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Investigating Synthetic Immune Recruitment by Proximity Inducing Molecules: Validation of Covalent Immune Recruiter (CIRs) Function Using the Octet® Platform

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Abstract

The recognition of cancer cells by the host's immune system forms the basis of modern tumor immunotherapy, a highly targeted treatment option with demonstrated ability to manage and cure previously lethal cancers. Immune recognition is minimally governed by the proximity of active phagocytic and cytotoxic immune cells to the site of target cancer cells. The development and validation of chemical tools that modulate the proximity of host immune cells with cancer cells is vital for furthering understanding of mechanistic aspects of the recognition process as well as the generation of useful immunotherapeutic design principles. One class of these chemical tools are covalent immune recruiters (CIRs), which function by binding and forming selective irreversible linkages to both tumor antigens highly expressed on the cancer cell surface and natural immune machinery (e.g., serum antibodies and Fc receptors). In this white paper, we describe the development of Octet® Bio-Layer Interferometry (BLI) assays that validate and characterize bi-functional small molecule CIR target binding and covalent reaction kinetics. We demonstrate how the Octet® system can be employed to efficiently characterize CIR binding affinities against both a prostate tumor antigen and human serum antibodies, as well as measure the selective covalent recruitment of these antibodies to the tumor antigen. These assays can accelerate the advancement of lead compounds to *in vivo* validation studies, and have additional utility in characterizing emerging classes of covalent inhibitor drugs.

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Introduction

As the subject of the 2018 Nobel Prize in Medicine, tumor immunotherapy has proven to be an exciting cancer treatment strategy. Most immunotherapies to date have focused on T cell function; however, more natural killer (NK) cell-based therapies that involve monoclonal antibodies (mAbs) that target tumor antigens are emerging[1]. mAbs have shown clinical success for the treatment of both hematological cancers and previously difficult to treat solid tumors[2]. One fundamental mechanism through which mAbs can kill tumor cells is antibody-dependent cellular cytotoxicity (ADCC). ADCC refers to when mAbs elicit an immune response through the activation of NK cells and is triggered by the bi-specific binding of an antibody to both a NK cell activation receptor (CD16α) and a target cancer cell protein antigen. This binding results in the release of cytotoxic molecules that lead to targeted cancer cell death (Figure 1)[2].

While promising, mAbs are potentially immunogenic, can degrade *in vivo* and experience difficulties trafficking to the site of solid tumors[3]. Furthermore, large doses of the mAb need to be administered intravenously (IV)[4]. This increases manufacturing costs, resulting in higher drug prices and limiting general patient accessibility[5].

In this white paper, we describe an approach to utilize tumor immunotherapeutic antibodies directly *in vivo* using small immune recruiting molecules coined as "covalent immune recruiters" (CIRs). CIRs selectively link to naturally abundant serum antibodies and redirect them to the surface of tumor cells. The resultant display of tumor coated antibodies activates stimulatory receptors on innate immune cells, such as CD16α on NK cells, and triggers an antitumor immune response. The efficacy of these CIRs as modulators of protein proximity can be characterized by the Octet® Bio-Layer Interferometry system.

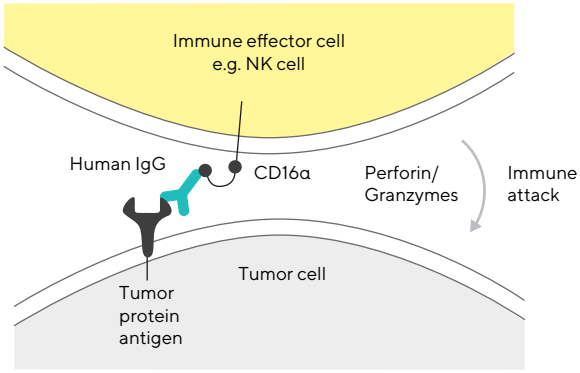

Figure 1: Schematic of antibody-dependent cell cytotoxicity (ADCC). An antibody recruits immune function to target a foreign cell, leading to its destruction.

Covalent Immune Recruiters (CIRs)

CIRs are comprised of the following functional domains:

1. An antibody or immune binding domain (ABD): a small molecule hapten domain that is recognized by antibodies naturally present in human serum. One such hapten is the dinitrophenyl (DNP) hapten, which is recognized by a class of naturally occurring serum antibodies known as anti-DNP antibodies (Ab). The origins of these antibodies are unclear but they are thought to represent a class of cross-reactive antibodies whose antigen-binding site contains abundant electron-rich aromatic amino acid residues.

