

Fast and accurate evaluation of oxidation-induced destabilization of mAbs

Mariam Mohamadi, Nuška Tschammer and Dennis Breitsprecher

NanoTemper Technologies GmbH, Floessergasse 4, 81369 Munich

Abstract

The applicability of monoclonal antibodies (mAbs) in therapeutic research continues to rise — they now account for almost 50% of protein-based drugs.¹ Monitoring their quality as well as binding properties are critical as these parameters provide insight into mAb functionality and efficacy as potential drugs.

Here we study trastuzumab, also known as the commercial drug Herceptin®, a monoclonal antibody that has been used to successfully treat patients with certain forms of breast cancer. Trastuzumab acts by binding to and interfering with the HER2/neu receptor in cancer patients. Using two complementary technologies, we examine how targeted oxidation affects trastuzumab structure and therefore its binding capabilities to protein A. First, a rapid analysis of the mAb preparation quality was performed using the Tycho™ NT.6 system. The same samples were then run on the Monolith® NT.115^{Pico} system to analyze how oxidation compromises mAb interactions. Stability directly translated into binding affinity, showcasing that the Tycho NT.6 affords researchers a fast and accurate characterization of trastuzumab sample quality

Introduction

Since the introduction of the first commercially available therapeutic antibody in 1986, focused efforts in industry labs to develop and optimize antibody-based therapeutic drugs have continued to expand. They now account for almost 50% of protein-based drugs¹ and a majority are used to treat diseases such as cancer and autoimmune disorders, as well as being used for treatment in cases of transplant

rejection.² For protein production researchers working on these antibody-based therapeutic drugs, optimizing manufacturing and storage conditions is a critical step in their process development.

All proteins, including antibodies, are susceptible to chemical and physical destabilization processes if improperly handled or stored.

Some of these degradation processes lead to the loss of biological activity or efficacy and even potentially cause adverse immunogenic reactions when administered in patients.³ Similarly, antibodies used in basic research as well as diagnostics studies can be rendered unreliable due to degradative processes. It has been shown that degradation at the antigen binding site as well as in the Fc region impact binding affinity towards the antigen and the FC-receptor, and therefore influence antibody function.^{3,4}

One step of the chemical degradation pathway is the oxidation of methionine (Met) residues in the constant region of antibodies. Met residues are readily oxidized, and it has been reported that Met oxidation affects the antibody's affinity or interaction with the neonatal Fc receptor and Fcγ receptor,⁵ as well as the binding to protein A⁶, by destabilizing the antibody.

A fast methodology to evaluate degradation and assess antibody functionality as a consequence of oxidation effects would enable protein production researchers to develop better antibody-based applications.

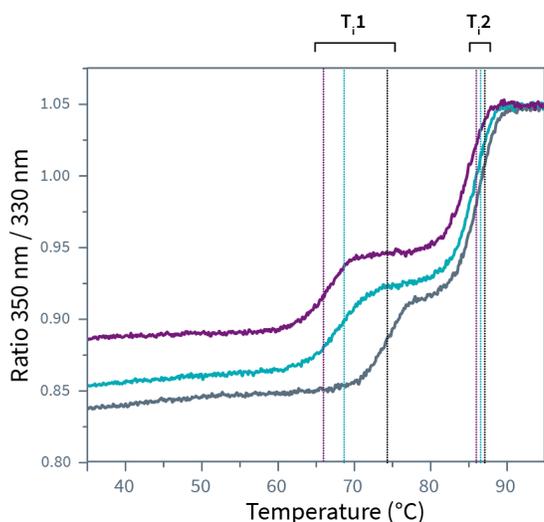
Trastuzumab, also known as the commercial drug Herceptin, is a therapeutic monoclonal antibody that has been used to successfully treat patients with certain forms of breast cancer for almost 20 years. Specifically, trastuzumab acts by binding to and interfering with the HER2/neu receptor in cancer patients. Having the ability to quickly monitor relative stability and functionality of a protein will result in better antibody development.

