

Therapeutic Antibody Drug Monitoring Assay Development Utilizing Nanopore Optical Interferometry

Summary

The SKi Pro System was utilized to develop assays for characterizing therapeutic antibodies Alemtuzumab (Campath[®]) and Rituximab (Rituxan[®], MabThera[®]). Peptides specific for the whole molecule antibodies and their Fab derivatives were selected by phage display and utilized to characterize antibody binding in several real time assay formats. Kinetic characterization of both whole molecule and Fab fragments demonstrated an avidity effect of the whole molecule to the peptide antigen.

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Introduction

Alemtuzumab (Campath) is a humanized monoclonal antibody that targets the CD52 antigen, which is expressed on the surface of normal and malignant lymphocytes. Administration of alemtuzumab results in rapid clearance of circulating normal and malignant T and B lymphocytes. Alemtuzumab is indicated as a single agent for the treatment of B-cell chronic lymphocytic leukemia (B-CLL). When given as treatment for refractory B-cell chronic lymphocytic leukemia (CLL), the overall response (OR) rates are 33–40%, while, in untreated patients, the OR rates are 87–89%. Other malignancies that express CD52 are potential targets for alemtuzumab therapy, including acute lymphocytic leukemia (ALL), hairy cell leukemia, and Waldenström's

macroglobulinemia. Viral, bacterial and fungal infectious complications are frequently encountered with patients receiving Alemtuzumab therapy due to the depletion of normal immune cells. [1]

Rituximab is a chimeric IgG1 monoclonal antibody that specifically targets the CD20 surface antigen expressed on normal and neoplastic B lymphoid cells. Rituximab binds to CD20 and is believed to work with the body's own immune system to attack and kill the CD20-positive, B-cells. Rituximab is currently used in the treatment of both follicular and aggressive B cell non-Hodgkin's lymphomas and in combination with methotrexate for the treatment of moderately to severely active rheumatoid arthritis. While generally well tolerated, serious side effects of Rituximab can include infusion reactions, tumor lysis syndrome, severe mucocutaneous reactions and progressive multifocal leukoencephalopathy. [2]

Therapeutic drug monitoring involves measuring the concentration of the therapeutic drug and its metabolites, typically in the bloodstream of patients receiving the drug. The absorption, metabolism and elimination of therapeutic drugs can exhibit significant inter- and intra-patient variability based upon age, general state of health, genetic makeup, and the interference of other medications. The goal of therapeutic drug monitoring is to guide drug therapy by providing data on therapeutic levels of drugs in order to maximize efficacy and minimize toxicity. [3]

Methods to monitor serum Campath levels include measuring serum antibodies bound to CD52 expressing cell lines, enzyme linked immunosorbent assays (ELISA) and flow cytometric bead based methods. [4, 5] Shortcomings of these methods include low-throughput for the cell line method, and the inability to discrim-

