Using asymmetric flow field-flow fractionation hyphenated with multiple detectors for the analysis of pharmaceutical bionanomaterials

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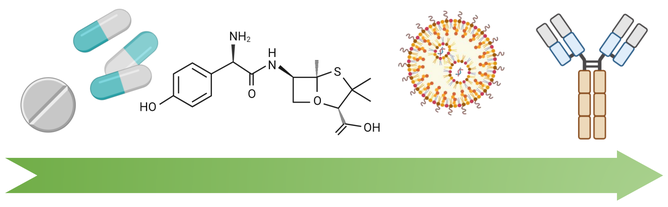
University of Strathclyde Science

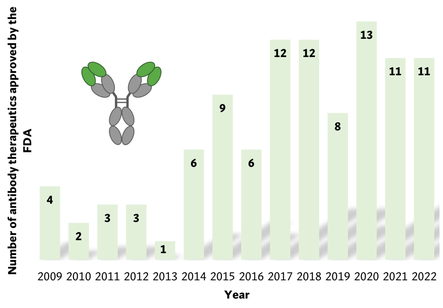
EPSRC Engineering and Physical Sciences Research Council

MMS Multiscale Metrology Suite for Next-Generation Health Nanotechnologies

Introduction

- ✓ Antibodies bind target antigens with high specificity and affinity.
- ✓ Antibodies have prolonged half-life and confined biodistribution in human bodies.
- ✓ Can activate immune response
- ✓ Low immunogenicity *in vivo*





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Analytical Challenges

- Physicochemical stability
- Evaluation of stability and behaviour in different matrices
- Method validation in different circumstances

Aims and objectives:

- To understand IgE physical stability at different pH and in response to environmental stressors encountered in the mAb product lifecycle.

Methodology

Asymmetric Flow Field Flow Fractionation (AF4) Method Development

IgG standard **AF4 –UV-Vis-MALS**

- **Channel composition:** Conventional, 350 µm spacer thickness
- **Membrane**: 10 kDa Regenerated cellulose amphiphilic
- **Cross flow rate:** 1-3 mL/min
- **Eluent:** PBS pH 7.4 (0-200 mM NaCl)

Immunoglobulin E (IgE) Response to formulation and environmental stresses

Formulation

pH 5.5, 6.5, 7.5

IgE

Dynamic Light Scattering (DLS)
- Z-average
- Polydispersity index (PDI)

Nanoparticles Tracking Analysis (NTA)
- Mean, mode size (nm)
- D10, D50, D90
- Total particles per mL

SEC –UV/Vis
- Size
- Recovery

AF4 –UV/Vis-MALS
- Size
- Molar Mass
- Conformation
- Recovery

Stresses

Ambient temperature (25 °C)

80 °C, 15 min

56 °C,24 hours

-80 °C, 1 hour

37 °C, 30 min

5x

Results & Discussion

AF4 Method Development

Immunoglobulin G (IgG)

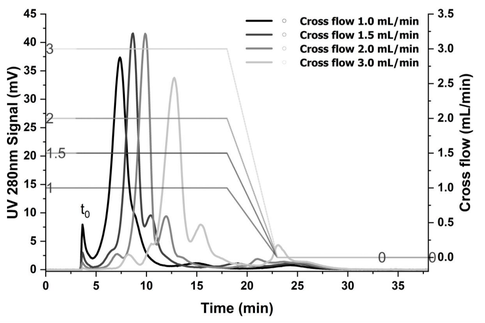


Figure 1 AF4 Fractogram at different cross flow rates (1-3 mL/min), 1 mg/mL IgG, 20 µL injection volume, eluent buffer: PBS (pH 7.4).

Table 1 A comparison of various cross flow rate; data represents mean(n=3) ± standard deviation; statistical analysis of percentage recovery was completed using a Tukey test at p < 0.05 (A, B group).

