Correlation of Discreet Drug-linker Variation on Antibody Drug Conjugates (ADC) to Binding Activity Using Surface Plasmon Resonance

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Introduction

Antibody-drug Conjugates (ADCs)

Antibody-drug conjugates (ADCs) are one of the fastest growing classes of oncology therapeutics. ADCs are hybrid innovative biotherapeutics, that combine the specificity of monoclonal antibodies (mAb) with highly potent cytotoxic small molecules conjugated via a bifunctional linker, which is a critical factor in determining the effectiveness of the ADC therapy. Characterization of ADCs, which includes site-specificity, potency of the drug, conjugation sites, binding properties, and Drug to Antibody Ratio (DAR) can be challenging owing to the complexity of this class of biologic.

ADCs rely on the target-binding specificity of a mAb to selectively deliver potent drugs to target cells. Binding interactions between IgG antibodies and Neonatal Fc receptor (FcRn) regulates half-life *in vivo* and is associated with the improvement of therapeutic efficacy. The efficacy of tumour targeting ADCs may also depend on antibody effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC), that are mediated via interactions between the antibody Fc region and components of the immune system, such as immune cell surface Fc gamma (Fcγ) receptors, and complement.

Trastuzumab (Tmab), a recombinant humanized IgG1 monoclonal antibody against the Receptor tyrosine-protein kinase erbB-2 (HER-2), is known to mediate ADCC activity by attracting the immune cells to tumours sites that overexpress HER-2 antigen. The most important contributors to ADCC activity *in vivo* are thought to be Natural killer (NK) cells, which express only Fc gamma receptor IIIa (FcγIIIa).

In our previous study, we have investigated the relationship between the nature of PEG linkers and Fc region mediated binding interactions using an ADC mimic. In this study we expanded the investigation into discreet linker and DM1 toxin variation, and the FcRn and FcγRIIIa binding activities using Surface Plasmon Resonance (SPR).

Library Conjugation

PEG-DM1 Procedure:

TmAb was reacted with the desired NHS-linker at 2 loadings (5 and 7 mol eq) for 90 min. DM1 was then added at the same equivalents of linker and reacted for 60 min. Reaction solutions were then transferred to an Amicon 30kd spin filter and exchanged 3 times with 1x PBS pH 7.4 to remove impurities. Retentates were removed and submitted for analyses.

Figure 1. PEG-DM1 Conjugation Process

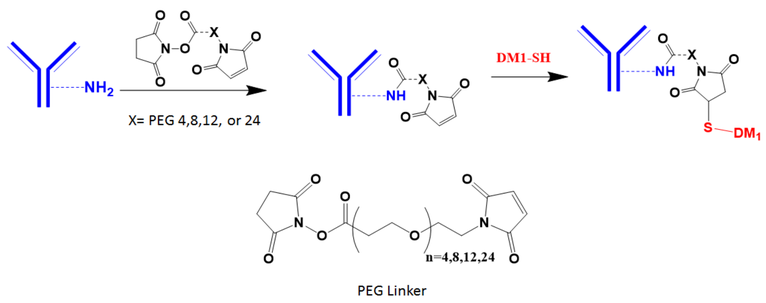
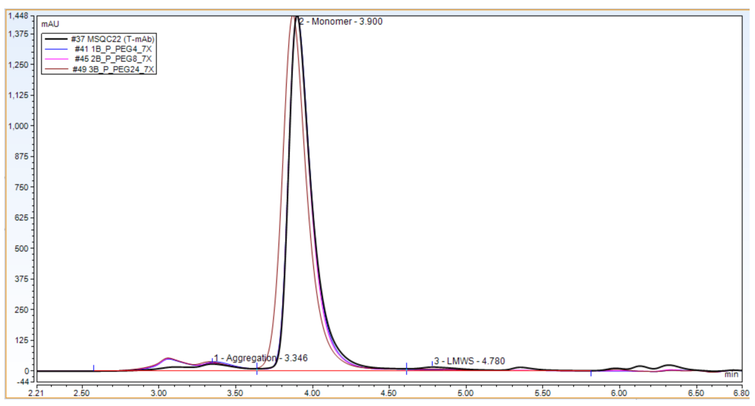


Table 1. Analytical Characterization Results

Sample	Drug Loading	Size Distribution		
	DAR	% Aggregation	% Monomer	% Free Drug
1A_P_PEG4_5X	1.48	7.22	91.59	ND
1B_P_PEG4_7X	1.67	7.36	91.74	ND
2A_P_PEG8_5X	1.55	7.01	92.28	ND
2B_P_PEG8_7X	1.64	7.2	92.1	ND
3A_P_PEG24_5X	1.32	7.05	92.09	ND
3B_P_PEG24_7X	1.39	7.32	91.86	ND

Figure 2. Size Distribution Chromatograms Comparison



Smaller PEGs retain on column similarly to unconjugated mAb, however as PEG length increases, the size shift is pronounced to a point where it can be detected beyond its proportional size. This may be due to changes in solubility, or physical width, or likely both.