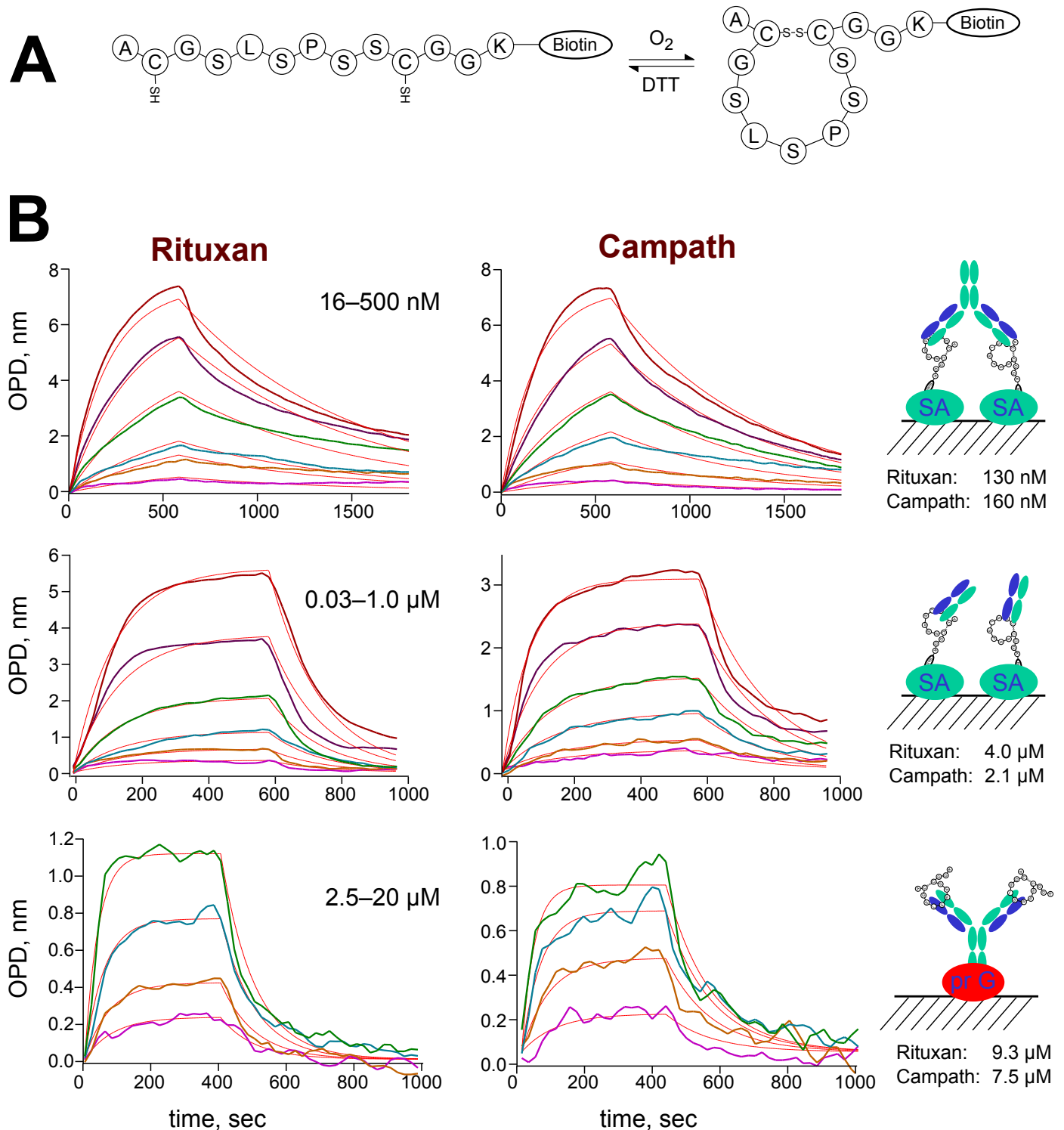


Figure 1: A Schematic of one of the two peptides studied here—the Campath peptide—and its equilibrium between the oxidized and reduced forms. B 2 \times dilution series sensorgram experiments described in the text together with two state fits. Data was taken by immobilizing peptide and interacting full antibody, by immobilizing peptide and interacting the Fab regions of the antibody, and by immobilizing the full antibody and interacting the soluble, unbiotinylated peptides. The two state fit for the antibody interacting with peptide case is poor due to the inherent heterogeneity of this interaction. A graphical summary of the affinity measured in each experiment is shown on the right.

inate the functional specificity of the antibody measured by the ELISA and bead method. The Silicon Kinetics SKi Pro system utilizes Nanoporous Optical Interferometry (NPOI) to measure antibody-antigen interactions in real time in a label-free format. In addition to determining antibody concentrations, SKi Pro can characterize the kinetics of the binding interaction, quantitating the on rate (k_{on}), off rate (k_{off}) and affinity (K_D) of the antibody to its antigen.

This application note demonstrates the use of the SKi Pro system to characterize whole molecule and Fab Campath and Rituxan antibodies utilizing novel peptides specific for these antibodies. The discriminating capability of this methodology is further illustrated by the determination of an avidity effect of the whole molecule antibody binding to the immobilized peptide antigen.

Method

Rituxan and Campath whole molecule antibodies were obtained from the University of San Diego Cancer Center pharmacy. Fabs of these antibodies were made utilizing the method described previously. [6] Peptides specific for the antibodies were selected utilizing phage display. [7] Cyclic structures were established through oxidation of cysteine amino acids with Sodium Tetrathionate at positions 2 and 10 in the 13 amino acid length. Peptides, biotinylated on their carboxy terminal lysines, were used in some experiments.

