Site-Specific Labeling of Antibodies for MicroScale Thermophoresis

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Abstract

Site-specific conjugation of a dye to antibodies is useful for producing highly homogeneous material for use in downstream affinity experiments. This application note describes a labeling procedure that combines the selective reduction of antibody interchain disulfides and their labeling with maleimide-based chemistry. Different antibodies conjugated by this approach yield high-quality MST affinity data for interactions with both antigens as well as Fc receptors.

Introduction

Determining the affinity of an antibody to its antigen, a carrier protein or an Fc receptor with MicroScale Thermophoresis (MST) requires the labeling of one binding partner with a fluorophore. Working with fluorescently-labeled antibody provides several advantages for the subsequent binding assays, especially if many different ligands (such as antigens and Fc receptors) are being tested for binding to an antibody, as is often performed during the development of biotherapeutics. The antigen, carrier protein or Fc receptor can be labeled using either conventional NHS- or maleimide-based labeling on lysine or cysteine residues, or site-specific His-tag labeling. However, labeling of antibodies requires greater planning regarding the protocol used and the position of dye conjugation. Most monoclonal antibodies (mAbs) do not contain free cysteine residues, which precludes classical maleimide labeling. Labeling of the numerous lysine residues, which are predominantly found in the Fc chain of antibodies, can result in a heterogeneously-labeled sample, with potential for interference with Fc receptor binding. Thus, the site-specific conjugation of fluorophores to antibodies is highly desired, as it allows for precise control over conjugation location and yields a more homogeneous material.

The approach described here is based on methods used for the production of antibody drug conjugates (ADCs) like Brentuximab vedotin (an FDA-approved ADC).1–3 This labeling procedure involves the selective reduction of the solvent-exposed disulfide bond with dithiothreitol (DDT) or tris(2-carboxyethyl) phosphine (TCEP), followed by modification of the resulting thiols with maleimide-containing drugs.

Figure 1: After reduction of the interchain disulfide bond with TCEP, NT-647-maleimide was attached to the now-accessible cysteine residues. Any of the interchain disulfides can be reduced and labeled.
This labeling strategy does not involve lysines or carbohydrates in the Fc segments of the antibody, which are important for interactions with Fc receptors; therefore, it is highly recommended for investigating interactions between antibodies and Fc receptors as shown here for the therapeutic mAbs, Herceptin (Trastuzumab) and Humira (Adalimumab). Overall, with this labeling method one can reliably measure the interactions of the Fc segment or the Fab domains of labeled antibodies with their respective binding partners/antigens.

Results and Discussion

Selective reduction of interchain disulfides of antibodies followed by the conjugation of free cysteines by NT-647-maleimide was performed as described in the Antibody Labeling Kit RED-maleimide (Cat. Nr. L007). TCEP and the dye were added to the antibody in the 5-fold molar excess. Labeled antibody was kept on ice during experimentation or at 4°C for 2-3 days. The affinities of the antibodies to Fc receptors and antigens were measured as described below.

As shown for the therapeutic mAbs Humira and Herceptin, gentle reduction of interchain disulfides induced only minor changes in the thermal unfolding profile of the antibodies, and thus did not significantly compromise their structural integrities (Figure 2).

Figure 2. Thermal stability of reduced and labeled (in blue) (A) Humira in comparison with the unlabeled antibody (in turquoise); and (B) Herceptin as determined by nanoDSF. The antibodies remain structurally intact after reduction and labeling.

**Binding of Fc receptors**

The affinity of FcγRIIA/CD32a and FcγRIIIA/CD16a for Humira and Herceptin was determined by MST in PBS, pH 7.4, supplemented with 0.05% Tween 20. The interaction between the FcRn receptor and the two mAbs was determined in PBS, pH 6.0, supplemented with 0.5% Tween 20. The binding of FcRn is strongly pH dependent; this receptor binds with high affinity to IgGs at pH 6.0 – 6.5, but shows no detectable binding at pH 7.5.4 The concentration of labeled antibody used in all assays was 1 nM.

