Supplementary for:

Multi-Detector Frit-Inlet Asymmetric Flow Field-Flow Fractionation Method Development for Nanoparticle Mixtures: Deeper Analysis Beyond ISO Quality Standards

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Table S1 *Comparison of descriptive statistics for entire Nanoparticle Tracing Analysis (NTA) descriptors for MC3-LNP for 0 hour (control) and incubated for 24 hours at 37 °C in the presence of 35 mg/mL BSA. Span describes the distribution width: (D90 – D10) / D50. Results are shown as mean ± S.D of three independent measurements.*

	MC3-PBS (0 hr)	**MC3-PBS (24 hr)**	**MC3-BSA (24 hr)**
Concentration (particles/mL)	3.6×10^{11} (± 6.8×10^{10})	2.4×10^{11} (± 2.0×10^{11})	1.4×10^{12} (± 5.9×10^{11})
Mean (nm)	80.4 (± 19.3)	96.1 (± 9.4)	80.7 (± 1.8)
Mode (nm)	60.2 (± 4.8)	69.9 (± 6.9)	66.2 (± 2.5)
D10 (nm)	51.4 (± 4.8)	61.7 (± 4.0)	59.3 (± 0.8)
D50 (nm)	68.2 (± 6.4)	79.2 (± 7.3)	73.1 (± 1.8)
D90 (nm)	122.6 (± 55.1)	150.1 (± 14.8)	104.0 (± 3.9)
Span	1.00 (± 0.62)	1.11 (± 0.04)	0.61 (± 0.04)

Table S2 *Physiochemical attributes describing hydrodynamic particle diameter (particle size) and polydispersity index (PDI) measured by DLS distribution algorithm analysis for BSA (control), MC3 (control) incubated in PBS (control) and in 35 mg/mL BSA. Results are shown as mean ± S.D, n= 3 independent replicates. Peak 1 indicates BSA peak, peak 2 indicates LNP peak and Peak 3 are aggregate particles. Measurement values indicate 0 hour (control) and 24 hours incubation at 37 °C. Peaks 1, 2 and 3 are labelled in* ***Figure 3****.*

			Particle diameter (nm)			
Sample	**Time-Point**	**Sample**	**Peak 1**	**Peak 2**	**Peak 3**	**PDI**
BSA (Contol)	0 hr	BSA-PBS	8.9 (± 1.0)	665.3 (± 0.0)	2597.3 (± 1466.6)	0.131 (± 0.009)
	24 hr		8.8 (± 0.5)	537.1 (± 0.0)	3074.8 (± 873.3)	0.175 (± 0.079)
MC3 (Control)	0 hr	MC3-PBS	-	90.1 (± 3.1)	1538.7 (± 66.5)	0.168 (± 0.012)
	24 hr	MC3-PBS	-	99.5 (± 20.9)	2928.7 (± 2537.1)	0.314 (± 0.173)
MC3-BSA	24 hr	MC3-BSA	7.5 (± 0.2)	92.7 (± 2.9)	518.0 (± 897.2)	0.540 (± 0.049)

