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“Refining the quality of monoclonal antibodies: grafting unique peptide-binding site in the Fab framework”

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Abstract

Monoclonal antibodies (mAbs) are a major therapeutic modality. Grafting the mediotope binding site onto mAbs, also known as mediotope-enabling, can extend the usefulness of mAbs by providing an additional protein-protein interaction surface without altering the stability or antigen binding. We have previously used this site for attaching dyes, cytotoxic drugs, and entire proteins. Here, we provide a simple protocol for mediotope-enabling mAbs, and verifying mediotope and antigen binding using flow cytometry (FACS).

Keywords

Mediotope; antibody-drug conjugate; bispecific antibody

1. Introduction

Antibodies are the largest class of therapeutic molecules and one of the most important tools in the biosciences[1]. Their utility can be extended through the addition of new proteins or small molecules, such as dyes[2,3]. One technology for such additions is the mediotope platform[4]. The mediotope is a 12 amino acid, cyclic peptide that binds to the cavity between the light and heavy chains of cetuximab. The binding site is unique to cetuximab, and completely absent from human antibodies[4]. However, we have successfully grafted the binding site onto several human monoclonal antibodies, including trastuzumab[4,5]. Once engineered, mediotope binding site acts effectively as a unique and highly specific but innocuous receptor site on the mAb. As such, there are multiple applications made available with this technology[6,7].

For instance, it is possible to use the technology for pre-targeted imaging. Here, mediotope-enabled mAbs targeting a specific antigen is administered to the patient. Over time, the mAb accumulates at the site of disease and clears the blood stream (typically two days). At that point, it is possible to add the mediotope functionalized with DOTA and a radionuclide. The radiolabeled mediotope can rapidly accumulate at the disease site decorated with mediotope-enabled mAb. Unbound material is rapidly filtered and excreted. Imaging of the disease can occur as soon as 2 hours. Two significant advantages to this approach are: 1) the

radionuclide undergoes fewer half-lives, thus there is more signal and 2) as the radio-labeled mediotope is rapidly cleared, there is less background as well as less radiation exposure for the patient. The increase in signal and the decrease in background significantly improves the overall image quality. Initial studies using the mediotope for pre-targeted imaging are very promising. Other applications include drug delivery, clustering through multivalent mediotopes, and the creation of multispecific immune engagers.

Herein, we provide a detailed approach to mediotope-enable and characterize any antibody, give the sequence is known.

2. Materials

2.1 Grafting

1. Sequence Alignment Software

2.2 Verification

1. Alexa647-Ac-CQFDLSTRRLQCGGSK mediotope (*see* Note 1)
2. Dye labeled secondary antibody for flow cytometry (*see* Note 2)
3. A flow cytometer capable of two color detection (*see* Note 3)
4. Washing Buffer (1x PSB with 1% BSA)

3. Methods

3.1 Grafting

1. Locate the amino acid sequence of the antibody of interest (*see* Note 4).
2. Download the fasta sequence for cetuximab (PDB ID 4gw1, DOI: [10.2210/pdb4GW1/pdb](https://doi.org/10.2210/pdb4GW1/pdb)), mediotope-enabled trastuzumab (PDB ID 4hjb, DOI: [10.2210/pdb4HJG/pdb](https://doi.org/10.2210/pdb4HJG/pdb)), and parental trastuzumab (PDB ID 4hkz, DOI: [10.2210/pdb4HKZ/pdb](https://doi.org/10.2210/pdb4HKZ/pdb)) from <https://www.rcsb.org/>. For all PDBs, the light chain is chain A, and the heavy chain is chain B.
3. In your alignment software, import the light chains from your antibody of interest, cetuximab, mediotope-enabled trastuzumab, and parental trastuzumab. *See* Fig. 1.
4. Align the sequences using clustalW.
5. Locate and substitute the following positions in your antibody of interest:
6. Export your mediotope-enabled light chain.
7. In your alignment software, import the heavy chains from your antibody of interest, cetuximab, mediotope-enabled trastuzumab, and trastuzumab.
8. Align the sequences using clustalW.
9. Locate and substitute the following positions in your antibody of interest:

10. Export your meditope-enabled heavy chain.
11. Synthesize codon-optimized DNA with substituted amino acid sequences or introduce substitutions into existing vector through mutagenesis (*see* Note 6).