Cross flow (mL/min)	Monomer (%)	Dimer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
1.0	74.53±1.01^{A}	-^{C}	14.91±0.39^{A}	11.05±0.65^{A}	83.02±0.52^{B}
1.5	70.23±0.17^{B}	12.54±0.10^{B}	5.60±0.07^{B}	11.62±0.08^{B}	82.98±0.37^{B}
2.0	67.52±0.13^{C}	14.70±0.09^{A}	5.08±0.04^{B}	12.70±0.17^{B,C}	82.87±0.20^{B}
3.0	65.88±0.24^{C}	14.54±0.10^{A}	4.31±0.07^{C}	15.26±0.21^{C}	90.14±2.50^{A}

Selected parameters:

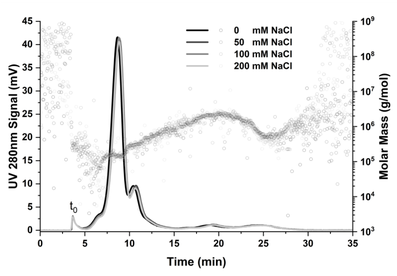
Cross flow: 1.5 mL/min , Injection volume: 20 µL, Elution buffer: 100mM NaCl, PBS (pH 7.4) were selected for quantifying the IgG at 1 mg/mL.

Table 2 A comparison of various percentage fragments and recovery from injection mass (µL); data represents mean(n=3) ± standard deviation; statistical analysis of percentage recovery and fractionation was completed using a Tukey test at p < 0.05 (A, B group).

Injection Mass (µg)	Monomer (%)	Dimer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
20 (1 mg/mL, 20 µL)	74.88 ± 0.78^{A}	12.84 ± 0.02^{A}	6.02 ± 0.14^{A}	6.25 ± 0.48^{A,B}	93.11 ± 0.78^{A,B}
50 (1 mg/mL, 50 µL)	74.44 ± 1.71^{A}	11.40 ± 0.14^{B}	5.51 ± 0.33^{A}	8.66 ± 1.69^{A}	96.22 ± 2.89^{A}
40 (2 mg/mL, 20 µL)	76.98 ± 0.30^{A}	13.23 ± 0.20^{A}	4.27 ± 0.36^{B}	5.52 ± 0.32^{B}	91.17 ± 0.37^{B}
100 (2 mg/mL, 50 µL)	74.80 ± 0.05^{A}	11.10 ± 0.08^{B}	5.54 ± 0.04^{A}	8.56 ± 0.05^{A}	97.43 ± 0.22^{A}

Table 3 The impact of ionic strength from NaCl addition (0-200 mM) ; data represents mean(n=3) ± standard deviation; statistical analysis of percentage recovery (%R) and fractionation was completed using a Tukey test at p < 0.05 (A, B, C group).

NaCl (mM)	Monomer (%)	Dimer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
0	70.23±0.17^{A}	12.54±0.10^{A}	5.60±0.07^{C}	11.62±0.08^{A}	82.98±0.37^{B}
50	69.02±0.22^{B}	12.29±0.16^{A}	6.98±0.06^{A}	11.71±0.29^{A}	85.24±1.13^{A}
100	70.82±0.20^{A}	11.85±0.05^{B}	6.32±0.11^{B}	11.01±0.04^{B}	82.99±0.30^{B}
200	70.38±0.17^{A}	11.93±0.04^{B}	6.40±0.26^{B}	11.29±0.04^{A,B}	80.81±0.21^{A}



Results & Discussion

AF4 Method Development

Immunoglobulin E (IgE)

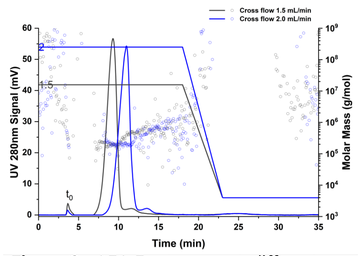


Figure 2 AF4 Fractogram at different cross flow rates (1.5-2 mL/min), 1 mg/mL IgE, 20 µL injection volume, elution buffer: PBS (pH 7.4).

Table 4 The effect of cross flow rate (mL/min) on the detected IgE monomeric purity and yield (1mg/mL IgE); statistical analysis of percentage recovery and fractionation was completed using a Tukey test at *p < 0.05; (ns) no significant.