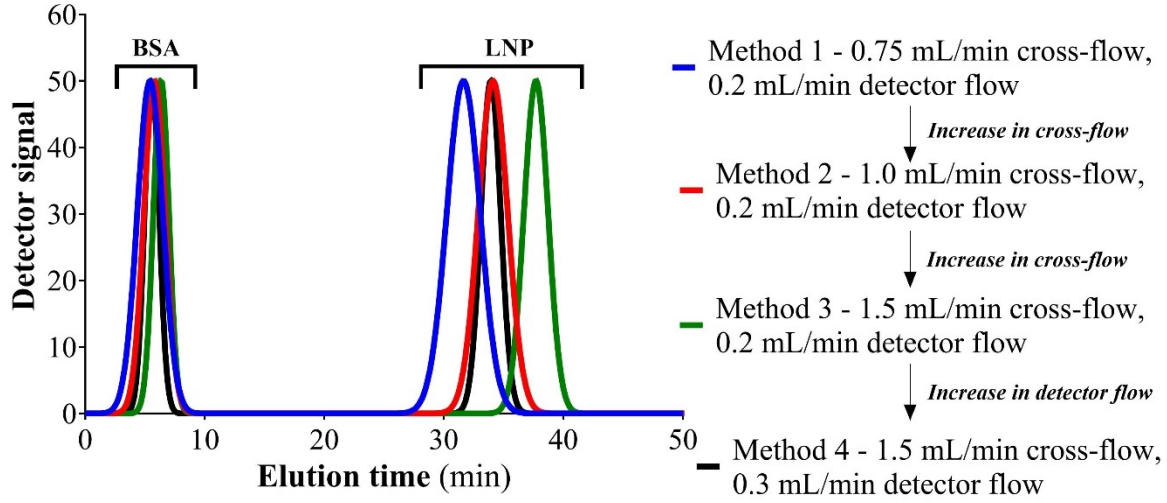

Figure S1 *FI-AF4 method simulation for MC3 LNP-BSA mixtures using the NovaAnalysis software. The simulated fractogram shows that all methods tested can separate Bovine Serum Albumin (BSA) and LNP(BSA) peaks, with a shift in elution time following the application of different exponential decay cross-flow and detector flow profiles. The parameters inputted on the software at % v:v ratio of 50%:50% (BSA:LNP), radius of hydration (R_h) of 3.8 nm and 46.4 nm (BSA and LNP respectively). These parameters were inputted following the particle sizes obtained using DLS for the MC3 LNP-BSA mixture.*

