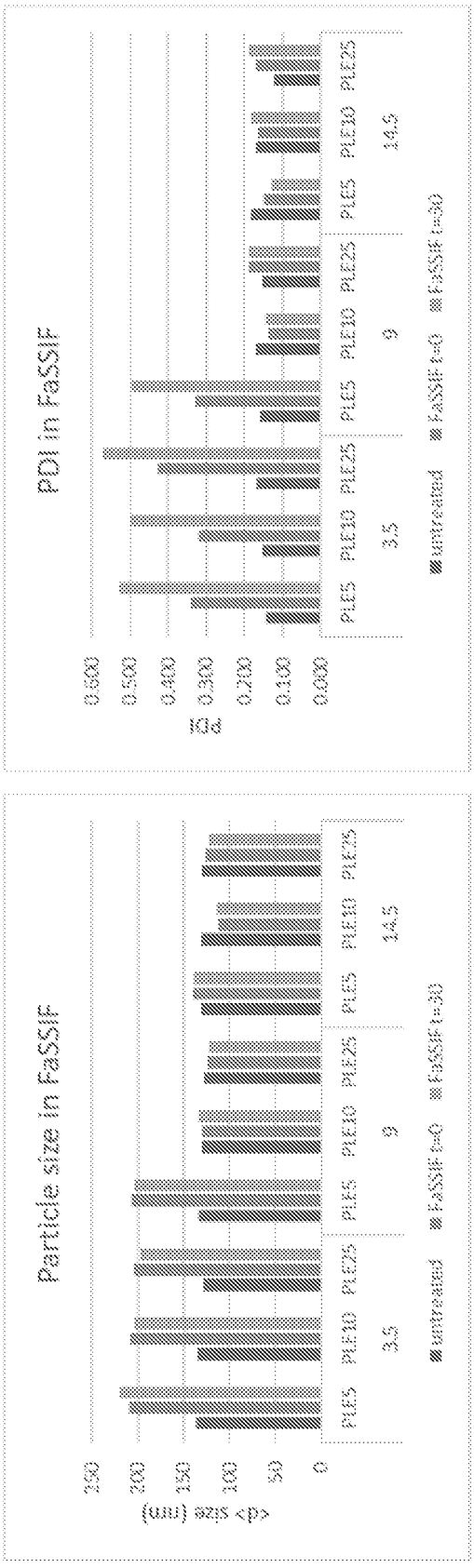
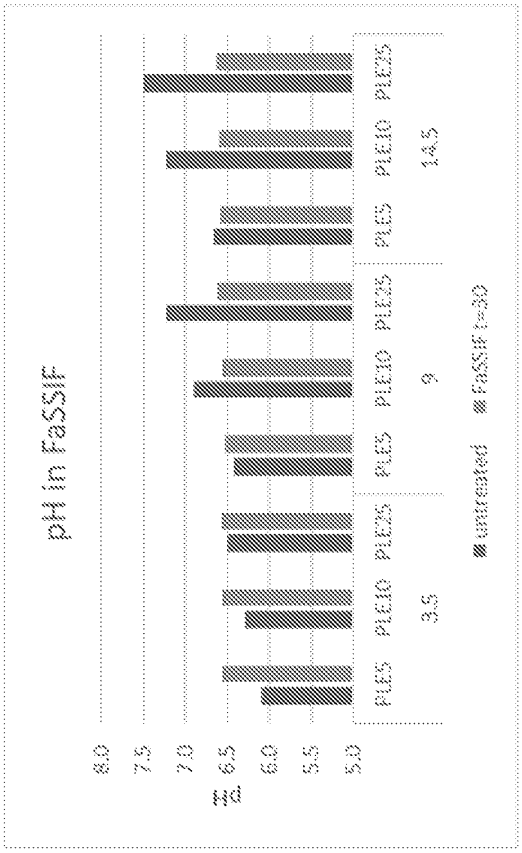
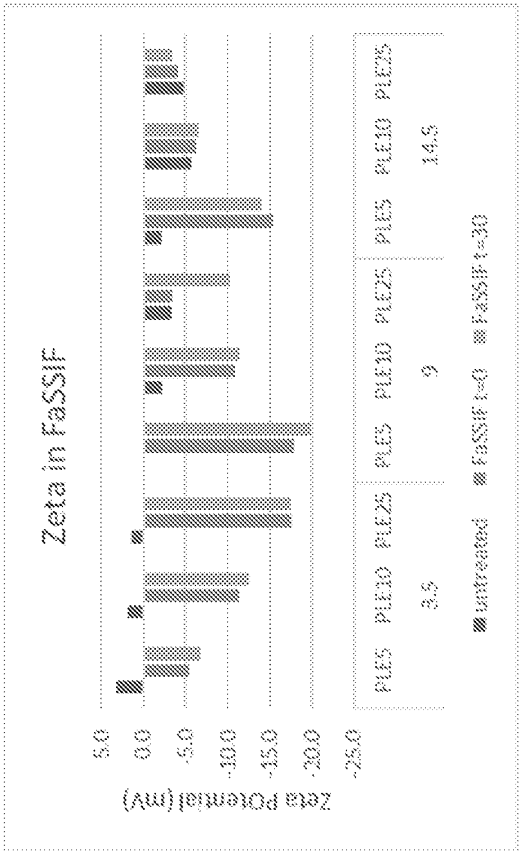

