

Field Flow Fractionation Analysis of Host-Guest Complexes of Peptides and Proteins Conjugates

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Summary

Host-guest chemistry allows for conjugation through non-covalent interactions of a hollow compound (host) to one or more molecules displaying recognition moieties (guests). The formed complex is not permanently linked and can therefore present stability issues during characterisation analyses.

Asymmetric Flow Field-Flow Fractionation (AFFF) allowed characterisation and separation of both the (large) guest alone, and—on account of the high concentrations achieved at the membrane—the host-guest complex. The millimolar concentration obtained during the separation step avoided dissociation of the conjugate, permitting a measure of the complex stoichiometry and obtaining a flavour of its stability in solution.

I. Introduction

Host-guest chemistry is a transient conjugation of a hollow compound (host) to one or more molecules (guests). The molecules are not permanently linked, which can provide beneficial characteristics such as stability in the bound state, without reducing the biological activity of the unbound molecule. Another advantageous property is slow release of the active product in the body. This approach can be applied to a range of molecules including peptides and antibody-drug conjugates; it permits specificity and stability within the body, while improving release upon a convenient stimuli (e.g. dilution, pH, temperature).

AFFF was used to study the conjugate forming between a cyclic RGD peptide, displaying a specific 1st guest moiety pre-complexed with the host cucurbit[8]uril (CB[8]), and a recombinant fibronectin type III domain of human tenascin C (Tn3, carrying an N-terminal tryptophan acting as 2nd guest). The complex forms as heteroternary with a stoichiometry of 1:1:1 (peptide:CB[8]:Tn3, see Figure 1).



Figure 1: Schematic of the conjugate formation. A peptide displaying a 1st guest is pre-complexed with the host CB[8] and then conjugated with a protein domain carrying a 2nd guest.

The affinity of the protein binding event is reasonably high (μM), but the off rate is also very high (360 s^{-1} at 37°C). AFFF was used as an orthogonal method to determine the binding ratio of the conjugate, but could also be used to monitor the stability of these complexes. [1]

II. Materials and Methods

An HPLC system (Agilent, 1100 series) was used to control flow to an **Eclipse Dualtec**; the eluent was 10 mM phosphate buffer, pH 7. The species were separated over a 1 kDa cut-off polyether sulfone (PES) membrane (Wyatt Technology, cat. no. 2725) with a 350 μm spacer. Aliquots consisting of 20 μL of Tn3 (125 μM) with various molar ratios of peptide-CB[8]-Tn3 were injected. The detector flow was set at 1 mL/min and the cross flow was set at 2.0 mL/min from 0 to 15 min, 2.0 to 0.2 mL/min from 15 to 20 min, and 0.2 mL/min from 20 min to 30 min. A 5 minute focus and inject step was used in the method. Elution of protein species was followed by absorbance at 280 nm (Agilent, 1100 series) in conjunction with multi-angle light scattering (MALS) analysis using **miniDAWN TREOS** and **Optilab T-rEX** detectors.

Analysis

The AFFF method was defined to allow the complex to stay bound during the run, and only dissociate after leaving the separation unit upon dilution. The **ISIS AFFF simulation software** was used to predict the concentration of the complex at the membrane, which was calculated to vary from 60 mg/mL to 20 mg/mL (Figure 2). The conjugate was injected at 2 mg/mL and maintained at a high concentration (~5 mM) at the membrane. The heteroternary complex did not dissociate during the separation, but only upon leaving the separation unit, where molecules at the membrane are mixed with the bulk liquid before entering the detector.



Figure 2. ISIS model of concentration distribution in the focusing zone. The plot shows the concentrations achieved at the membrane during the separation.

III. Results and Discussion

AFFF confirmed the binding stoichiometry of the pre-complex peptide-CB[8] and Tn3, as evidenced by the 1:1 ratio eluting with the maximum delay and no further increase upon injection of higher pre-complex/protein ratios (Figure 3). No change in **molecular weight** of the peaks due to dissociation of the complex after AFFF separation was observed, as determined by static light scattering (FFF-MALS).