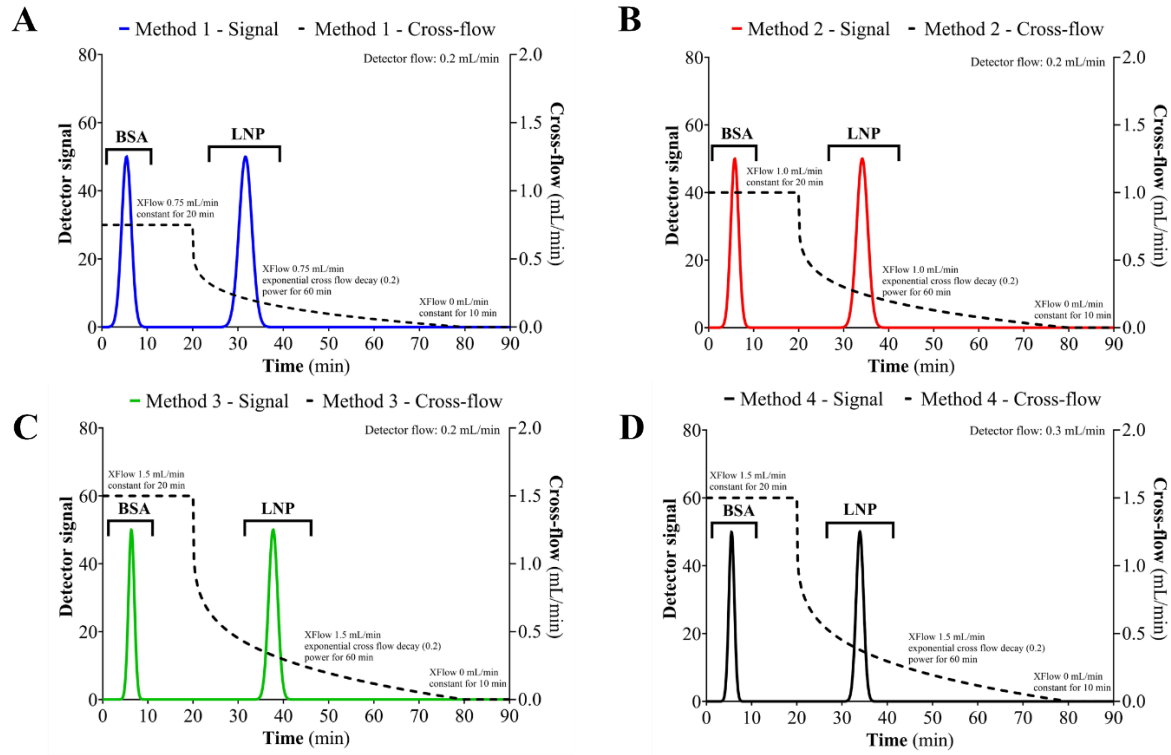

Figure S2 *FI-AF4 method simulation for MC3 LNP-BSA mixtures using the NovaAnalysis software. Shown is a fractionation profile of Bovine Serum Albumin (BSA) and Lipid Nanoparticle (LNP) peaks for (A) Method 1 (B) Method 2 (C) Method 3 and (D) Method 4. The profile obtained by simulating a linear cross-flow (XFlow) gradient with an initial flow rate within 20 min and dropping to 0 mL/min*

over 45 min at an exponential decay at power 0.2 with the final elution at 0 mL/min cross flow for 10 min. Dotted line shows the cross-flow profile over the run time duration.

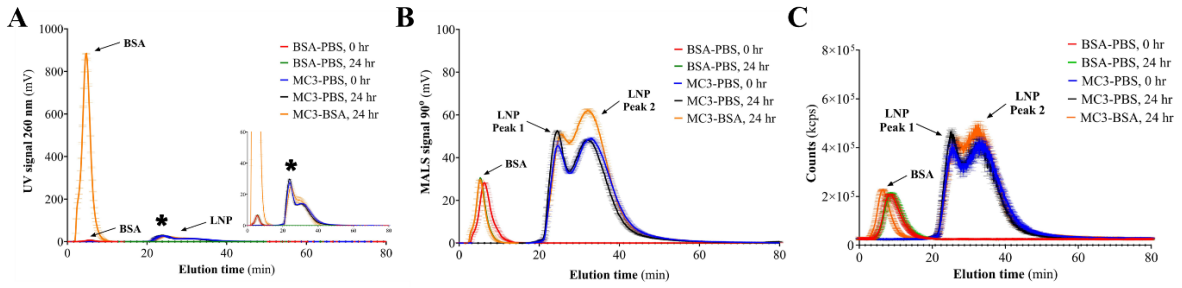

Figure S3 *Fractograms for (A) FI-AF4-UV at 260 nm (B) FI-AF4-MALS (90°) (C) FI-AF4-DLS for Bovine Serum Albumin (BSA) (control), LNPs (control) and MC3-LNPs incubated in 35 mg/mL BSA. Time-points indicate 0 hour (control) and 24 hours incubation at 37 °C. The arrows in each fractogram represent BSA, LNP Peak 1 and LNP Peak 2 eluting at different time-points of the elution profile. Error bars represent ± S.D mean of triplicate injections, BSA-PBS (24 hr) using FI-AF4-DLS represent duplicate injections. The peaks marked with a black asterisk (*) show enlarged peaks.*