Particle size in FaSSIF
<d> size (nm)
250
200
150
100
50
0
PLE5
PLE10
PLE25
3.5
PLE5
PLE10
PLE25
0
PLE5
PLE10
PLE25
14.5
untreated
FaSSIF t=0
FaSSIF t=30
PDI in FaSSIF
PDI
0.600
0.500
0.400
0.300
0.200
0.100
0.000
PLE5
PLE10
PLE25
3.5
PLE5
PLE10
PLE25
0
PLE5
PLE10
PLE25
14.5
untreated
FaSSIF t=0
FaSSIF t=30

Fig. 34


pH in FaSSIF
pH
8.0
7.5
7.0
6.5
6.0
5.5
5.0
PLE5
PLE10
PLE25
3.5
PLE5
PLE10
PLE25
9
PLE5
PLE10
PLE25
14.5
untreated
FaSSIF t=30


Zeta in FaSSIF
Zeta Potential (mV)
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
PLE5
PLE10
PLE25
3.5
PLE5
PLE10
PLE25
9
PLE5
PLE10
PLE25
14.5
untreated
FaSSIF t=0
FaSSIF t=30

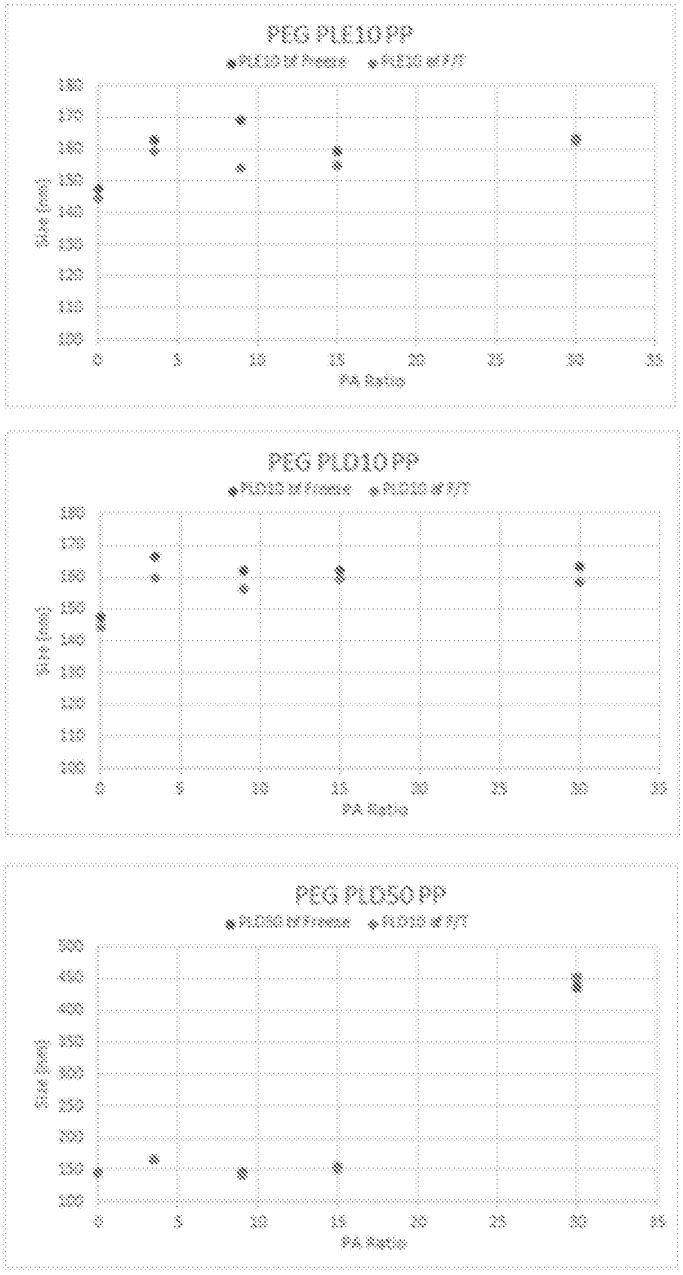


Fig. 35

Fig. 36

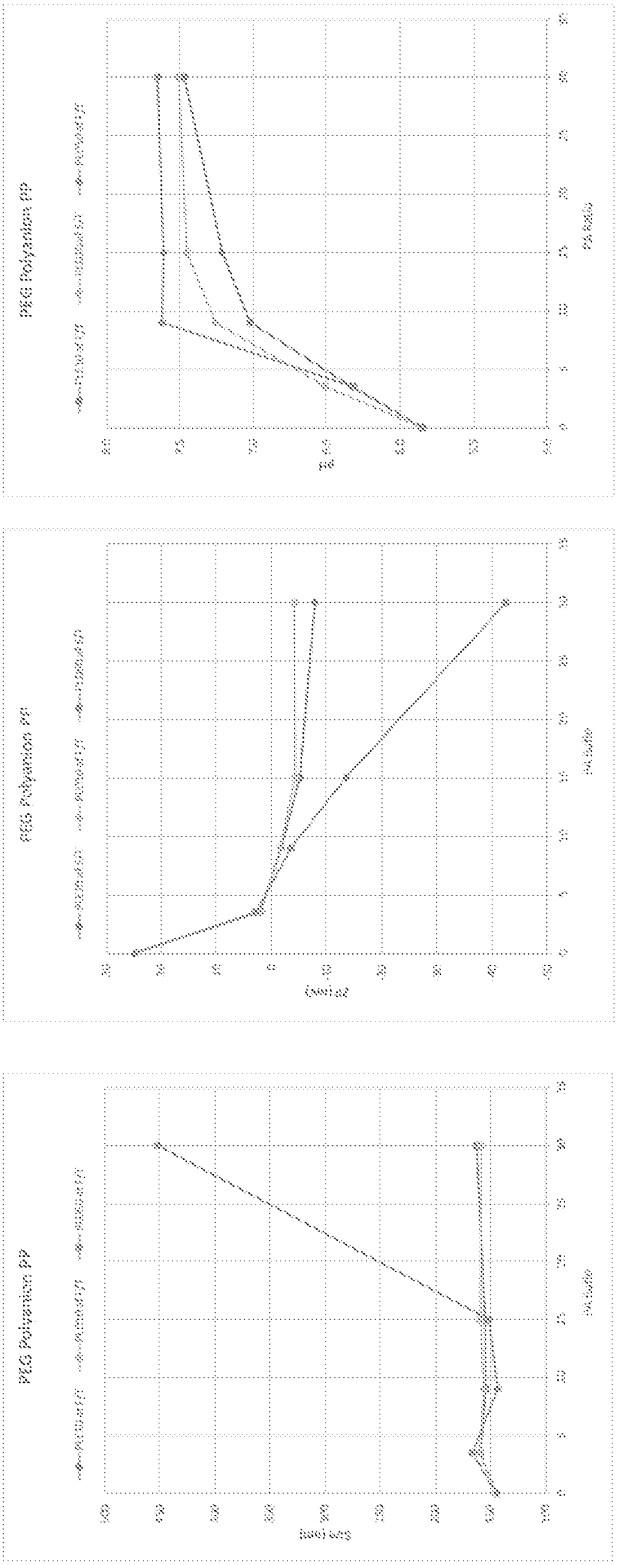


Fig. 37