2. A target binding domain (TBD): e.g., a synthetic ligand selective towards a highly expressed protein antigen on cancer cells such as glutamate urea lysine. Glutamate urea lysine binds prostate-specific membrane antigen (PSMA), which is highly expressed on the surface of prostate cancer cells[6]. It is notably associated with low nM affinity for PSMA.

3. An antibody or immune labeling domain(ALD): a reactive group that enables the CIR to irreversibly link to the antibody, following an initial reversible CIR:Ab binding step, through proximity induced affinity labeling chemistry.

We synthesized a unique class of CIRs that can selectively bind to and react with endogenous anti-DNP Ab in order to permanently label the antibody with a cancer targeting ligand. In this report, the following CIR derivatives (Figure 2) will be characterized using an Octet® assay to probe for selective target protein binding and antibody labeling kinetics (Figure 3):

1. CIR1: a proof-of-concept CIR which contains a DNP hapten, an amine-reactive ester, and desthiobiotin (DTB). DTB binds to Streptavidin Biosensors and acts as an ultra-high affinity tumor antigen model.

2. CIR3: which substitutes DTB with glutamate urea to target PSMA.

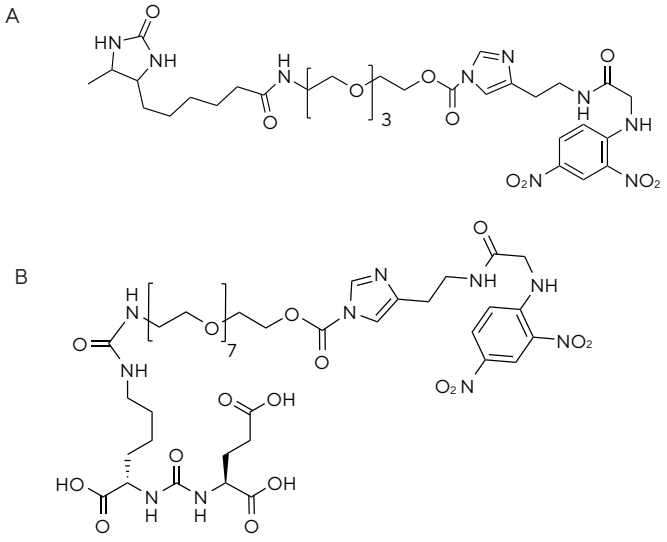

Figure 2: Chemical structure of (A) DTB covalent immune recruiter CIR1 and (B) glutamate urea substituted CIR3. CIR1 is designed to covalently modify serum anti-DNP Ab through its ABD (DNP hapten-right) and recruit these antibodies to surfaces presenting streptavidin using its TBD (DTB-left); DTB is associated with pM affinity for streptavidin. In the center is an electrophilic acyl imidazole ester moiety (ALD) which is positioned strategically close to a lysine residue on the anti-DNP Ab upon Ab binding to the DNP hapten. Non-covalent immune recruiting control molecules (NCIR) also presented in this report lack this electrophilic acyl imidazole moiety and therefore can only reversibly bind to both tumour antigen and Ab. CIR3 differs in the substitution of DTB with a longer linker appended to the PSMA binding ligand glutamate urea lysine, which possesses low nM affinity for PSMA.

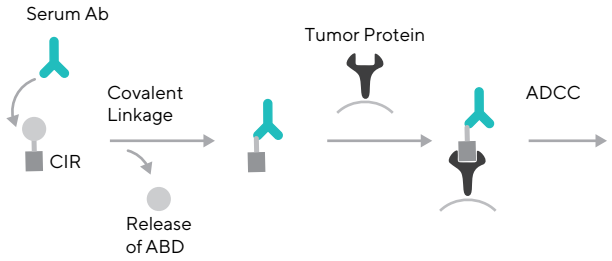

Figure 3: Schematic representation of CIRs modulating the immune system for targeted cancer-killing. Depiction of endogenous Ab with no intrinsic tumor targeting capabilities being recruited by a CIR to the surface of tumor cells and leading to immune cell activation via ADCC. In this example, the CIR must bind selectively to both the Ab and the tumor antigen and form a selective covalent bond with the antibody for function.

Octet® Assays Determine CIR Covalent Labeling Kinetics

Octet® systems are capable of real-time, high-throughput analysis of small molecule and biomolecule binding kinetics under equilibrium conditions. Due to its flexibility and compatibility for alternative assay format arrangements, it is a powerful tool for characterizing the CIR dependent binding equilibrium (e.g., tumor antigen:CIR:Ab) in addition to its use in determining CIR-antibody covalent recruitment kinetics. The label-free and highly sensitive nature of the Octet® system also enables analysis under dilute conditions, conserving expensive biologic reagents and attenuating aggregation phenomena intrinsic to isothermal titration calorimetry (ITC) and fluorescence polarization (FP) assays. Octet® systems also present a unique method to simultaneously characterize multiple protein binding and covalent labeling processes and discern reversible binding from a covalent reaction.

