A template mediated regio-selective labeling method for preclinical imaging of therapeutic antibodies

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SUMMARY

Here we present a new **regio-specific conjugation method of payloads to monoclonal antibodies** based on an affinity peptide, which guides a reactive payload toward the **Fc region** of the antibody. The method was initially developed for the conjugation of small metal chelators and fluorophores of high polarity and it has been extended to the conjugation of cytotoxic payloads.

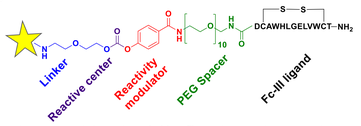

Figure 1: Schematic representation of an AbYlink™ reactive peptide derivative and its key components.

INTRODUCTION

The conjugation method exploits the affinity of the Fc-III peptide to the IgG-Fc region of antibodies. After mixing a reactive peptide with an antibody (or an ADC) at room temperature in a buffer solution at pH 9 for 2h, the **conjugation occurs spontaneously in a single reaction**. The Fc-III derived targeting moiety can then be liberated upon **acidification followed by standard purifications methods** (diafiltration or size exclusion chromatography).

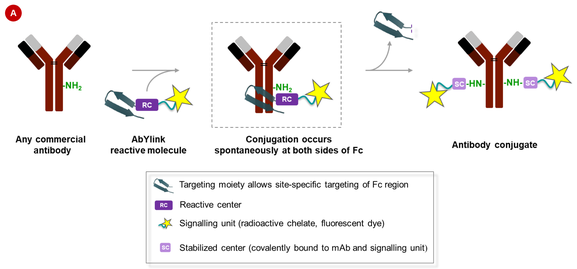

Figure 2: Schematic representation of the reactive peptide conjugation to an IgG-Fc domain

We recently published[1] our **generation 1 technology,** which delivered **moderate to good selectivity of metal chelator conjugation** to the IgG-Fc domain, mainly at K317.

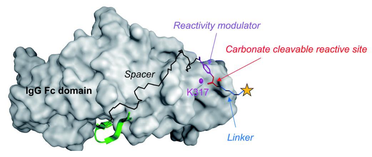

Figure 3: Schematic representation of the binding and reaction sites involved in AbYlink™ conjugation

In this case study, we applied **AbYlink™ technology** to produce [^{111}In]In-DTPA-trastuzumab for a **SPECT imaging study** in mice bearing SK-OV-3 tumors.

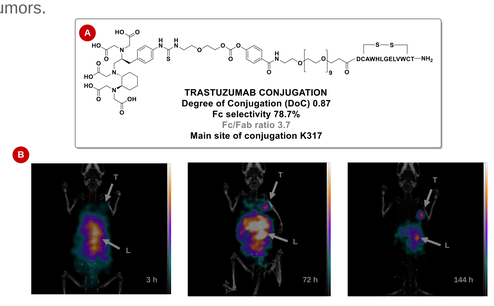

Figure 4: (A) Structure of DTPA-PEG$_{10}$-Fc-III and conjugation results to Trastuzumab (B) Coronal MIP fused SPECT and CT images at 3, 72, and 144 h post-injection of 18 MBq [111In]In-DTPA-trastuzumab in the same mouse bearing a SK-OV-3 tumour (HER2+).

METHODS

Synthesis
Reactive peptides were synthetized utilizing our reported[1] procedure.
Conjugation
A stock solution of reactive peptide (2.0 eq) in DMSO is added to a room temperature solution of antibody (5 g/L) in sodium carbonate solution at pH 9 (adjusted by addition of HCl). The reaction is stirred at room temperature for 2h before quenching by acidification to pH 4 with concentrated glycine buffer.
Purification
Quenched reaction mixture are loaded onto PF10 column pre-equilibrated with glycine buffer. Elution is carried according to manufacturer instructions. Eluates are collected in tubes containing neutralizing buffer (0.5M phosphate buffer pH 8.5). Collected fraction are buffer exchanged in Amicon 50 kDa centrifugation unit using PBS pH 7.4.
SEC Analysis
Aggregate content was controlled by SEC analysis using a Yarra 3000 column. All conjugates displayed a monomeric purity above 95%.
DoC determination by MS
The antibody conjugates were deglycosylated with Endo S enzyme prior to LC-FTMS experiments which were performed on a QExactive HF-HT-Orbitrap-FT-MS instrument. The average DoC values were calculated using the intact mass LC-FTMS data.
Fc/Fab selectivity loading determination by MS
Cleavage of antibody conjugates into Fab and Fc fragments was achieved by digestion with GingisKHAN or FabRICATOR proteases from Genovis prior to LC-FTMS experiments. The Fc/Fab selectivity was calculated as a ratio of DoC (Fc) to DoC (F(ab')$_2$) based on the MS data.
Reaction site (lysine) determination by middle-down analysis
The FabRICATOR cleaved samples were analyzed by middle-down mass spectrometry (LC-FT-MS/MS, conjugation site localization) by separation onto a C4 column followed by elution of proteoforms on a QExactive HF-HT-Orbitrap-FT-MS instrument.

RESULTS

With encouraging conjugations and imaging results obtained with our generation 1 peptide, further work was undertaken to optimize the technology. The aim was to improve the Fc/Fab selectivity by **targeting K248 instead of K317**. Indeed, **K248 is positioned inside the antibody binding pocket** targeted by Fc-III. Rational design allowed to better position the reactive center (RC) at the vicinity of K248.