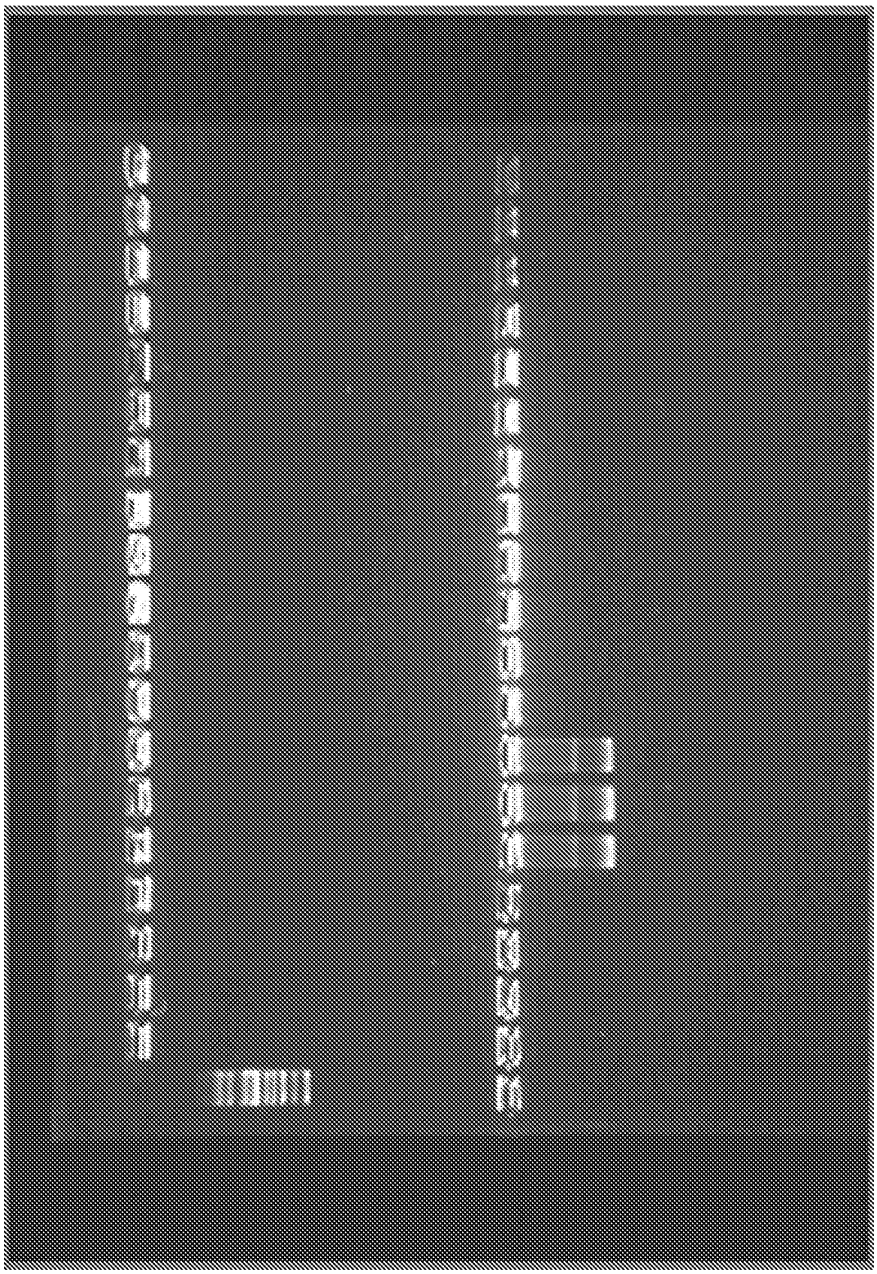


Fig. 38

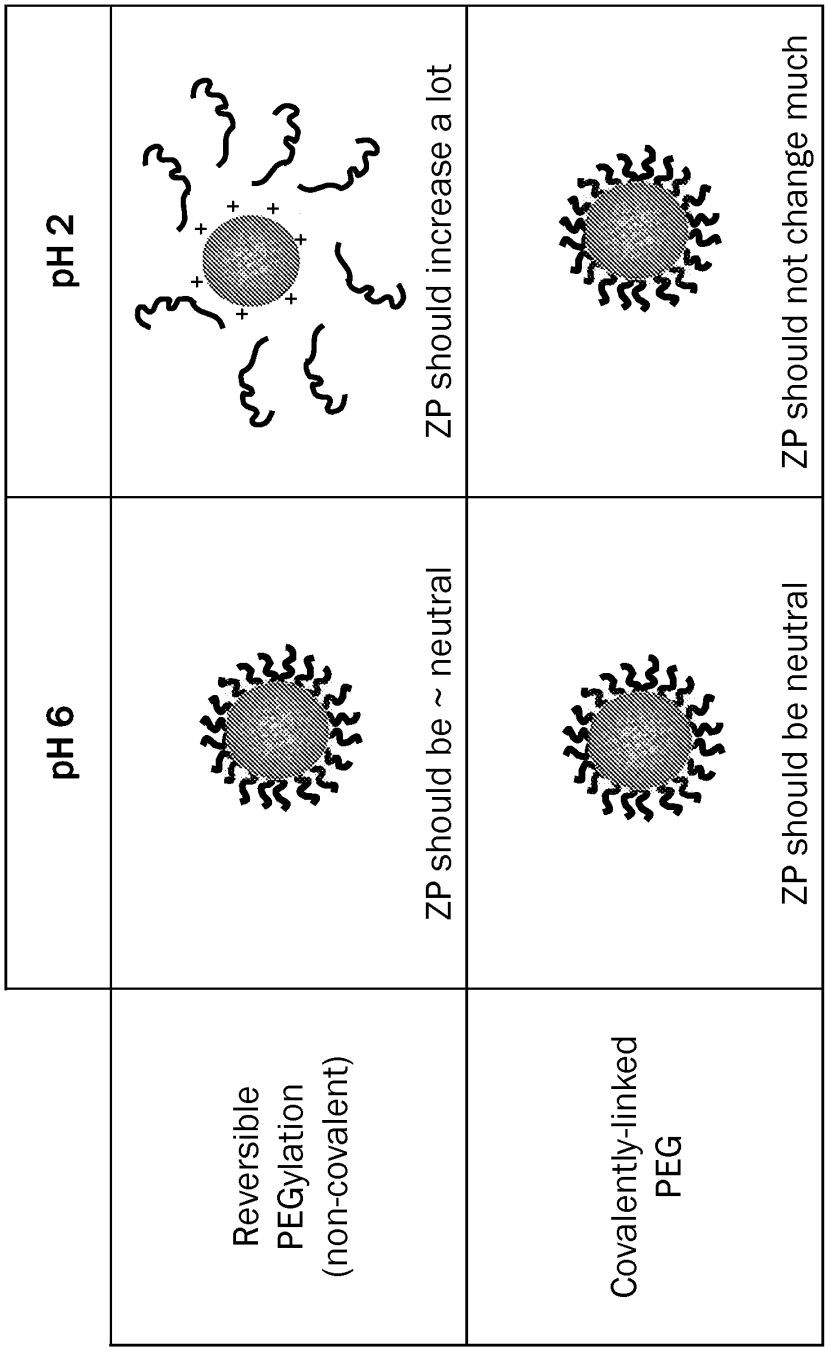

pH 6
pH 2
Reversible PEGylation (non-covalent)
ZP should be ~ neutral
ZP should increase a lot
Covalently-linked PEG
ZP should be neutral
ZP should not change much