Materials

- CIR1
- NCIR4
- CIR3
- CIR4
- NCIR7
- DMSO
- DNP-Glycine competitor (DNP-Gly)
- Anti-DNP Ab (Thermo Fisher Cat. # A6430)
- 10X Kinetics Buffer from Sartorius (Cat. # 18-5032)
- Octet® R8 instrument (Sartorius)
- 2 black 96-well flat bottom plates from Greiner (Cat. # 655209)
- Streptavidin (SA) Biosensors from Sartorius (Cat. #18-5019)
- PSMA-Biotin
- 10 mM Glycine-HCl (pH 2.2)
- PBS
- Skim Milk Powder

Experimental

Experiment 1a: General Assessment of the Effects of Covalent Antibody Linkage on Total Antibody Recruitment to Target (Immobilized) Protein

Description: This experiment monitors the association and dissociation of CIR1 and non-reactive control molecule NCIR4 when incubated with anti-DNP IgG antibodies[7]. The CIR1/NCIR4 was pre-loaded onto the Streptavidin Biosensor, where streptavidin is used as a proof-of-concept target tumor protein antigen that binds to CIR/NCIR, and then associated with anti-DNP Ab. After some time, the biosensor was submerged in a buffer solution to monitor dissociation.

Assuming that rapid equilibrium is established between the antibody and CIR/NCIR before a slower covalent reaction, we hypothesized that adding sub-saturating concentrations of antibody to the biosensors pre-loaded with CIR1 or NCIR4 would yield two distinct association phases. The first rapid association phase reflects the fast equilibrium formation of non-covalent CIR/NCIR:Ab complex defined by the K_D, and the second slower association phase reflects the subsequent "covalent" recruitment of remaining free antibody to biosensors pre-loaded with CIR only (Figure 4). Since no change in biolayer thickness accompanied conversion of non-covalent (CIR:Ab) to covalent complex (CIR-Ab), performing the above experiment depends on working under sub-saturating concentrations of antibody where a fraction of antibody remains unbound at equilibrium. We also anticipated Ab bound reversibly to NCIR4 (lacking covalent labeling capability) or to CIR1 prior to reaction completion would dissociate upon submerging the biosensor in dissociation buffer. This is analogous to the Covalent Inhibitor sensorgram generated in Application Note 23, Commitment to Covalency: Kinetics of Irreversible Inhibitors With a Regenerable Streptavidin on the Pioneer FE System.

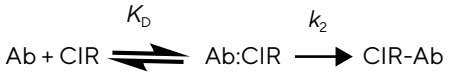

Figure 4: Proximity induced covalent labeling of Ab. Ab:CIR indicates non-covalent binding and CIR-Ab indicates covalent binding. Determining the selectivity and rate of Ab:CIR conversion into CIR-Ab is critical to validating the potential *in vivo* function of CIRs.

Protocol

1. Incubate Streptavidin Biosensors for 15 minutes with a 1X Kinetics Buffer, 1% DMSO solution.

2. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.

3. Load the biosensors with a solution containing 200 nM of CIR1 or NCIR4 in a 1X Kinetics Buffer, 1% DMSO solution, for 5 minutes.

4. Quench with 5% (w/v) milk in a 1X Kinetics Buffer, 1% DMSO solution, for 3 minutes. Milk is used as an abundant source of non-specific binding proteins and is often used in ELISA formats as a blocking agent for available hydrophobic surface areas.

5. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.

6. Associate with 40 nM anti-DNP Ab in a 1X Kinetics Buffer, 1% DMSO solution for 2 hours at room temperature.

7. Dissociate the biosensors with a 1X Kinetics Buffer, 1% DMSO solution at room temperature in the presence or absence of 1 mM DNP-Gly.

Results

Strikingly, the anticipated two-phase association representing fast binding and a slow covalent reaction for the NCIR and CIR case was not observed. This suggests all available sites on the biosensor were saturated with the antibody at equilibrium, which is inconsistent with the literature value binding affinity (K_D) for polyclonal antibodies for DNP (80 nM). Additionally, Ab recruitment to both CIR and NCIR pre-loaded biosensors appeared largely inert to dissociation in the absence of a DNP-Gly competitor. We attributed this observation to the bivalent nature of IgG antibodies leading to an avidity enhancement that greatly decreased the apparent K_D (Figure 5).