Cross flow (mL/min)	Void (%)	Fragment (%)	Monomer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
1.5	1.47	0.68	76.13	3.43	3.70	86.07
2.0	0.52*	1.06 (ns)	81.56*	3.56 (ns)	4.09 (ns)	91.38*

A 2 mL/min cross-flow rate was selected for IgE.

Recovery and percentage fractionations depend on the rate of cross-flow. The method with the highest method performance criteria was carried forward.

Immunoglobulin E (IgE) Response to formulation and environmental stresses

Formulation

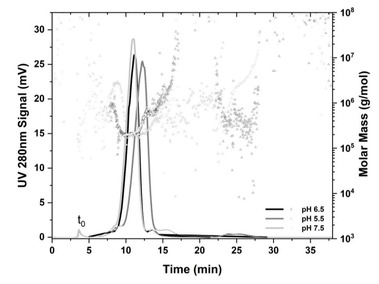


Figure 3 AF4 Fractogram measured at UV 280 nm at different pH conditions on IgE, including untreated baseline control samples (pH 6.5) and buffer exchanged samples (pH 5.5 and 7.5); 20 µL injection volume with cross flow at 2.0 mL/min, elution buffer: 1xPBS (pH 7.4).

IgE is stable in pH 6.5 buffer, with minimal particle formation in comparison to pH 7.5 and 5.5.

pH 7.5 and 5.5 formulations result in higher molecular weight species and increased particle formation.

Table 5 Corresponding AF4-UV/Vis-MALS & SEC-UV-Vis measuring the impact of different pH conditions on IgE, including untreated baseline control samples (pH 6.5) and buffer exchanged samples (pH 5.5 and 7.5); Data represents mean(n=3) ± standard deviation; statistical analysis was completed using a Tukey test* = p < 0.05, ** = p<0.01, *** = p=0.001, **** = p < 0.001 , ns – not significant, () - versus pH 6.5, [] – versus pH 5.5..

AF4-UV/Vis-MALS	Fragment (%)	Monomer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
pH 6.5	1.11 ± 0.06	90.23 ± 1.30	2.51 ± 0.32	3.06 ± 0.37	85.41 ± 1.72
pH 5.5	3.04 ± 0.87	92.23 ± 0.54 (ns)	2.89 ± 0.52	0.17 ± 0.11	101.35 ± 0.78
pH 7.5	3.36± 0.14	89.68 ± 0.51 (ns) [*]	2.32 ± 0.28	2.33 ± 0.21	97.71 ± 1.30

SEC-UV/Vis	Fragment (%)	Monomer (%)	Aggregate (%)
pH 6.5	4.08 ± 4.24	93.72 ± 4.99	2.20 ± 1.23
pH 5.5	10.42 ± 0.50 (ns)	87.66 ± 0.92 (ns)	1.92 ± 1.22 (ns)
pH 7.5	4.59 ± 0.94 (ns) [ns]	96.01 ± 2.51 (ns) [ns]	1.03 ± 0.19 (ns) [ns]

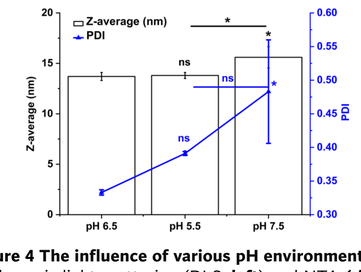


Figure 4 The influence of various pH environments; Z-average and polydispersity index (PDI) measured by dynamic light scattering (DLS, **left**) and NTA **(right)** for IgE samples (1 mg/mL, 0.2M arginine buffer, pH 6.5) subjected to buffer exchanged samples (pH 5.5 and 7.5). One-way ANOVA multiple comparisons, *p<0.05 relative to pH 6.5, ns: not significant.