Fig. 39

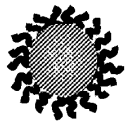
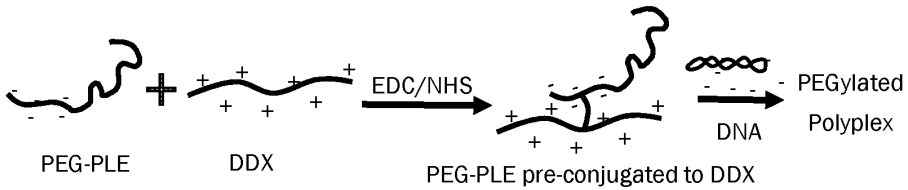
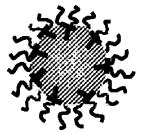
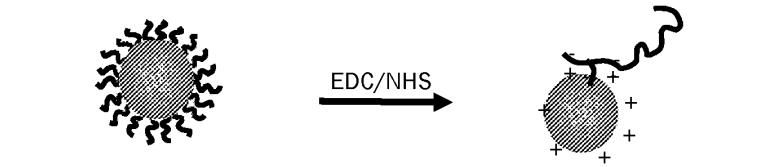
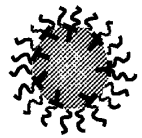
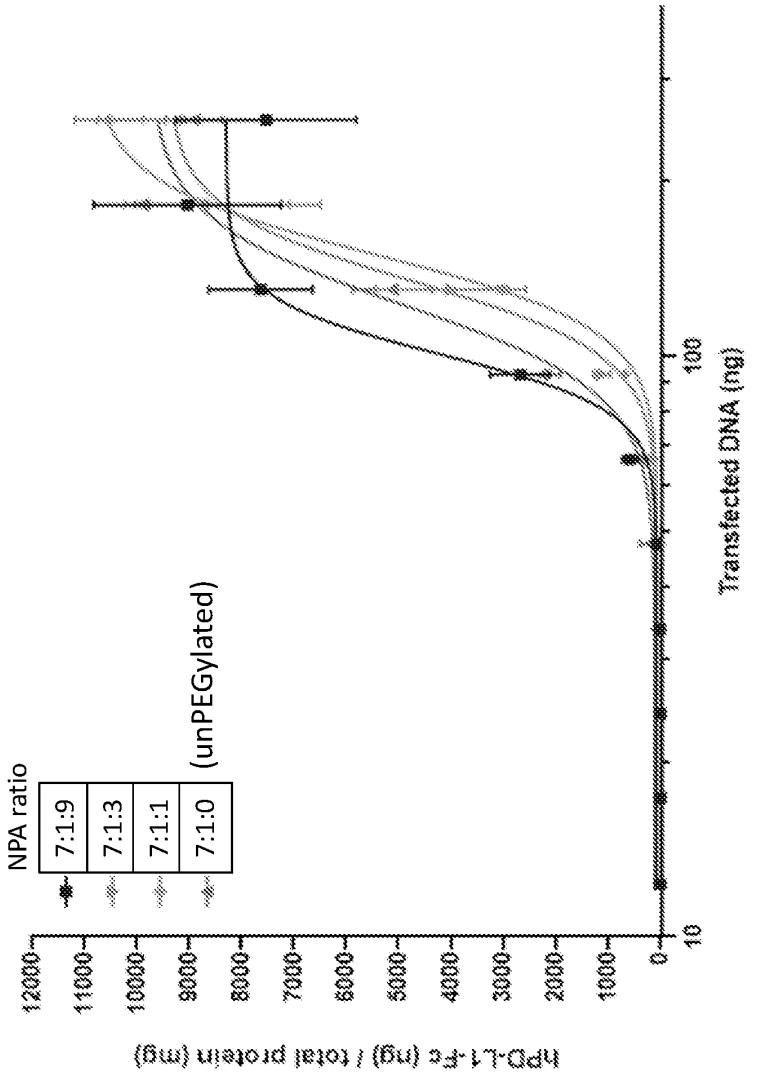
	Nanoparticle Structure	ZP @ pH6	ZP @ pH2	Δ ZP	PAA challenge
Reversible PEGylated DDX/DNA polyplex, NPA=7:1:9		-2.6 mV	+28.0 mV	↑ 30.6 mV	pDNA released
		0 mV	+ 9.2 mV	↑ 9.2 mV	pDNA released
		+2.6 mV	+6.8 mV	↑ 4.2 mV	pDNA remained bound

Fig. 40



NPA ratio	EC50	Bottom	Top	HillSlope
7:1:9	100.4	72.0	8291.3	9.0
7:1:3	134.3	59.8	9398.1	6.8
7:1:1	140.3	47.0	10043.3	6.8
7:1:0	122.4	99.4	9744.2	5.7