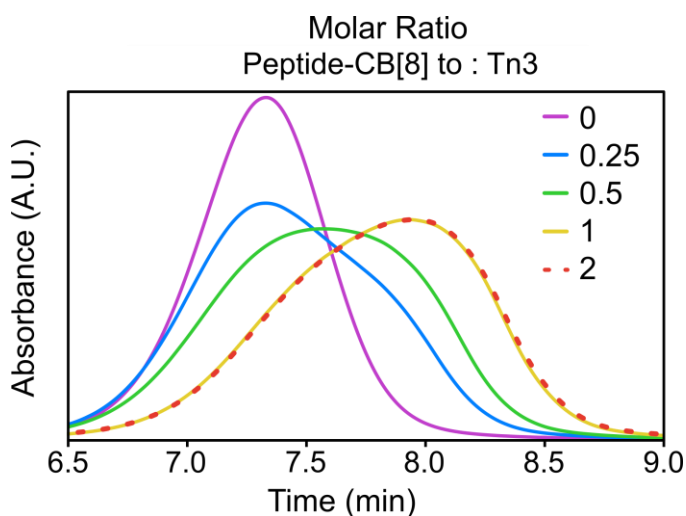


Figure 3. The change in molecular size upon peptide-protein conjugation is demonstrated by AFFF elution curves of Tn3 in presence of increasing molar ratios of peptide-CB[8]:Tn3. Data at ratio 2 are shown as dashed line to allow for the visualisation of data at ratio 1.

The molecular weights of Tn3 and peptide-CB[8]-Tn3 conjugate were determined by batch light scattering mode with direct infusion into a MALS detector, resulting in 14.2 kDa and 16.4 kDa, respectively. Given the absence of dissociation during AFFF elution, these values were used within the ISIS software to predict the difference in elution time of the protein only and the complex. ISIS modeling resulted in a 0.1 min difference, whereas the experimental value was 0.75 min, suggesting a more open structure than the globular protein model used by the modeling software (Figure 4).

This modeling was also applied to evaluation of the stability of peptide-CB[8] complexed to an antibody Fc region (with an N-terminal tryptophan residues acting as 2nd guest). In this instance, the ISIS software predicted a 0.05 min difference in retention time, but no significant difference in the elution time could be detected experimentally, even though other calorimetric and spectroscopic methods had demonstrated the formation



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of the heteroternary complex. The globular prediction model used by ISIS for this complex was likely more accurate and such a tiny difference of 0.05 min elution time cannot be accurately detected by AFFF-MALS.

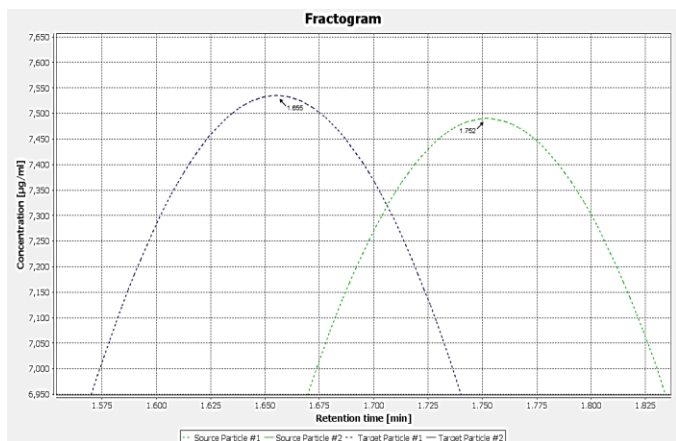


Figure 4. ISIS software prediction of the elution times for globular proteins of 14.2 kDa and 16.4 kDa mirroring Tn3 and peptide-CB[8]-Tn3, respectively. Elution times are reported without the focus and injection stages which account for an addition of ~5 min. Predicted resolution of the separation was 0.1 min.

IV. Conclusions

This work demonstrates that AFFF in combination with MALS can be used effectively to evaluate stability and stoichiometry of host-guest complexes, when the size and/or shape of the molecules forming the conjugates allows for a significant difference in the elution times of the bound versus unbound species. In this respect AFFF-MALS serves as an orthogonal method to traditional interaction analysis techniques.

V. Reference

[1] R. J. Gubeli, S. Sonzini, A. Podmore, P. Ravn, O. A. Scherman, C. F. van der Walle. *Chem. Commun.* **2016**, 52, 4235-4238. Selective, non-covalent conjugation of synthetic peptides with recombinant proteins mediated by host-guest chemistry. *Supporting Information*.



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