Stresses

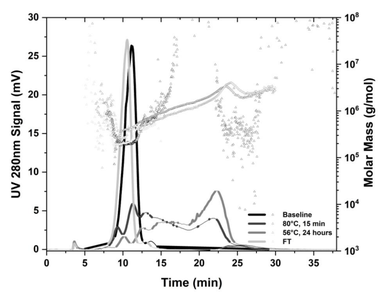


Figure 5 AF4 Fractogram at different stress conditions on IgE formulated at 1 mg/ml and pH 6.5, including untreated (Baseline), thermal stress 80°C (80°C, 15 min), thermal stress 56°C (56°C, 24 hours), and freeze-thaw stress (FT), 20 µL injection volume with cross flow at 2.0 mL/min, elution buffer: 1xPBS (pH 7.4).

Table 6 Corresponding AF4-UV/Vis-MALS & SEC-UV/Vis measuring the impact of. different stress conditions on IgE formulated at 1 mg/ml and pH 6.5, including untreated (Baseline), thermal stress 80°C (80°C, 15 min), thermal stress 56°C (56°C, 24 hours), and freeze-thaw stress (FT); Data represents mean(n=3) ± standard deviation; statistical analysis was completed using a Tukey test* = p < 0.05, ** = p<0.01, *** = p=0.001, **** = p < 0.001 relative to baseline, ns – not significant, nd – not detected.

AF4-UV/Vis-MALS	Fragment (%)	Monomer (%)	Dimer (%)	Oligomer (%)	Aggregate (%)	Recovery (%)
Baseline	1.11 ± 0.06	90.23 ± 1.30	2.51 ± 0.32	nd	3.06 ± 0.37	85.41 ± 1.72
56°C, 24 hours	0.75 ± 0.03	4.06 ± 0.10 (****)	8.52 ± 0.33	25.68 ± 3.51	59.95 ± 3.67	93.46 ± 0.48
80°C, 15 min	0.40 ± 0.01	6.50 ± 0.11 (****)	17.20 ± 0.51	40.81 ± 0.23	34.10 ± 0.46	91.91 ± 0.68
Freeze-Thaw	1.88 ± 0.12	90.55 ± 0.30 (ns)	2.67 ± 0.14	nd	2.74 ± 0.20	77.57 ± 1.21

SEC-UV/Vis	Fragment (%)	Monomer (%)	Aggregate (%)
Baseline	4.08 ± 4.24	93.72 ± 4.99	2.20 ± 1.23
56°C, 24 hours	1.28 ± 0.6 (ns)	nd	98.72 ± 0.60
80°C, 15 min	1.61 ± 1.35 (ns)	nd	98.39 ± 1.35
Freeze-Thaw	1.84 ± 0.95 (ns)	96.93 ± 1.09 (ns)	1.23 ± 0.14 (ns)

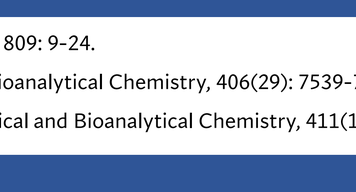


Figure 6 The impact of the environment stresses, including thermal stresses, and freeze-thaw; Z-average and polydispersity index (PDI) measured by dynamic light scattering (DLS, **left**) and NTA **(right)** for IgE samples (1 mg/mL, 0.2M arginine buffer, pH 6.5) subjected to thermal stress 56°C (Thermal 1), thermal stress 80°C (Thermal 2), and Freeze-Thaw stress (FT). One-way ANOVA multiple comparisons, *p<0.0001 relative to baseline, ns: not significant.

A significant change in Z-average and PDI observed following thermal stress.

NTA data show the emergence of submicron particles following thermal stress (no particles detected in the baseline sample).

Significant loss of monomeric purity observed in response to thermal stress.

A consideration for formulation, storage and shipment conditions for formulation selection.

Conclusion

AF4 serves as an orthogonal, gentle separation method for conducting high-resolution analysis of antibody stability, providing orthogonal characterisation compared to other techniques.

FFF run parameters, the formulation, and the environment can significantly affect the efficacy of FFF in resolving higher molecular weight species.

Sequence engineering strategies, including adjustments to electrostatic and hydrophobic regions, alongside isoelectric manipulation, offer potential for designing stable IgE molecules beyond formulation changes.

References

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