Supplementary Material

Field-flow fractionation for molecular-interaction studies of labile and complex systems: A critical review

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Table 1. Examples of interaction parameters estimated from FFF information

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Dissociation constant (K_d)							
Analytes	FFF technique	Detection	Eluent Conditions	Amounts of reagents	Estimated K_d	Main comments	REF.
IgG antibody-FcRn receptor	AF4	UV/Vis, MALS, RI	Phosphate-buffed saline (PBS) at various pHs	FcRn (0.125–20 μM) were incubated with a fixed concentration of IgG (2.5 μM) at pH 5.8, 7.4	3.74 ± 0.07 μM	• The estimated K_d was comparable with previously published results by SPR, gel filtration, ITC • Stoichiometry of the FcRn/HSA/IgG complex was also obtained based on the AF4 elution times	[73]
IgE-Weigand DNA aptamer & streptavidin-streptavidin DNA aptamer	AF4	UV/Vis, FLD	Phosphate-buffered saline (PBS), pH 7.4 & Tris-buffered saline, 2 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4	Increasing amounts of protein were added to 2 nM Weigand aptamer (IgE) or 10 nM of the streptavidin aptamer	16.3 ± 0.5 nM & 20.9 ± 1.8 nM	• The buffer conditions can greatly affect the affinity between aptamer and target • The obtained K_d values were higher or lower than those reported in literature by approx. 60% in both cases • Addition of a fluorophore to the aptamer may disrupt the binding • Immobilization of streptavidin for SPR measurements may lead to conformation differences and, hence, differences in affinity	[120]
Aggregation kinetics							
Analytes	FFF technique	Detection	Eluent Conditions	Amounts of reagents	Interaction parameters	Comments	REF.
Heat-stressed anti-streptavidin (anti-SA) IgG1 antibody	AF4	UV/Vis, MALS, dRI	Phosphate buffer, pH 6.25	Amount injected 20 μg	Lumry-Eyring nucleated polymerization (LENP) model for non-native protein aggregation fit the AF4 data	• AF4 data used to determine the aggregation kinetics for species greater than 100 nm • The LENP model suggested a potential mechanism involving slow nucleation and aggregate-aggregate condensation	[73]
Milk protein with surfactant-stabilized oil-in-water emulsions	SdFFF	UV/Vis	Distilled deionized water, 0.1% (v/v) FL-70 detergent, 0.02% (w/v) NaN_3		The kinetics of oil-droplet aggregation at elevated temperatures was studied by determining the aggregation rate constants for each emulsion	• The aggregation rate constants for each emulsion were calculated from the particle-size measurements • Comparison of the weight-average diameter of the droplets determined by SdFFF with that found from SEM pictures showed a 0.4% deviation, indicating the validity of the SdFFF in studying the kinetics of aggregation for the oil-in-water emulsions in the presence of milk proteins and/or surfactants	[77]
Drug loading and release kinetics							

Analytes	FFF technique	Detection	Eluent Conditions	Amounts of reagents	Interaction parameters	Comments	REF.
Liposomes and poorly soluble and lipophilic drugs	AF4	UV/Vis, MALS, dRI	Tris buffer, pH 7.4	Injected amount 10-20 μg	The time course of transfer between the model drug and the liposomal fractions was found to be described by a 1st order exponential function; $\boldsymbol{Y = Y_{EQ} - Ae^{-kt}}$ - Y; the relative amount of model compound transferred between the liposomal fractions at time t - Y_{EQ}; the relative amount transferred at equilibrium and marks the height of the plateau - A; the pre-exponential Coefficient - k; the rate constant of transfer	• Quantification of the model drug compound was performed off-line (based on HPLC-UV/Vis) and on-line (based on the change in the measured UV/VIS extinction of the donor fraction in the incubation mixtures relative to the UV/VIS extinction of corresponding donor liposomes) • There was agreement between the on-line and off-line analysis both following a 1st order exponential function kinetic model	[192, 193,240]
Binding stoichiometry- bioconjugation efficiency							
Analytes	FFF technique	Detection	Eluent Conditions	Amounts of reagents	Interaction parameters	Comments	REF.
Leptin receptor binding leptin antagonists (LR_{ecto}:leptin complexes)	AF4	UV/Vis, MALS, dRI	-	LR_{ecto}, and its complexes with leptin antagonists in a concentration range of 18 to 50 mM were analysed	Stoichiometry of the non-covalent quaternary complexes LR_{ecto}:leptin	• Based on the molecular weight obtained by MALS the stoichiometry of the complex was estimated as 2:2 • The non-covalently bound complex was found to dissociate during SEC analysis	[78]
Polyphosphazene immunoadjuvants and proteins of various isoelectric points	AF4	UV/Vis and batch-mode DLS	Phosphate-buffered saline (PBS), pH 7.4	-	Estimation of %protein binding and K_d of the complex (0.2-0.4 μM) based on the quantity of the free protein at different protein:polymer ratios	• The absence of the protein peak in its formulation with the polymer suggests complete binding to the adjuvant • Fluorescent labelling of the protein enables its detection in the visible part of the spectrum (495 nm). At this wavelength the polymer is not detected • The "stained" protein-polymer complex elutes at the exact position with the bare adjuvant	[241, 242]

Monitoring conformation changes							
Analytes	FFF technique	Detection	Eluent Conditions	Amounts of reagents	Interaction parameters	Comments	REF.
Enzyme-loaded polymersomes	AF4	VWD, MALS, DLS, dRI *variable wavelength detector (VWD^{i})	Phosphate-buffered saline (PBS	-	Scaling plot $R_g = K \cdot M^v$ -Rg; the radius of gyration -M; molecular weight -K; constant -v; slope of scaling plot; scaling parameter	• Increase of the scaling parameter (v) after loading of the enzyme indicated that the spherical shape of the polymersomes membrane remained unchanged but the membrane surface changed from smooth and well-defined to more rough • This change of membrane surface suggested strong interaction between the enzyme and the membrane	[78]
IgE-Weigand DNA aptamer & streptavidin-streptavidin DNA aptamer	AF4	UV/Vis, FLD	Phosphate-buffered saline (PBS), pH 7.4 & Tris-buffered saline, 2 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4	Increasing amounts of protein were added to 2 nM Weigand aptamer (IgE) or 10 nM of the streptavidin aptamer	Calculation of the percentage of aptamer molecules in their folded state. Based on R_g (obtained by molecular-dynamics simulations) and measured retention times of the aptamers, the theoretical elution times of folded (t_F) and unfolded (t_{UF}) forms were estimated. $\%Folded = \frac{t_{UF} - t_M}{t_{UF} - t_F} \times 100\%$	• Differences in rigidity between the folded and unfolded ssDNA were based on the estimate of the scaling parameter (v) • Combining information from molecular-dynamics (MD) simulations (R_g, free-energy drop during folding) and the %Folding (AF4 retention times) for the different aptamers it was concluded that aptamer molecules with a larger free-energy drop exhibit higher %Folding. • The folded structure was considered to be beneficial to maintain the binding between aptamer and their target	[120]