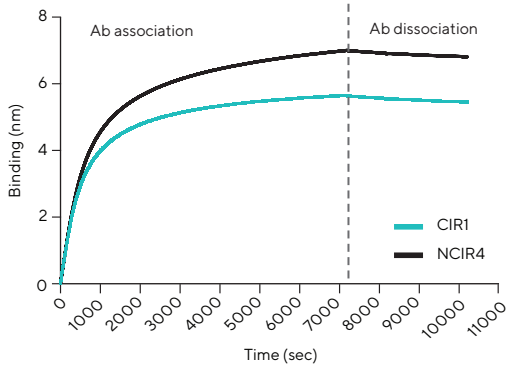

Figure 5: Initial comparison of Ab association/dissociation with CIR1 or NCIR4 pre-loaded on Streptavidin Biosensors. This sensorgram shows the association of Ab with CIR1 or NCIR4 (to the left the dotted line), and the dissociation (to the right of the dotted line). As clearly shown, there is virtually no dissociation for CIR1 or NCIR4 observed. The CIR/NCIR association with the biosensors only is not shown and gives rise to very low signal amplitudes due to correspondingly small changes in biosensor thickness.

Notably, avidity effects leading to stronger apparent binding affinities are likely general to the analysis of antibodies using immobilized antigens/haptens in the context of biosensor binding assays, especially when the assay is designed to test a bivalent analyte. We caution the interpretation of solution K_Ds based on calculations generated with a similar assay design. The apparent affinities calculated, however, may hold special relevance in the context of antibody binding to antigens immobilized on the cell surface that are present in high copy number and therefore lead to high binding avidity. Since antibodies bind CIR and NCIR with such apparent high affinity, it follows that only one association phase was observed, as all available biosensor binding sites were saturated with antibody at equilibrium ([Ab] >> K_D) and the antibody binding could not be distinguished from a covalent reaction.

We hypothesized that avidity binding could be disrupted via dilution of the biosensor surface by lowering the CIR loading densities through lower association times and decreased concentrations. This would increase the average intermolecular spacing between CIRs on the biosensor. Although this would lead to lower overall signal, we anticipate that the Ab would only be able to bind to one CIR (via the DNP molecule ABD). Unfortunately, we were unable to find loading conditions that would abolish the apparent binding avidity without losing all measurable signal and sought alternative strategies to interrogate the molecular origins underlying the apparent ultra-high affinity binding of anti-DNP to probe immobilized CIR/NCIR. We believe the dilution experiments were compromised by a diffusional barrier that exists between the biosensor surface and bulk solvent. This leads to a much higher apparent antibody concentration at the biosensor surface which promotes rapid “re-binding”.

Experiment 1b: Deconvoluting Antibody Binding Avidity From Covalent Reaction Using a Dissociation Promoting Competitor Molecule (Free DNP Hapten)

Description: We set out to compare the stability of Ab recruited to Streptavidin Biosensors pre-loaded with CIR1 versus NCIR4 to dissociation in the presence of free DNP-Gly competitor molecule. This allows us to investigate the avidity binding nature of anti-DNP Ab to CIR/NCIR pre-loaded onto the biosensors and attempt to discern covalent recruitment versus non-covalent binding of Ab. If the Ab is bound non-covalently but with high avidity, rapid antibody re-binding will be inhibited by the DNP competitor. This manifests as an increased dissociation rate governed by a dissociation rate constant (k_{off}) that describes the monomeric antibody binding interaction. In contrast, Ab "bound covalently" would remain inert to the competitor (Figure 6). As such we envisioned the change or decrease in "dissociable" Ab with time could be taken as a measure of the extent of the covalent reaction (i.e., the amount of irreversible binding complexes formed compared to NCIR).

Our Hypothesis: With longer Ab association times (reaction times), biosensors loaded with CIR will result in increased covalent labeling of the target protein that will lead to less Ab dissociation from the biosensor compared to that observed for biosensors loaded with NCIR.

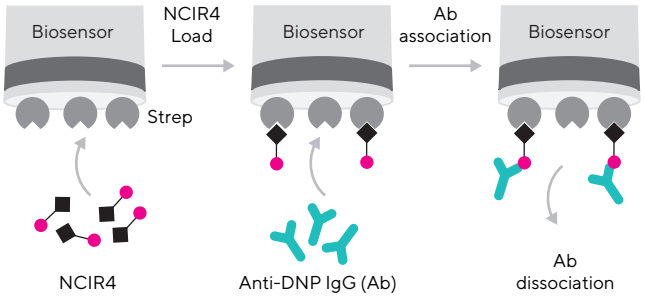

Figure 6: CIR1/NCIR4 is first loaded onto the Streptavidin Biosensors through its DTB terminus. Next, Ab binds to the CIR1/NCIR4 through the appended DNP comprising the ABD (dark grey). Over time, CIR1 is covalently attached, whereas NCIR4 is not. During the dissociation phase, the free DNP-Glycine inhibitor promotes dissociation of any non-covalent Ab complex.