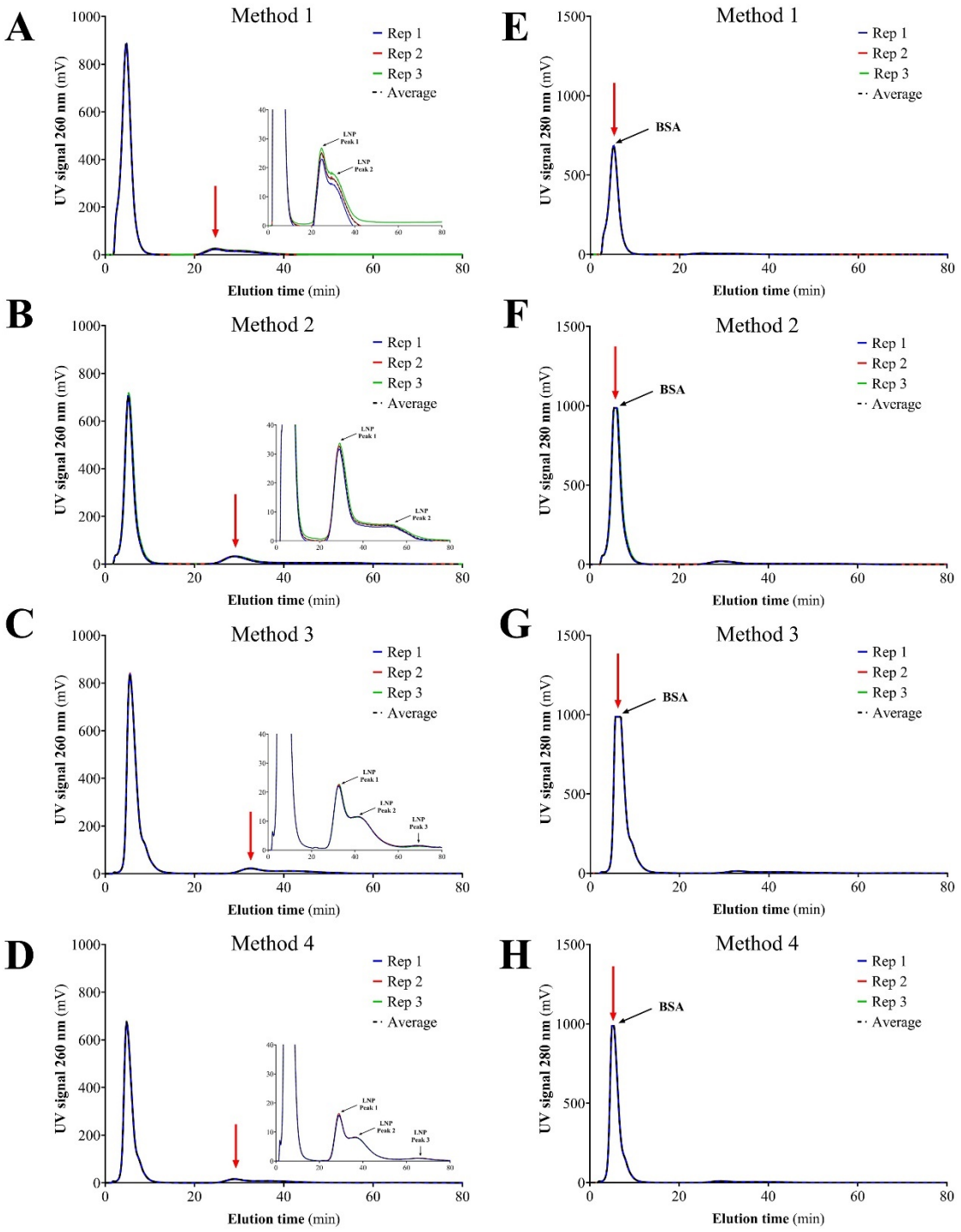

Figure S4 *UV detector coupled to FI-AF4 Flow-mode for MC3-LNPs incubated in 35 mg/mL BSA for 24 hours at 37 °C. Plots (A) to (D) represent Methods 1 to 4 respectively for UV-Vis detector absorbance at 260 nm for investigating the Lipid Nanoparticle (LNP) (LNP peaks 1-3 depicting the different LNP subpopulations). Plots (E) to (H) represent Methods 1 to 4 respectively for UV detector absorbance at 280 nm for investigating Bovine Serum Albumin (BSA). The red arrows represent the peak of interest for the specific UV absorbance wavelength (260 nm or 280 nm). Reps 1 to 3 represent triplicate injections with an average calculated.*

Table S3 *Average FI-AF4-UV signal intensities (mv) measured at 260 nm and 280 nm for Lipid Nanoparticles (LNP) and Bovine Serum Albumin (BSA) respectively. Refer to* ***Figure S1*** *and* ***Figure S2*** *for details of FI-AF4 Methods 1 – 4 and* ***Figure S4*** *for peak identity. Methods 1 and 2 did not identify Peak 3 (-). RSD, relative standard deviation.*