Fig. 41

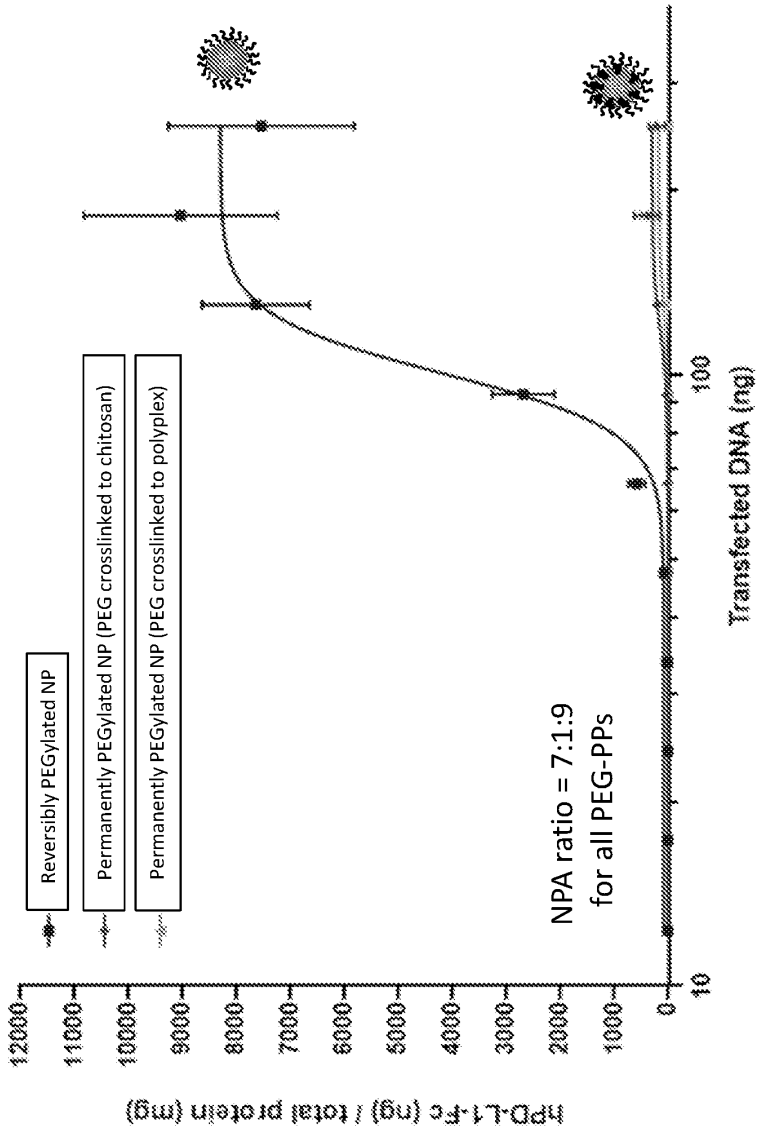

Reversibly PEGylated NP
Permanently PEGylated NP (PEG crosslinked to chitosan)
Permanently PEGylated NP (PEG crosslinked to polyplex)
NPA ratio = 7:1:9
for all PEG-PPs
hPD-L1-Fc (ng) / total protein (mg)
Transfected DNA (ng)
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
10
100