The affinity of FcγRIIIA/CD16a for Humira was determined to be 8.58 ± 1.60 nM (Figure 3), which is higher than the affinity measured by SPR (447 nM)5, where FcγRIIIA/CD16a was immobilized on a sensor chip. For FcγRIIIA/CD32a, an affinity of 0.33 ± 0.04 nM was determined. As expected, the FcRn receptor showed no detectable binding to Humira at pH 7.4. The affinity of FcRn for
Humira was $6.61 \pm 1.61$ nM at pH 6.0, which corresponds to published values.\textsuperscript{6}

The affinity of Herceptin to FcγRIIIA/CD16a was estimated at $29.02 \pm 7.91$ nM (Figure 4), which is higher than the reported affinity (252 nM, SPR).\textsuperscript{7}

For FcγRIIA/CD32a, an affinity of $0.52 \pm 0.28$ nM was determined. As expected, the FcRn receptor showed no detectable binding to Herceptin at pH 7.4. The affinity of FcRn for Herceptin was $17.50 \pm 5.80$ nM at pH 6.0, which is in accordance with published values.\textsuperscript{7}

\textbf{Figure 3:} Binding of (A) Fcγ RII/CD32, (B) Fcγ RIIIA/CD16 and (C) FcRn to Humira by MST. The resulting dose-response curves were fitted to a one-site binding model to extract $K_d$ values. MST experiments were performed at LED 20\% and high MST power.

\textbf{Figure 4:} Binding of (A) Fcγ RIIIA/CD16, (B) Fcγ RII/CD32 and (C) FcRn to Herceptin by MST. The resulting dose-response curves were fitted to a one-site binding model to extract $K_d$ values. MST experiments were performed at LED 20\% and high MST power.
Antigen binding

The affinity of TNFα (tumor necrosis factor alpha) for Humira and the affinity of Her2 (human epidermal growth factor receptor 2) for Herceptin was determined in PBS supplemented with 0.05% Tween 20. The binding of TNFα to Humira induced significant changes in fluorescence. Thus, the raw fluorescence was used to determine the affinity of TNFα for Humira, which yielded a $K_d$ value of $6.85 \pm 1.50$ nM. The literature reports affinities between 8.6 pM to 483 pM for this interaction. The affinity of Her2 for Herceptin was 0.98 ±0.23 nM, which is in the range of values reported in the literature (from 0.1 nM to 5 nM).

Figure 5: The binding of (A) TNFα to Humira and (B) Her2 to Herceptin. For the binding of TNFα to Humira, significant ligand-induced fluorescence changes were observed, thus the change in fluorescence was used to determine the $K_d$ value. The resulting dose-response curves were fitted to a one-site binding model to extract $K_d$ values. MST experiments were performed at LED 20% and high MST power.

Conclusions

Selective reduction of intrachain disulfides and subsequent conjugation with the NT647-maleimide dye provides an excellent approach for efficient labeling of antibodies for MST binding assays. Labeled antibodies can be used to determine the affinities of various binding partners, irrespective of their binding site (Fc or Fab segments) with the same experimental settings. This greatly facilitates antibody interaction analysis and circumvents potential shortcomings of conventional labeling and immobilization approaches for antibodies, such as modifying amino acid residues at the interaction interfaces.

Materials and Methods

Selective reduction and labeling of antibodies

The antibodies were labeled according to the protocol provided in the Monolith®-NT-Antibody Labeling Kit RED-maleimide (L007). Briefly, the antibodies were diluted to a concentration of 2 µM in the Reduction and Labeling (RL) buffer supplemented with 10 µM TCEP and incubated at 37°C for 2 hours. After this, 10 µM of NT647-maleimide was added to the mixture and incubated on ice for 30 min. Afterwards the free dye was removed from the labeled antibody with the Column B provided with the Antibody Labeling Kit.
Phosphate buffered saline (PBS) was used as the elution buffer. For the MST experiments 1 nM of labeled antibody was used.

nanoDSF experiments

Thermal unfolding experiments of labeled and unlabeled antibodies were conducted using the NanoTemper Technologies Prometheus NT.48 instrument and PBS as the assay buffer. The samples (10 μL) were loaded into High Sensitivity Prometheus NT.48 Capillaries, nanoDSF-grade. To determine the unfolding transition points, the melting curves were obtained at a temperature ramp of 1°C/min from 20°C to 95°C and the shift of intrinsic tryptophan fluorescence at emission wavelengths of 330 nm and 350 nm was monitored. The data were acquired and analyzed using the PR.ThermControl software.

MST binding experiments

The interactions between the antibodies and their binding partners were measured in Monolith® NT.115 Standard Treated Capillaries. The measurements were performed in phosphate buffered saline supplemented with 0.05% Tween-20 (PBS-T). Before the MST measurements, samples were centrifuged for 10 min at 4°C and 14000 g. The ligands for the binding studies were dissolved in PBS-T at double the concentration indicated in the figures. The measurements were performed on a NanoTemper Technologies Monolith® NT.115Pico instrument. A final antibody concentration of 1 nM yielded fluorescence intensities of around 6000 counts at an LED power of 20 %. The samples were measured at high MST power with an MST-on time of 20 s. The data were analyzed using MO.Affinity Analysis Software.

References

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