In this study, we evaluate the effects of oxidative stress on the thermal unfolding profile of the humanized monoclonal antibody IgG1_κ trastuzumab using the Tycho NT.6 system. We show that trends in the unfolding profile quickly determined by the Tycho NT.6 correlate with the antibody's capability to bind to protein A, as seen using the Monolith NT.115^{Pico} system. The results provide insight into the effectiveness of this mAb to act as a therapeutic binding protein once degradation due to improper storage and handling has occurred.

Results and Discussion

Quick analysis of oxidized samples shows differences in unfolding profiles

To compare different oxidation levels of a test antibody, recombinant mAb trastuzumab was exposed to 0.3 % H₂O₂ for 3 and 18 hours, as described previously.^{6,7} Samples oxidized for different time periods were analyzed for thermal unfolding using the Tycho NT.6 (Figure 1).



	Initial ratio	T _{1,1} (°C)	T _{1,2} (°C)
Native	0.837	74.2	87.4
3 h oxidation	0.853	68.0	86.9
18 h oxidation	0.885	66.5	86.3

Figure 1: H₂O₂-triggered oxidation for 3 and 18 hours on trastuzumab results in different folding profiles as measured on the Tycho NT.6 system.

Initial ratio is the ratio of the 350 nm/330 nm fluorescence signal detected at the start of the experiment. Inflection temperature (T) is the transition point along the curve which represents an unfolding transition of the sample due to thermal treatment. Two inflection temperatures of 74.2 °C and 87.4 °C were detected for the native protein. Tycho NT.6 analysis of the 3- and 18-hour oxidation treatments showed a lower T_{1,1} for both (68 °C and 66.5 °C respectively) as compared to the native protein.

Oxidation has two marked effects on the unfolding profile of trastuzumab. Firstly, the unfolding profile changes significantly with prolonged incubation under

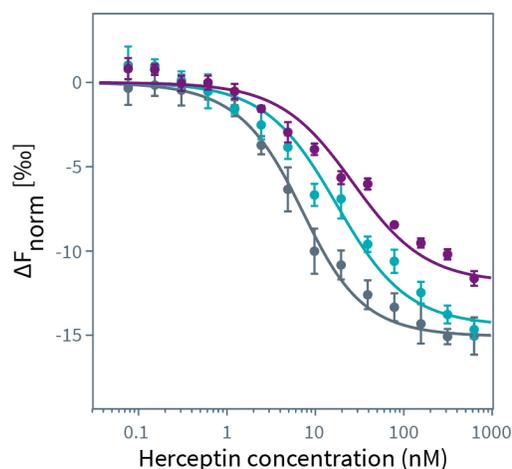
oxidative conditions. This is indicated by the shift towards higher values of the initial ratio of detected fluorescence signal. Secondly, a left-shift in the T_i values is correlated to the length of oxidation treatment. The shift in T_i is larger for the first unfolding event, suggesting that oxidation causes major structural changes particularly in the corresponding domain.

A T_i value is the inflection point along the curve which represents an unfolding event due to thermal treatment.

In IgG antibodies, the C^H2 domain is usually the first to unfold, followed by the Fab and C^H3 domains as temperature increases.⁸ Results from the Tycho NT.6 would suggest that oxidation primarily acts on the C^H2 domain.

Binding affinity is influenced by oxidation treatment

Since protein A is known to bind IgGs between the C_H2 and C_H3 domains,⁹ it was used in a binding affinity experiment to confirm that the C_H2 domain of trastuzumab has been affected by oxidation. Binding affinities were identified by using MicroScale Thermophoresis (MST) performed on the Monolith NT.115^{Pico} system. A dose response curve was generated by titrating the concentration of trastuzumab in the presence of a set concentration of protein A (Figure 2). The resulting K_d values show that the higher the oxidation level of trastuzumab, the lower its affinity to protein A. Thus, trastuzumab oxidation, and the resulting destabilization, directly correlated with a decrease in the antibody's ability to bind protein A.