Protocol

1. Incubate Streptavidin Biosensors for 15 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
2. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
3. Load the biosensors with 200 nM of CIR1/NCIR4 in a 1X Kinetics Buffer, 1% DMSO solution, for 5 minutes.
4. Quench the biosensor with 5% (m/v) milk in a 1X Kinetics Buffer, 1% DMSO solution, for 3 minutes.
5. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
6. Associate the biosensors with saturating concentrations of anti-DNP Ab (500 nM) in a 1X Kinetics Buffer, 1% DMSO solution for 1 hour. Note that saturating concentrations of Ab are intended to maximize the concentration of non-covalent CIR/NCIR:Ab formed on the biosensor, and therefore the rate of covalent reaction.
7. Dissociate the biosensors with 1 mM DNP-Gly in a 1X Kinetics Buffer, 1% DMSO solution for 50 minutes.
8. Repeat steps 1–5.
9. Associate the biosensors with 500 nM of anti-DNP Ab in a 1X Kinetics Buffer, 1% DMSO solution for 15 minutes.
10. Dissociate the biosensors with 1 mM DNP-Gly in a 1X Kinetics Buffer, 1% DMSO solution for 50 minutes.

Results

The presence of a competitor in the dissociation buffer was able to disrupt the non-covalent binding to Ab, corresponding to an increase in the dissociation rate (Figure 7). In addition to demonstrating potential utility as a method to characterize covalent antibody recruiting and deconvolute reaction from equilibrium binding, the Octet® platform delivers an efficient method that simultaneously confirms binding to two target proteins and discerns monovalent affinity versus multivalent avidity binding. Using the above competition dissociation method, K_D values for DNP on NCIR4 binding to polyclonal anti-DNP Ab in agreement with literature values determined in solution were obtained. Obtaining this wealth of biophysical information by conventional ITC and FP methods is difficult, if not impossible. Additionally, Dip and Read Biosensor architectures in Octet® platforms allow for the analysis of multiple conditions or CIRs simultaneously.

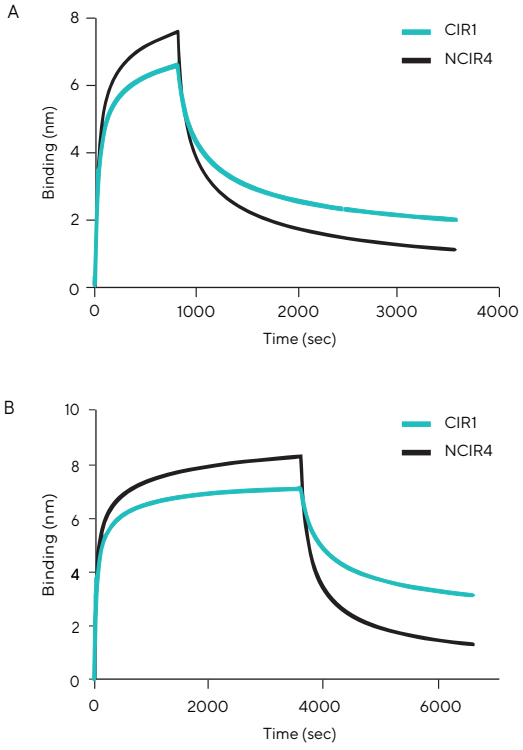

Figure 7: Using a DNP competitor to differentiate Ab recruited by CIR1 vs. NCIR4. (A) CIR1 and NCIR4 incubation with Ab on biosensors (association) for 15 minutes result in similar dissociation profiles. (B) CIR1 and NCIR4 incubation with Ab for 1 hour show a smaller dissociation amplitude (higher final signal) for biosensors loaded with CIR compared to those loaded with NCIR consistent with covalent recruitment in the case of the former.

Experiment 1c: Determining CIR1 Labeling Reaction Kinetics

Description: This experiment compares the dissociation profiles observed for Ab recruited to CIR pre-loaded biosensors (bound to target protein SA) at 6 different CIR:Ab association times followed by dissociation in the presence of DNP-Glycine. Decreases in the dissociation amplitude with, corresponding to covalent bond formation (CIR-Ab), was measured and plotted to extract the reaction rate constant describing CIR:Ab → CIR-Ab.