The respective Campath and Rituxan specific peptides were immobilized on the SKi Sensor streptavidin biosurface. Rituxan and Campath whole molecule antibodies were injected under flow rates of 10 $\mu\text{L}/\text{min}$ and binding interactions to the immobilized peptides recorded in real time. As can be seen in Figure 1, a 2 \times concentration series of the respective antibodies were analyzed and the k_{on} , k_{off} and K_D values were calculated from the obtained sensorgrams using SKi Report.

Next, the Campath and Rituxan Fab's were injected under similar flow rates and binding interactions to the immobilized peptides recorded in real time. k_{on} , k_{off} and K_D values were similarly calculated from the obtained concentration series sensorgrams utilizing the SKi Report software.

Finally, whole molecule Campath and Rituxan antibodies were separately immobilized on the SKi Sensor carboxy surface as follows. Carboxy surfaces were activated with EDC/S-NHS in water (at 50 mM and 200

mM respectively), and 100 $\mu\text{g}/\text{mL}$ protein G was exposed 5 minutes to the activated surface. The surface was then blocked with 1M pH 8.5 ethanolamine solution for 20 minutes and equilibrated into PBS running buffer. The sample binding surface was prepared by interacting the antibodies with the protein G (200 $\mu\text{g}/\text{mL}$, 2 $\mu\text{L}/\text{min}$ flow rate) in PBS/0.05% BSA buffer, whereas the reference surface was left unchanged. A concentration series of the respective peptides were analyzed and the k_{on} , k_{off} and K_D values were calculated from the obtained sensorgrams using SKi Report.

Discussion

The current work illustrates the capability of the SKi Pro system to finely characterize the binding of therapeutic antibodies to novel peptides specific for the antibodies. Campath and Rituxan were chosen for this demonstration. Both antibodies are a significant part of the current cancer and autoimmune disease armamentarium. Developing specific and sensitive assays to monitor these therapeutic antibodies will improve patient management and patient care.

Label free methods for characterizing biomolecular interactions are useful for measuring clinically relevant serum proteins. The capability of such technologies for measuring both low and high affinity antibody interactions has led to widespread adoption for immunogenicity testing, where serum antibodies reactive to therapeutic proteins are characterized and quantitated. [8] In the described work we seek to demonstrate the utility of a new method for measuring biomolecular interaction analyses, NPOI, for characterizing the interactions of therapeutic antibodies to small peptide antigens. NPOI utilizes a novel 3D nanoporous silicon biosurface and an optical interferometry design to deliver sensitive and accurate measurements of biomolecular interaction analyses. (Figure 2.)

Three assay formats were developed to study Campath and Rituxan whole molecule and Fab binding to novel 13 mer peptides developed through peptide phage display selection. In the first format, the respective biotinylated peptides were immobilized on the SKi sensor streptavidin nanoporous silicon biosurface, and the binding of the whole molecule antibodies analyzed in real time. In the second format, Campath and Rituxan Fabs were characterized for their binding to these immobilized peptides. In the third and most challenging assay format, whole molecule Campath and Rituxan antibodies were immobilized, and the respective 13 mer

peptides characterized for binding to these antibodies.

A comparison of the obtained on rate, off rate, and affinity values for the whole molecule antibodies versus their respective Fabs demonstrates an avidity effect for both whole molecule Campath and Rituxan. Further, the accurate characterization of these 1kD peptides binding to the immobilized 150kD antibodies supports this finding.

Table 1: Results of the two state kinetic fit to the Rituxan and Campath experiments here.

	$k_{on}, M^{-1} sec^{-1}$	k_{off}, sec^{-1}	$K_D, \mu M$
Rituxan			
peptide/Ab	7.5×10^3	9.8×10^{-4}	0.13
peptide/Fab	1.6×10^3	6.5×10^{-3}	4.0
Ab/peptide	7.7×10^2	7.1×10^{-3}	9.3
Campath			
peptide/Ab	8.1×10^3	1.3×10^{-3}	0.16
peptide/Fab	2.5×10^3	5.1×10^{-3}	2.1
Ab/peptide	1.0×10^3	7.6×10^{-3}	7.5

Previous work had demonstrated the resistance of the 3D nanoporous silicon biosurface to the nonspecific binding of proteins in complex solutions such as plasma. [9] We therefore expect to extend the demonstrated capability of the SKi Pro system for characterizing therapeutic antibodies, to measuring antibody titers in serum, plasma and other biological fluids.

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