Peak identity	**Replicate injections**	**Method**			
		1	**2**	**3**	**4**
		Signal intensity (mV)			
BSA	Rep 1	607.4	739.0	986.3	985.3
	Rep 2	609.9	987.2	985.9	986.1
	Rep 3	257.9	987.3	986.0	986.1
	Average ± RSD	597.7 (± 3.2)	904.5 (± 15.8)	986.1 (± 0.0)	985.8 (± 0.0)
LNP Peak 1	Rep 1	22.9	31.4	22.0	15.2
	Rep 2	24.9	32.5	22.5	16.3
	Rep 3	26.8	31.7	22.7	16.3
	Average ± RSD	24.9 (± 7.8)	31.9 (± 1.8)	22.4 (± 1.7)	15.9 (± 3.8)
LNP Peak 2	Rep 1	12.3	4.6	11.2	7.4
	Rep 2	14.3	4.7	11.3	7.2
	Rep 3	15.8	5.0	11.0	7.2
	Average ± RSD	14.1 (± 12.5)	4.8 (± 4.0)	11.2 (± 1.3)	7.3 (± 2.0)
LNP Peak 3	Rep 1	-	-	1.5	0.9
	Rep 2			1.6	1.0
	Rep 3			1.4	1.0
	Average ± RSD			1.5 (± 8.5)	1.0 (± 3.5)