3.2 Verification of binding by FACS

1. 5×10^6 cells expressing the target antigen are grown prior to FACS (*see* Note 7).
2. On the day of FACS, 5×10^6 cells are brought to final volume of 1 mL in washing buffer. Cells are then washed by centrifuging at 300 g for 5 minutes, removing the supernatant, and resuspending with fresh washing buffer. This wash is repeated twice.
3. Split the cells into 5 tubes of 200 microliters, corresponding to 1×10^6 cells per tube. Label the tubes 1) parental antibody, 2) meditope-enabled antibody (memAb), 3) meditope alone, 4) secondary alone, and 5) untreated.
4. To the parental antibody sample, add the original parental antibody to a final concentration of 100 nM. To the memAb sample, add the memAb to a final concentration of 100 nM. The other three samples are not treated at this time.
5. Incubate all samples on ice for 30 minutes.
6. Wash the samples with washing buffer as above to remove unbound antibodies.
7. Add the fluorescently labeled secondary antibody, according the manufacturer's guidelines, to the parental, memAb, and secondary alone control samples.
8. Add the fluorescently labeled meditope to a final concentration of 50 nM to the memAb and meditope control samples.
9. Incubate all samples on ice for 30 minutes.
10. Wash the samples with washing buffer as above to remove unbound meditope and antibodies.
11. Perform flow cytometry on all five samples recording the fluorescence channels for both the meditope and secondary antibody dyes.
12. The parental mAb should show binding to the target cells. The memAb sample should show the same binding to the target cells as the parental mAb when comparing labeling using the secondary antibody. The memAb sample should also show signal from the meditope, indicating meditope binding. The control samples (secondary alone, meditope alone, and untreated) should not show any labeling. Weak non-specific binding is sometimes observed from the Alexa647-meditope. *See* Fig. 2.

4. Notes

Note 1. We normally use N-terminally acetylated CQFDLSTRRLQCGGSK as a starting peptide, and conjugate Alexa647 to the terminal lysine through NHS chemistry (e.g. Cat. # A37573, ThermoFisher). This can readily be purchased from peptide synthesis companies.

Note 2. We use F(ab')₂-Goat anti-Human IgG Fc Secondary Antibody, Alexa Fluor 488 (Cat. # H10120, ThermoFisher). Depending on your species and Fc, you will need to pick an appropriate secondary.

Note 3. The dye combination for the meditope and secondary must be compatible for two color FACS on your FACS machine. Alternative dyes can be used on either the meditope or secondary. We prefer to keep Alexa647 on the meditope as we have seen less non-specific binding with this dye compared to Alexa488.

Note 4. This procedure has been successfully tested on κ -LC antibodies based on 4d5 (trastuzumab) constant region framework.

Note 5. In our original memAb grafting, we replaced position 83 with isoleucine. After additional optimization, we have switched position 83 to glutamic acid. The affinity improves from 1000 nM to 20 nM with this substitution[8].

Note 6. The affinity for meditope in meditope-enabled antibodies varies slightly from antibody to antibody[5].

Note 7. We recommend FACS as an initial verification for meditope and antigen binding. It is generally relatively easy to find a cell line expressing the target antigen. We recommend further verification of memAb properties using size exclusion chromatography and surface plasmon resonance (SPR). We routinely mix memAb with equimolar concentrations Alexa647-meditope, and run the mixture on a size-exclusion column. If the meditope binds, it will co-elute with the memAb. If the antigen is available or can be produced, we will test antigen binding using SPR. To date, every memAb tested has had indistinguishable binding from the parental mAb.

5. References

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Fig. 2.

FACS binding analysis of memAb. By comparing the binding of the Alexa488, anti-Fc secondary between the parental mAb and memAb, we can see that antigen binding is not altered by mediotope-enabling. From Alexa647-meditope sample, we can see that only in the presence of the memAb will the mediotope bind to the target cells, indicating specific mediotope-memAb interactions. Slight non-specific binding the Alexa647-meditope to the cells is observed, likely from the sticky nature of Alexa647. In the above experiment, the parental mAb is trastuzumab and memAb is me-trastuzumab.

Table 1:

Light Chain mutations

Trastuzumab number	Substitution
LC S9	I
LC S10	L
LC K39	R
LC P40	T
LC G41	N
LC K42	G
LC A43	S
LC K45	R
LC F83	E (<i>see Note 5</i>)
LC T85	D
LC Q100	A

Table 2:

Heavy Chain mutations

Trastuzumab number	Substitution
HC A40	S
HC V93	I

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