Assay design/Overview

Surface Plasmon Resonance (SPR)

SPR has become the leading technology to analyse antibody binding interactions mediated by both Fc and Fab regions, since it enables real-time information on the binding association (k_{on}), dissociation (k_{off}) rates, and determination of the equilibrium dissociation constant (K_D) between interacting molecules. Therapeutic mAb – Fc receptor interactions can be investigated using full kinetics analysis to characterise the underlying binding mechanism.

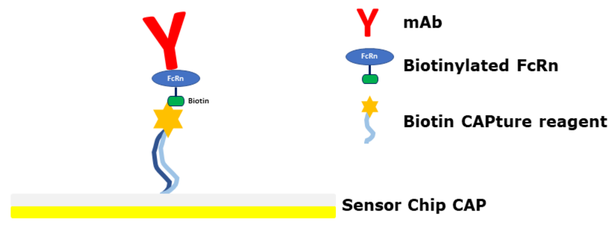
Binding assays were developed to perform real time kinetic analysis of the binding interaction between the Fc region of therapeutic mAbs and human FcγRIIIa and FcRn using SPR. The assays were developed using a Biacore T200 instrument, and a utilize ligand capture approach (recombinant FcγRIIIa or FcRn) followed by kinetic binding analysis of multiple concentrations of the mAb in order to generate equilibrium dissociation constant (K_D) values.

Neonatal Fc Receptor (FcRn)

Rehydration and conditioning of Sensor Chip CAP was performed to optimize the binding capacity prior to the binding analysis. 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween20 at pH 6.0 was used as running buffer and dilution buffer.

Multi cycle kinetics for each sample were performed by injecting a range of concentrations of TmAb analyte flowing over a FcRn captured surface (Sensor chip CAP, Figure 3). Unconjugated TmAb was analysed as the Reference Standard/Control within the assay. The resulting data were fitted using a a Two state reaction binding model using Biacore Evaluation software (Cytiva) to determine the K_D value.

Figure 3. An overview of the binding assay format to study the interaction of modified and unmodified TmAb with FcRn.

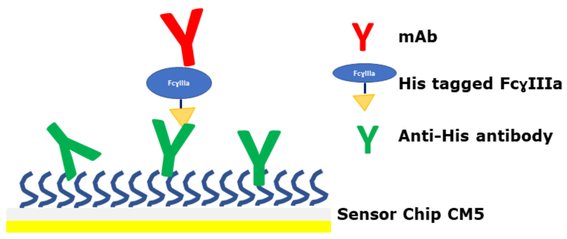


FcγRIIIa

10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween20 at pH 7.4 was used as running buffer and dilution buffer. CM5 sensor chip was primed using running buffer prior to immobilization of Anti-His antibody at 25 °C, 50 µg/mL in 10 mM sodium acetate pH 4.5 using standard amine coupling procedure.

Multi cycle kinetics for each sample were performed by injecting a range of concentrations of Tmab analyte over a FcγRIIIa captured surface (CM5, Figure 4). Unconjugated TmAb was analysed as the Reference Standard/control within the assay. The resulting data were fitted using a two-state reaction binding model using Biacore™ Insight Evaluation Software (Cytiva) to determine the K_D value.

Figure 4. An Overview of the Binding Assay Format to Study the Interaction of Modified and Unmodified TmAb with FcγRIIIa.



Summary

SPR binding assays allowed real time kinetic analysis of interactions between Fc receptors and mAb-based therapeutics including ADCs. We have demonstrated rapid analysis of different constructs using SPR which provides preliminary indication of linker selection on Fc binding activity. Our results suggested some loss in affinity for FcRn but a general increase in FcγRIIIa binding activity for Tmab using PEG linkers. Further investigation is required to understand the impact on the linker conjugation to the sensitivity of target binding and effector function. The goal of ADC Express™ is to deliver research ready, ADC construct diversity sets to assist customers with the selection of appropriate candidates for clinical scale production. Working with our partners, we can provide any and all diversity concepts from varied mAb constructs to conjugation methodologies to linkers and payloads. These libraries can be used to promote confident, data-driven candidate selection at the pre-production development phase to maximize therapeutic outcomes in the clinic. Our aim is to offer a commercially viable diversity set(s) that can become the industry standard tool for pre-clinical candidate selection.