Protocol

1. Incubate Streptavidin Biosensors for 15 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
2. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
3. Load the biosensors 200 nM of CIR1 in a 1X Kinetics Buffer, 1% DMSO solution, for 5 minutes.
4. Quench the biosensor with 5% (m/v) milk in a 1X Kinetics Buffer, 1% DMSO solution, for 3 minutes.
5. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
6. Associate with 500 nM Anti-DNP Ab in a 1X Kinetics Buffer, 1% DMSO solution for 1 hour.
7. Dissociate the biosensors with 1 mM DNP-Gly in a 1X Kinetics Buffer, 1% DMSO solution for 50 minutes.
8. Repeat steps 1–7, with a 30 minute, 1 hour, 2 hour, 8 hour and 15 hour association of a fixed concentration of antibody with biosensors pre-loaded with CIR, measured in real-time.
9. Repeat steps 1–7 with NCIR4.

Results

Increasing the time for CIR covalent reaction with anti-DNP Ab decreases the final dissociation amplitude (Figure 8). We were also pleased to observe that an increase in association time always resulted in an increased dissociation amplitude. After a 15-hour incubation, there was nearly zero decrease in dissociation consistent with reaction completion. Interestingly, incubation of NCIR with Ab over extended durations of time also leads to the increased formation of unique non-covalent complexes that cannot be as rapidly dissociated with free DNP competitor. We propose that this represents the formation of kinetically stable, high avidity NCIR:Ab non-covalent complexes.

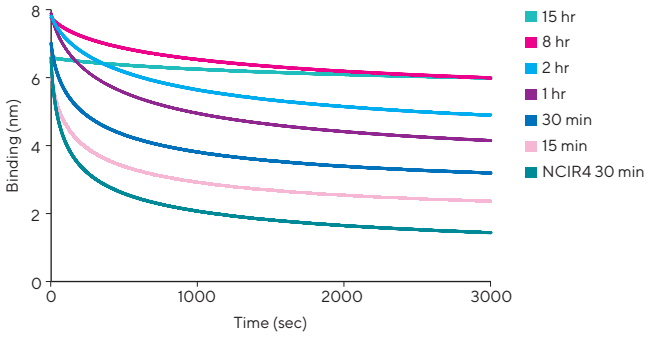

Figure 8: Disassociation curves of CIR1 with different pre-incubation time points. Increasing the association (reaction) time enables less Ab dissociation, indicating a covalent reaction. Notably, the NCIR shows extensive dissociation consistent with non-covalent antibody recruitment.

Experiment 1d: Determining the Kinetics of the CIR4

Description: In this experiment, we compare the kinetics of a CIR derivative (CIR4) to CIR1. The former positions the reactive ALD (acyl imidazole ester) at a position closer to the DNP binding site which is potentially more favorable for covalent labeling (Figure 9 and 10).

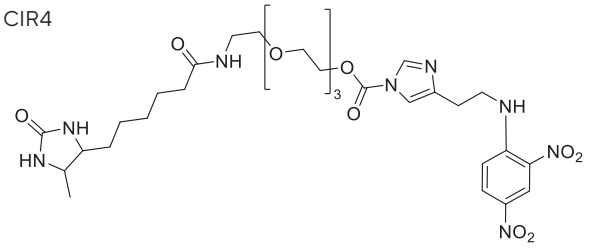

Figure 9: The chemical structure of CIR4 is identical to CIR1 except for a shorter more rigid linker (lacking glycine spacer unit) connecting the ALD to the ABD.

Protocol

1. Incubate Streptavidin Biosensors for 15 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
2. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
3. Load the biosensors with 200 nM of CIR4 in a 1X Kinetics Buffer, 1% DMSO solution, for 5 minutes.
4. Quench the biosensors with 5% (m/v) milk in a 1X Kinetics Buffer, 1% DMSO solution, for 3 minutes.
5. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
6. Associate CIR-preloaded biosensors with 500 nM Anti-DNP Ab in a 1X Kinetics Buffer, 1% DMSO solution for 1 hour measured in real-time.
7. Dissociate the biosensors with 1 mM of DNP-Gly in a 1X Kinetics Buffer, 1% DMSO solution for 50 minutes.
8. Repeat steps 1–7, with a 30 minute, 1 hour, 2 hour and 8 hour association.

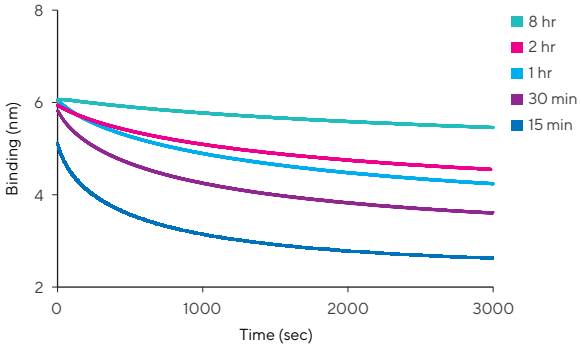

Figure 10: Disassociation curves of CIR4 with different association (reaction) times.