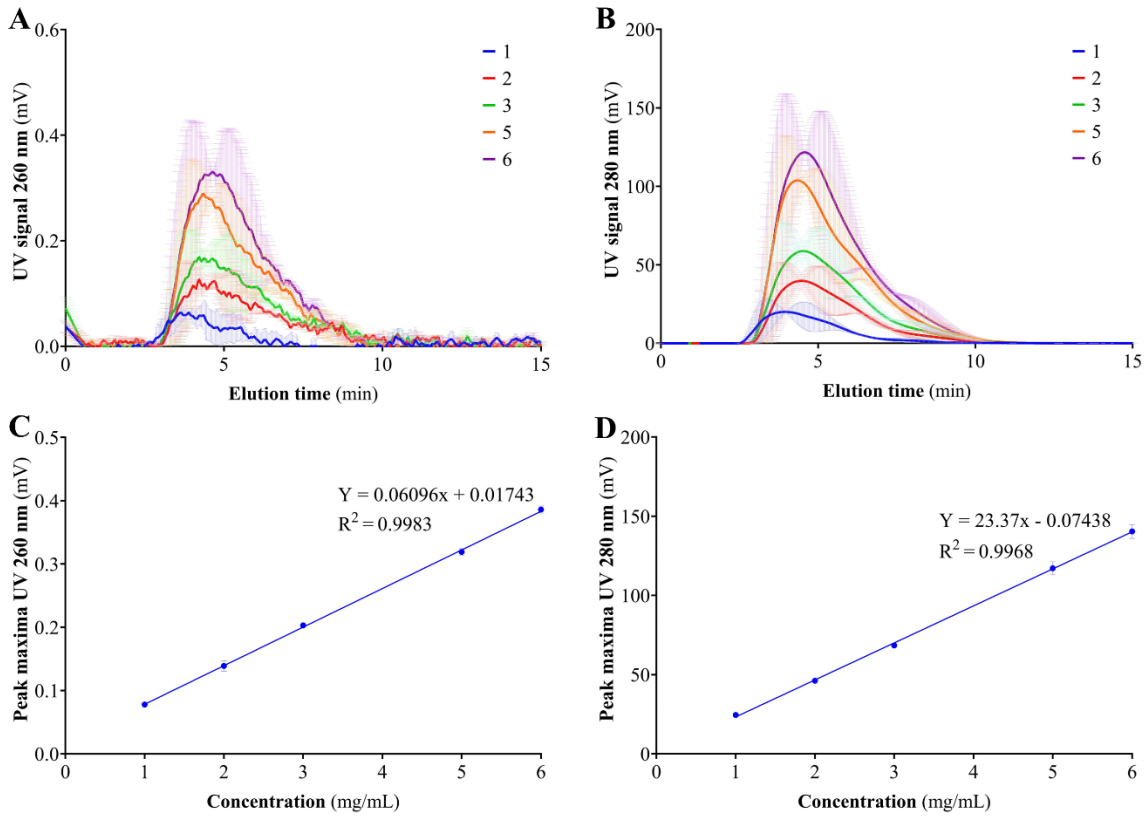

Figure S5 *Regression of linear function of UV detector signal peak maxima. Plots represent AF4-UV fractogram for BSA (1-5) mg/mL for (A) UV 260 nm (B) UV 280 nm and the corresponding linearity for (C) 260 nm and (D) 280 nm.*

Table S4 *Coefficient of Determination (R^2) and normalised Root Mean Square Error (RMSE) calculated for the predicted and actual values for MALS scattering fit models (Zimm, Coated Sphere,*

Random Coil and Debye). The predicted and actual values are extracted from Nova FFF software version 2.2.0.1. The R^2 and RMSE undefined refer to metrics could not be calculated as the slope of the $R(\theta)$ versus $\sin^2(\theta/2)$ plot is zero indicating an invalid model fit.

Peak Identity	Method	Zimm	Coated Sphere	Random Coil	Debye
BSA	1	RMSE and R^2 undefined	RMSE 0.03 (± 0.000) R^2 0.22 (± 0.09)	RMSE and R^2 undefined	RMSE 0.002 (± 0.001) R^2 0.33 ± 0.00
	2		RMSE 0.006 (± 0.001) R^2 0.41 (± 0.08)	RMSE 0.006 (± 0.000) R^2 0.43 (± 0.02)	RMSE 0.006 (± 0.000) R^2 0.48 (± 0.01)
	3		RMSE 0.008 (± 0.003) R^2 0.52 (± 0.28)	RMSE 0.009 (± 0.003) R^2 0.51 (± 0.28)	RMSE 0.007 (± 0.001) R^2 0.30 (± 0.08)
	4		RMSE 0.006 (± 0.001) R^2 0.50 (± 0.10)	RMSE 0.006 (± 0.001) R^2 0.57 (± 0.03)	RMSE 0.006 (± 0.000) R^2 0.59 ± 0.03
LNP Peak 1	1	RMSE and R^2 undefined	RMSE 0.006 (± 0.005) R^2 0.97 (± 0.03)	RMSE 0.004 (± 0.000) R^2 0.98 (± 0.00)	RMSE 0.003 (± 0.000) R^2 0.99 (± 0.00)
	2		RMSE 0.008 (± 0.002) R^2 0.96 (± 0.02)	RMSE 0.000 (± 0.000) R^2 0.95 (± 0.02)	RMSE 0.009 (± 0.001) R^2 0.96 (± 0.01)
	3		RMSE 0.011 (± 0.004) R^2 0.98 (± 0.01)	RMSE 0.011 (± 0.004) R^2 0.98 (± 0.02)	RMSE 0.009 (± 0.002) R^2 0.89 (± 0.06)
	4		RMSE 0.008 (± 0.000) R^2 0.95 (± 0.00)	RMSE 0.008 (± 0.000) R^2 0.95 (± 0.01)	RMSE 0.007 (± 0.000) R^2 0.96 ± 0.00
LNP Peak 2	1	RMSE and R^2 undefined	RMSE 0.005 (± 0.000) R^2 0.99 (± 1.00)	RMSE 0.005 (± 0.000) R2 1.00 (± 0.00)	RMSE 0.004 (± 0.000) R^2 1.00 ± 0.00
	2		RMSE 0.017 (± 0.003) R^2 1.00 (± 0.00)	RMSE 0.025 (± 0.004) R^2 0.99 (± 0.00)	RMSE 0.048 (± 0.006) R^2 0.98 (± 0.00)
	3		RMSE 0.084 (± 0.031) R^2 0.98 (± 0.01)	RMSE 0.073 (± 0.008) R^2 0.99 (± 0.00)	RMSE 0.009 (± 0.002) R2 0.94 (± 0.09)
	4		RMSE 0.007 (± 0.000) R^2 0.99 (± 0.00)	RMSE 0.008 (± 0.000) R^2 0.99 (± 0.00)	RMSE 0.006 (± 0.001) R^2 1.00 (± 0.000)
LNP Peak 3	1	-	-	-	-
	2				
	3	RMSE and R^2 undefined	RMSE 0.096 (± 0.043) R^2 0.98 (± 0.01)	RMSE 0.075 (± 0.010) R^2 0.98 (± 0.00)	RMSE 0.189 (± 0.019) R^2 0.90 (± 0.02)
	4		RMSE 0.107 (± 0.005) R^2 0.97 (± 0.00)	RMSE 0.072 (± 0.020) R^2 0.99 (± 0.00)	RMSE 0.244 (± 0.010) R^2 0.86 (± 0.01)