	K_d (nM)
Native	1.4
3 h oxidation	4.7
18 h oxidation	11.7

Figure 2: Oxidation treatment of trastuzumab influences its affinity for binding to protein A.

Dose-response curves for the interaction of trastuzumab with fluorescently labeled protein A was generated using MicroScale Thermophoresis (MST) run on the Monolith NT.115^{Pico} system. Error bars indicate standard deviation from triplicate measurements. The dissociation constant (K_d) is a measure of the concentration of a ligand where the binding site on a particular protein is half occupied. Oxidation of the mAb resulted in reduced K_d values as compared to the native sample.

In summary, trastuzumab's unfolding profile is influenced by oxidation treatment, which also translates into reduced binding capabilities (Table 1).

	Initial ratio	T_{i1} (°C)	T_{i2} (°C)	K_d (nM)
native	0.837	74.2	87.4	1.4
3 h oxidation	0.853	68.0	86.9	4.7
18 h oxidation	0.885	66.5	86.3	11.7

Table 1: Summary of key parameters from Tycho NT.6 experiment.

Using the Tycho NT.6 to rapidly monitor the unfolding profile of an antibody (or any kind of protein) can provide information on the functionality of the target protein and can also be used to identify critical steps in the downstream processing workflow.

Following up this analysis using the Monolith NT.115^{Pico} system provided detailed confirmation of the influences of oxidation on antibody interactions. Having the tools to rapidly and accurately monitor and discern the unfolding characteristics of an antibody when subjected to external challenges such as oxidation, will enable researchers to gain a better understanding of the functionality of their targets and may ultimately accelerate development of better therapeutics.

Materials and Methods

Forced antibody oxidation

The recombinant monoclonal antibody trastuzumab was diluted with phosphate buffered saline (PBS) to 15 mg/mL. To trigger artificial oxidation, the sample contained hydrogen peroxide (H_2O_2) at a final concentration of 0.3% and incubated at room temperature in the dark for 3 and 18 hours. The native antibody sample contained 15 mg/mL of antibody in PBS without addition of H_2O_2 and was also incubated at room temperature in the dark for 3 and 18 hours. The reaction was terminated by buffer exchange with cooled (4 °C) PBS supplemented with 0.05%

Tween-20. Up to three buffer exchange steps were carried out with protein desalting columns to remove excess hydrogen peroxide.

Tycho NT.6 experiments

The recovered antibody samples, after 3 and 18 hour incubations with hydrogen peroxide, were diluted with PBS supplemented with 0.05% Tween-20 to a concentration of 1 mg/mL for Tycho NT.6 experiments. Samples were loaded as duplicates into Tycho NT.6 Capillaries (NanoTemper Technologies, Cat# TY-C001) and thermal unfolding profiles of native and oxidized trastuzumab were recorded. Temperature inflection values (T_i) were obtained by automated data analysis.

Monolith NT.115Pico experiments

1 mg/mL of protein A (Thermo Fisher Scientific) was labeled with the fluorescent dye RED-NHS 2nd Generation according to the the Monolith NT Protein Labeling Kit RED-NHS protocol (NanoTemper Technologies, Cat# MO-L001). Subsequently, interaction of labeled protein A to native and oxidized trastuzumab was measured in PBS supplemented with 0.05% Tween-20. The concentration of labeled protein A was constant at 5 nM, and trastuzumab was added in a serial dilution at final concentrations between 500 nM and 30 pM. Samples were loaded into Monolith NT.115 Premium Capillaries (NanoTemper Technologies, Cat# MO-K025) and measured on a Monolith NT.115^{Pico} system using 5% excitation power and high MST power to determine K_d values. Data was analyzed using MO.Affinity Analysis software version 2.2.4.

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