Data Analysis

To determine the kinetics of CIR1 and CIR4, the fractional product formation (Equation 1) with time was determined by measuring the final (CIR_{FT}) and initial dissociation amplitudes (CIR_{IT}). These measurements were compared to those observed for the analogous NCIR. The relative fraction of product formed vs. time was fit using a first-order, one-phase association rate equation (Equation 2), to calculate k_{obs}. k_{obs} is equivalent to k_2, which is the pseudointramolecular antibody labeling rate constant when antibody concentrations are saturating and no free CIR exists at equilibrium.

Equation 1:
Fractional product formation = $(CIR_{FT}\text{-}NCIR_{FT})(CIR_{IT}\text{-}NCIR_{IT})$

Equation 2:
$Y = Y_o\ (Plateau - Y_o)/(1\text{-}e^{(-K\text{-}X)})$.
Where X = time, Y_o is the Y-intercept and *K* is the observed rate constant.

Results

We observed a k_2 of 1.107/h for CIR1 labeled anti-DNP Ab while CIR4 showed a modest enhancement in reaction rate with a k_2 of 2.732/h, supporting our hypothesis that a shorter linker connecting the ALD to ABD on CIR accelerates proximity-based covalent labeling of the antibody (Figure 11). We predict that Octet® systems can serve as a robust and efficient high-throughput platform to rapidly assess future CIR structure-activity relationships (SAR) studies involving operations that manipulate linker lengths and rigidities, as well as the nature of the ALD chemistry.

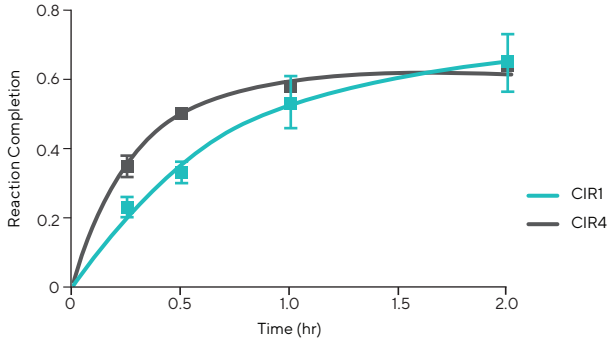

Figure 11: CIR1 and CIR4 Reaction Kinetics

Experiment 2: Demonstrating Covalent Immune Recruitment to a Bonafide Tumor Protein Antigen via CIR3

Description: While DTB substituted CIRs serve as a model compound with irreversible affinity for the target protein, it does not demonstrate the utility of our approach for weaker affinity binding protein antigens on the surface of tumor cells. Towards this end, we developed CIR3, which substitutes the DTB ligand with a glutamate urea lysine. CIR3 is a ligand known to bind prostate specific membrane antigen (PSMA), a highly expressed antigen on prostate cancer cells[6]. As a control for covalent reactions, a non-reactive analog of CIR3 (NCIR7) was developed where the acyl imidazole ester was replaced by an amine stable carbamate bond.

Attempts to characterize CIR3 labeling kinetics where Streptavidin Biosensors were loaded with biotinylated PSMA were unsuccessful. In contrast to the essentially irreversible DTB-streptavidin bond, the glutamate urea-PSMA binding interaction is in the double-digit nM range, leading to problems with CIR3 dissociation from the biosensor over the analysis time. This led to a loss of signal and complexities that complicated the kinetic analysis. This format could, however, uniquely confirm the double-digit binding affinity (K_D) and kinetics (k_{on} and k_{off}) of CIR3 for PSMA.

To circumvent these challenges, we modified the assay format by incubating equimolar concentrations of 100 nM Ab and 200 nM CIR3 in solution for varying amounts of time, followed by quenching with 1 mM DNP-Glycine. This halted the reaction by dissociating all non-covalent complexes. Following reaction quenching, each reaction solution was associated with biosensors loaded with biotinylated PSMA. We anticipated the rate of signal increase and final signal amplitude would be proportional to the relative amount of covalent product (CIR3-Ab) formed in solution. We further anticipated the incubation of CIR and Ab away from the biosensor in this modified assay format would minimize the formation of high avidity, non-covalent complexes which obscures the measurable difference between covalent and non-covalent antibody recruitment.