Table S5 *Comparison Radius of Gyration (R_g) obtained from the different MALS fit models for FI-AF4 methods 1- 4. Data is shown as ± S.D mean of at least two injections. Refer to* ***Figure 7*** *for definition of the peaks BSA and LNP Peaks 1 - 3. A hyphen (–) indicates no peak identified.*

Model	Method	R_g (nm)			
		BSA	LNP Peak 1	LNP Peak 2	LNP Peak 3

Coated Sphere	**1**	4.3 (± 0.4)	19.3 (± 0.2)	31.9 (± 0.6)	-
	2	7.8 (± 0.1)	21.3 (± 0.1)	58.7 (± 0.7)	-
	3	6.9 (± 0.9)	17.9 (± 0.7)	31.1 (± 0.7)	79.7 (± 1.9)
	4	8.8 (± 0.4)	19.5 (± 0.2)	32.1 (± 0.7)	83.3 (± 0.7)
Zimm	**1**	3.9 (± 0.1)	20.0 (± 0.1)	35.0 (± 0.4)	-
	2	7.8 (± 0.0)	22.6 (± 0.1)	93.3 (± 1.0)	-
	3	7.2 (± 0.7)	18.8 (± 0.8)	35.3 (± 0.4)	407.6 (± 163.3)
	4	8.8 (± 0.3)	20.6 (± 0.2)	36.1 (± 0.3)	313.2 (± 17.8)
Random Coil	**1**	5.5 (± 0)	19.5 (± 0)	32.5 (± 0)	-
	2	7.8 (± 0.1)	21.4 (± 0)	72.7 (± 1.3)	-
	3	7.1 (± 0.6)	18.1 (± 0.8)	33.0 (± 1.1)	130.3 (± 4.7)
	4	8.8 (± 0.3)	19.9 (± 0.3)	33.9 (± 0.4)	142.8 (± 4.5)
Debye	**1**	4.3 (± 0.6)	18.8 (± 0.1)	29.6 (± 0.1)	-
	2	7.7 (± 0.0)	20.9 (± 0.1)	47.4 (± 0.5)	-
	3	7.1 (± 0.7)	17.6 (± 0.7)	29.3 (± 0.5)	55.3 (± 0.3)
	4	8.7 (± 0.3)	19.2 (± 0.3)	30.1 (± 0.1)	56.1 (± 0.2)