Fig. 42

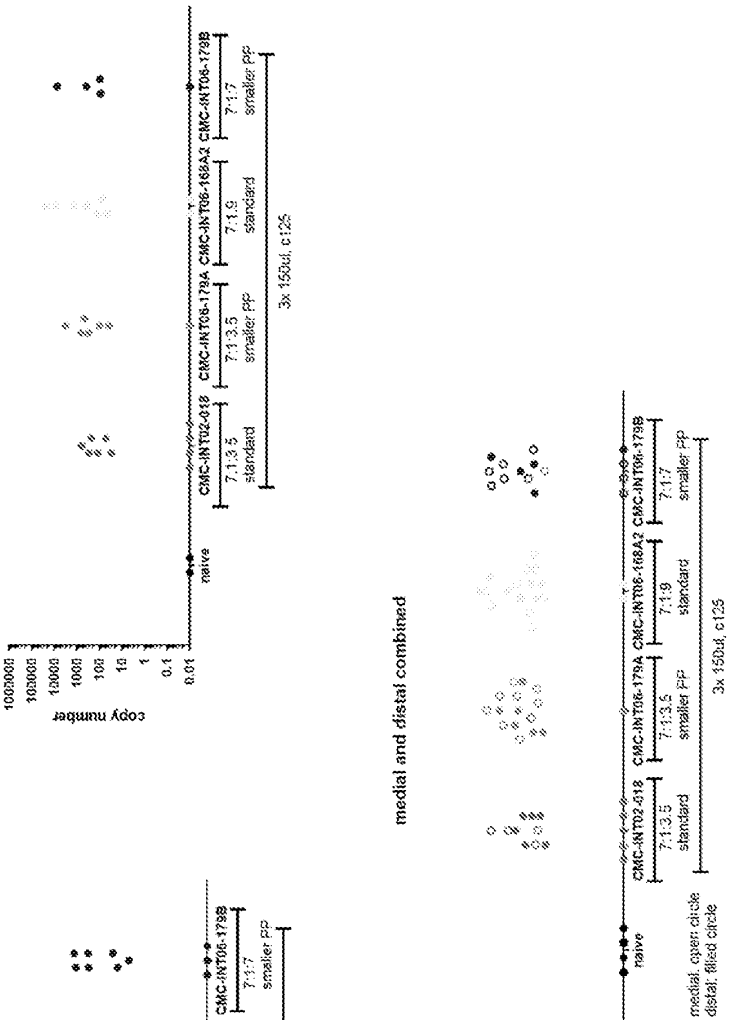
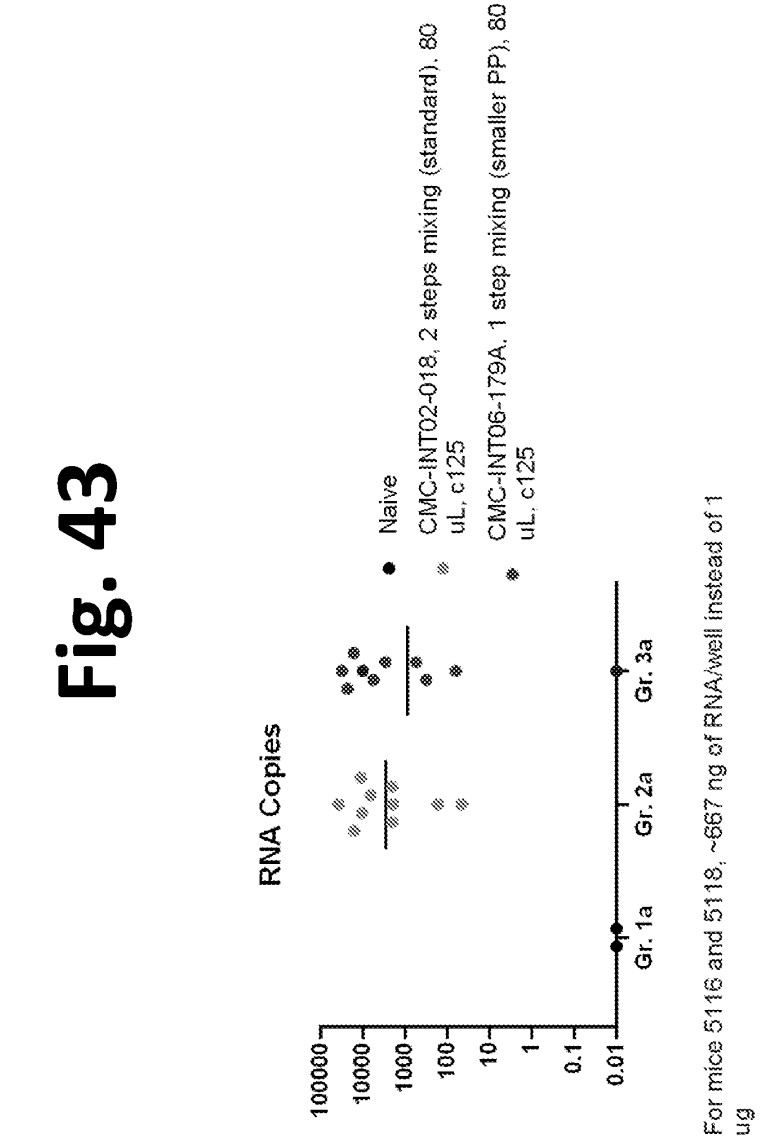

distal
medial
medial and distal combined
copy number
naive
CMC-INT02-018
CMC-INT06-179A
CMC-INT06-168A2
CMC-INT06-179B
7:1:3.5
standard
smaller PP
7:1:9
7:1:7
3x 150ul, c125
medial, open circle
distal, filled circle

Fig. 43


RNA Copies
100000
10000
1000
100
10
1
0.1
0.01
Gr. 1a
Gr. 2a
Gr. 3a
Naive
CMC-INT02-018, 2 steps mixing (standard), 80 uL, c125
CMC-INT06-179A, 1 step mixing (smaller PP), 80 uL, c125
For mice 5116 and 5118, ~667 ng of RNA/well instead of 1 ug