Figure 5: Schematic representation of the improvement of AbYlink™ reactive peptides

Guided by modelization, several attachment possibilities were tested in conjugation reactions. The ideal attachment point gave excellent **conjugation results with FITC.**

Conjugation method	mAb	DoC	Selectivity Fc/Fab	
AbYlink™ Conjugation *with Optimized FITC-carbonate-Fc-III*	Trastuzumab	1.87	>99.5%	Excellent
Random Lysine Conjugation *with FITC*	Trastuzumab	3.68	45.1%	No

Table 1: Comparison of FITC conjugation to Trastuzumab with AbYlink™ to a random conjugation approach

FITC conjugation with Abylink™ was also applied to T-DM1 (Kadcyla) to demonstrate that an **ADC can be labeled using AbYlink™ technology.**

Conjugation method	mAb	DoC	Selectivity Fc/Fab	
AbYlink™ Conjugation *with Optimized FITC-carbonate-Fc-III*	T-DM1 (Kadcyla)	1.87	93.7%	Excellent
Random Lysine Conjugation *with FITC*	T-DM1 (Kadcyla)	3.68	44.4%	No

Table 2: Comparison of FITC conjugation to T-DM1 with AbYlink™ to a random conjugation approach

The main interest of a selective conjugation to the Fc region resides in the **absence of labeling near the epitope in order to avoid the alteration of the the binding** to cells targeted by the antibody. Competitive binding experiments at the surface of BT-474 cells with Trastuzumab-FITC or Kadcyla-FITC labeled **using AbYlink™ retained higher binding affinity** compared to the corresponding conjugates obtained with random labeling.

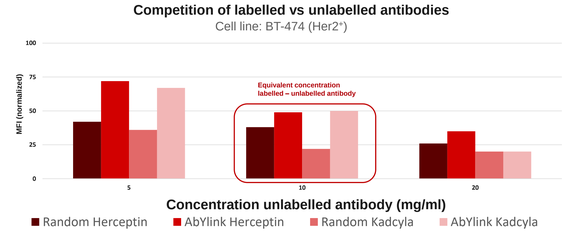

Figure 4: Competitive binding between FITC conjugates and naked Trastuzumab at 3 concentrations of competitor (trastuzumab).

The conjugation **was succesfully tested with a variety of metal chelators**, which are ideally suited for the chelations of radiometals compatible with **PET or SPECT imaging**.

Chelator	mAb	DoC	Selectivity Fc/Fab	
DOTA	Trastuzumab	1.87	97.6%	Excellent
CHX-DTPA		2.05	97.2%	
NODAGA		1.81	>99.5%	

Table 3: AbYlink™ conjugation of various chelators to Trastuzumab

Our conjugation method was also succesfully tested with different IgG formats. The labeling procedure is **compatible with naked antibodies, fusion proteins and ADCs derived form IgG1, IgG2 and IgG4**.

Chelator	mAb	DoC	Selectivity Fc/Fab	
DOTA	Trastuzumab (IgG1)	1.87	97.6%	Excellent
	Atezolizumab (IgG1)	2.12	97.2%	
	Panitumumab (IgG2)	2.13	99.4%	
	Pembrolizumab (IgG4)	1.90	98.8%	
	Fc only	1.90	-	

Table 4: Conjugation of DOTA to various IgG subclasses

AbYlink™ can also be optimized for the **conjugation of payload-linker systems.** Good results were obtained for the conjugation of Val-Cit-PAB-MMAE linkers. More lipophilic constructions such as maytansinoïd containing linkers could also be conjugated with a slightly reduced DoC and Fc/Fab selectivity.

Payload	mAb	DoC	Selectivity Fc/Fab	
VC-PAB-MMAE	Trastuzumab	1.91	97.0%	Excellent
SPDB-DM4		1.49	94.7%	

Table 5: Conjugation of various payloads to Trastuzumab

CONCLUSION

Here, we have developed a generic technology to label any antibody with a **radionuclide chelator in a single step chemistry**. Through engineering the Fc-III peptide ligand with a carbonate reactive site, a chelator or linker-cytotoxic payload, we have shown that the resulting pre-activated Fc-III conjugate is capable of efficiently and selectively labelling different IgGs with the concomitant release of Fc-III. **Successful labeling was achieved with several dyes, metal chelators and cytotoxic payloads**. We demonstrated that our conjugation leads to **homogeneous labeling of antibodies with a DoC close to 2 in a regiospecific manner**. This will greatly facilitate the analytics required by the regulatory authorities to approve GMP production of the API for clinical applications.

In comparison to the many other site-specific conjugation chemistries that have been developed to date, we believe that our one step Fc-targeted labeling approach is a major advancement in the field because (i) it requires no prior purification or modification of the antibody, (ii) the chemical reaction occurs spontaneously upon mixing the antibody with the Fc-III-reactive conjugate and is completed within 2 hours, and (iii) there is no need of any additional chemical step.

NEXT STEPS

Given that Fc-III binds to any antibody isotypes in a region that is not involved in antigen binding, we believe that this will greatly facilitate bioconjugation, since any chelators, and as a matter of fact many payloads, can be chemically pre-activated with Fc-III. **This paves the way to a broad array of applications in antibody engineering such as diagnostics, ADCs, Fc-fusion proteins, and so on.** Other important perspectives of this technology may be the *in vivo* non-invasive determination of tissue distribution, tumor accumulation and retention, and target engagement of ADCs.

REFERENCES

(1) Postupalenko et al., Chem. Sci., 2022, 13, 3965–3976

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FEATURES OF ABYLINK™

AbYlink™ is a new proprietary technology to rapidly, selectively and covalently label any off-the-shelf antibody in a single step for diagnostic purposes and ADC preparation

1. **Defined and invariable conjugation sites with no impact on antigen-binding regions**
2. **Homogenous antibody conjugation**
3. **Conjugation process completed in less than 2 hours**
4. **No prior antibody purification or modification necessary**
5. **Compatible with most imaging strategies (radiolabeled, fluorescent)**
6. **Compatible with all IgG formats**
7. **GMP-compatible technology**

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