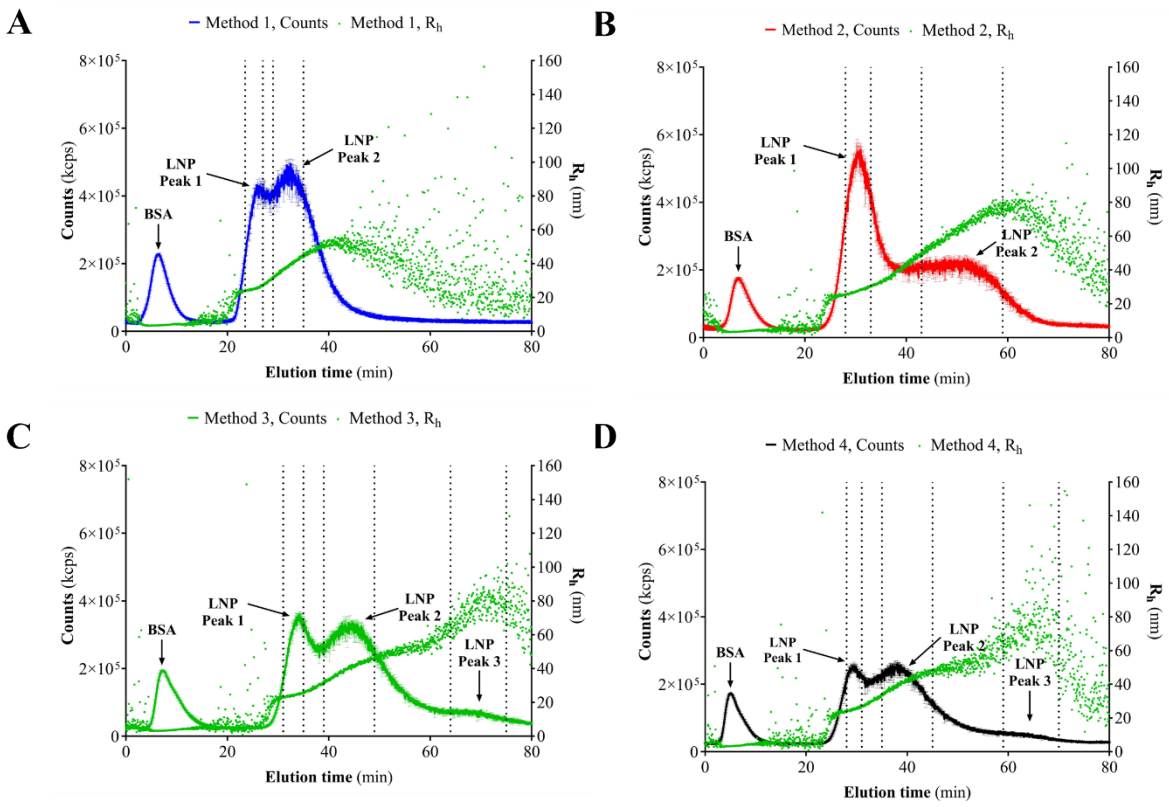

Figure S6 *Flow-mode (FI-AF4 separation with in-line DLS) for MC3-LNPs incubated in 35 mg/mL BSA for 24 hours at 37 °C. Plots represent (A) Method 1, (B) Method 2, (C) Method 3, (D) Method 4. The Hydrodynamic Radius (R_h) was measured across the entire flow trace (green circles) using the Malvern Zetasizer. The peaks represent an initial Bovine Serum Albumin (BSA) peak and Peaks 1-3 represents different LNP subpopulations of increasing hydrodynamic radius (R_h) with elution time. Error bars represent ± S.D mean of triplicate injections. Vertical dashed lines indicate peak region of interest.*

Table S6 *Average shape factor (R_g/R_h) calculated from peaks integrated in* ***Figure 7*** *and* ***Figure S6*** *for MC3-LNPs incubated in 35 mg/mL Bovine Serum Albumin (BSA) for 24 hours at 37 °C. Methods 1 and 2 did not identify Peak 3 (-).*

	LNP Peak Identity		
Method	**Peak 1**	**Peak 2**	**Peak 3**
1	0.735 (± 0.007)	0.794 (± 0.003)	-
2	0.792 (± 0.008)	0.853 (± 0.011)	-
3	0.709 (± 0.027)	0.765 (± 0.009)	1.069 (± 0.040)
4	0.741 (± 0.019)	0.794 (± 0.007)	1.263 (± 0.116)