Results

The affinities of the conjugated Tmab variants for recombinant human FcγRIIIa and FcRn were determined and compared to those obtained for unconjugated TmAb (Table 2). The results indicated a general increase in FcγRIIIa binding affinity (lower K_D, higher relative K_D) for all TmAb variants, relative to the unconjugated TmAb. This could indicate the potential for increased ADCC activity, regardless of the length of linker used.

Interestingly, conjugation of linker of any length was associated with decreased affinity for FcRn binding (3.7 – 17.4 %) relative to the unconjugated TmAb. In our previous study, a general increase in FcRn binding with the PEG ADC mimics was observed, however these constructs were not conjugated to toxin. An increase in circulating half-life has often been associated with improved therapeutic efficacy for ADCs. Our results suggested a general reduction in FcRn binding activity after conjugation, but no clear correlation between the size of linker, and drug antibody ratio (DAR), and relative K_D (Figure 5). It is also possible that the effect of linker conjugation, size, and the nature of toxin combined may have ADC-specific influence of Fc region mediated binding activity.

The HER-2 binding activity will be investigated to understand the full impact of variation of linkers and DAR.

Table 2. Result summary of SPR Binding Activity of Tmab Varients.

Sample	FcγRIIIa		FcRn	
	K_D (nM)	Relative K_D (%)	K_D (nM)	Relative K_D (%)
MSQC22 (TmAb)	340	100	65.4	100
1A_P_PEG4_5X	251	135.6	75.1	87.2
1B_P_PEG4_7X	243	139.8	76.0	86.1
2A_P_PEG8_5X	246	137.9	68.2	95.9
2B_P_PEG8_7X	254	133.5	69.6	94.0
3A_P_PEG24_5X	250	135.9	76.9	96.3
3B_P_PEG24_7X	261	130.2	79.2	82.6

Figure 5. Relative Activity Summary

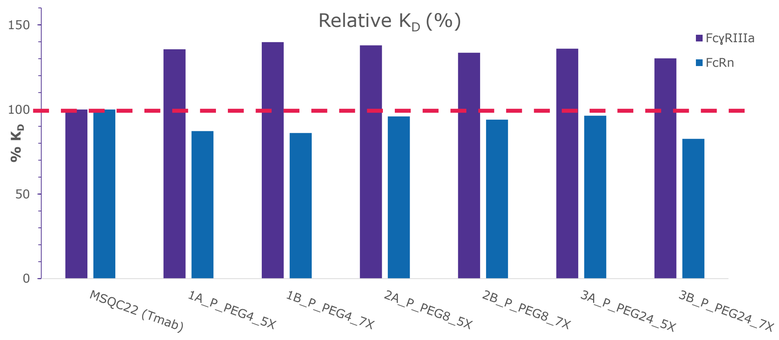
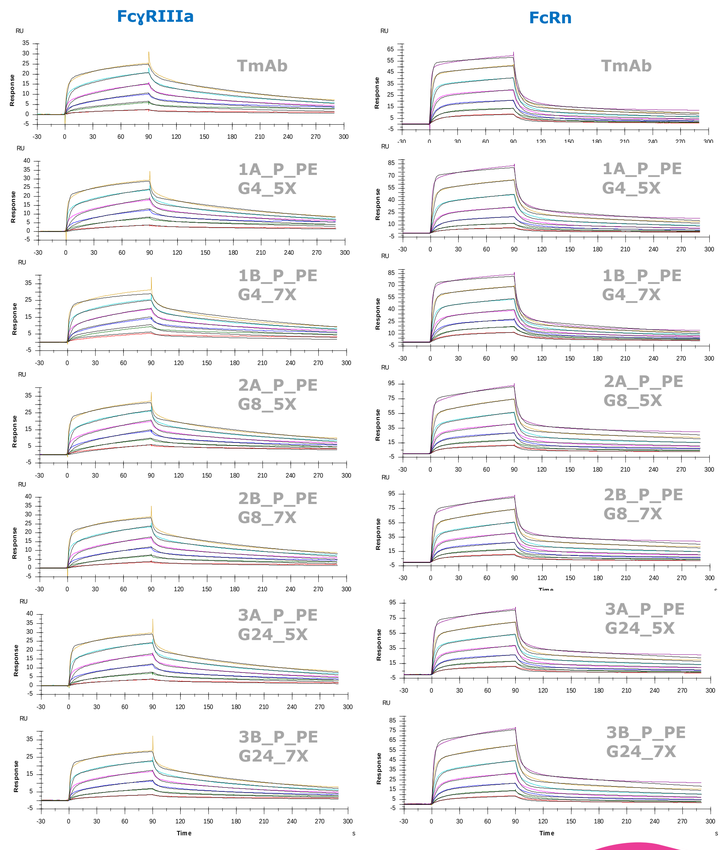


Figure 6. Senorgrams for FcγRIIIa-ADC and FcRn-ADC Interactions.



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