Protocol

1. Incubate Streptavidin Biosensors for 15 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
2. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
3. Load the biosensors with 100 nM PSMA-Biotin* in a 1X Kinetics Buffer, 1% DMSO solution, for 5 minutes.
4. Quench the biosensors with a 5% (m/v) milk in a 1X Kinetics Buffer, 1% DMSO solution, for 3 minutes.
5. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
6. (Optional) Regenerate the biosensors using 3, 5 second cycles of 10 mM Glycine-HCl (pH 2.2) and PBS.†
7. Baseline the biosensors for 3 minutes with a 1X Kinetics Buffer, 1% DMSO solution.
8. Associate with various timed incubation solutions of Ab+CIR3 and Ab+NCIR7.
9. (Optional), Regenerate the biosensors using 3, 5 second cycles of 10 mM Glycine-HCl (pH 2.2) and PBS.†
10. (Optional) Repeat steps 8 and 9, to generate data for replicates or new time points.

* It is essential that the receptor-biotin bond is stable. NHS-PEG4-Biotin provides such stability by generating an amide linkage to the protein. It is also essential not to overly biotinylate a receptor and aim for a range of approximately 0.5-2 biotins/ receptor.

† Regeneration is a process in which global non-covalent complexes are dissociated from biosensor, enabling its reuse, presuming immobilized protein is not denatured irreversibly. Regeneration requires a pre-regeneration step (step 6), and careful analysis /optimization to ensure no significant loss of signal over subsequent regenerations. For more information see Technical Note 14 Regeneration Strategies for Streptavidin Biosensors on the Octet® Platform.

Results

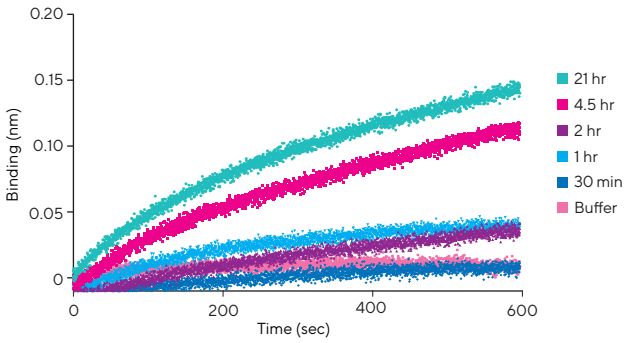

Figure 12: Kinetics of CIR3-Ab formation in solution using PSMA loaded biosensors. Increasing the solution CIR3 with Ab incubation time leads to higher nm shifts, indicating more covalent complex. The reaction appears to be complete by hour 21. Note that the NCIR7 amplitude is negligible due to dissociation mediated by excess DNP-Gly competitor.

Data Analysis

To assess the rate of the solution covalent labeling reaction between CIR3 and anti-DNP IgG, the initial slope of the association curve for each reaction time point was determined and taken to be proportional to the fractional conversion to covalently linked antibody to form CIR3-Ab. Although Ab concentrations are non-saturating, k_{obs} could still be obtained with satisfactory fits using a first-order integrated rate equation, and reflected solution labeling kinetics in close agreement with that determined "on biosensor" above using CIR1.

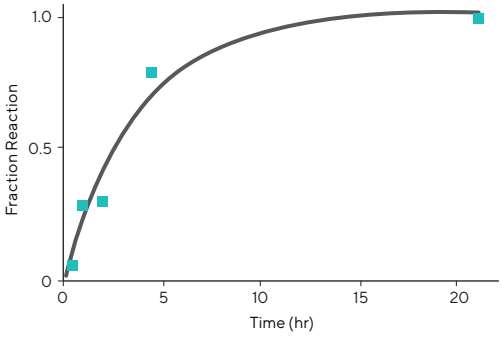

Figure 13: Evaluation of the solution CIR3-Ab covalent labeling reaction. Octet® instrument analysis using a versatile alternative assay format enables robust validation of CIR target tumor antigen and antibody binding for further deconvolution of antibody labeling kinetics. Notably, this format could not be used to assess CIR1 due to observed diffusional phenomena hypothesized to arise from the rapid and irreversible association of free CIR1 with Streptavidin Biosensors. As such, CIR1 more quickly binds the biosensor than Ab complexes acting as an effective inhibitor.

Summary

We demonstrate the utility and efficiency of the Octet® platform at characterizing covalent immune recruiter molecule function and their efficacy as modulators of protein proximity. Efficient *in vitro* validation further enables the potential acceleration of CIR translation to *in vivo* model validation and the clinic as a new class of synthetic tumor immunotherapeutic. Importantly, the Octet® validation results presented in this white paper were consistent with the kinetics and selectivity analysis carried out in parallel fluorescence SDS-PAGE gel and flow cytometry experiments (see the ACS Chemical Biology publication[7]) where CIRs further demonstrated the ability to affect immune recognition and targeted destruction of tumor antigen-expressing cells.

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