Analysis of “Magneto-nanosensor platform for probing low-affinity protein-protein interactions and identification of a low-affinity PD-L1/PD-L2 interaction (Lee et al.)”

Scientific Poster on Analysis of Research Knowledge

Presented by Heeae Noh and Seo Jin Lee

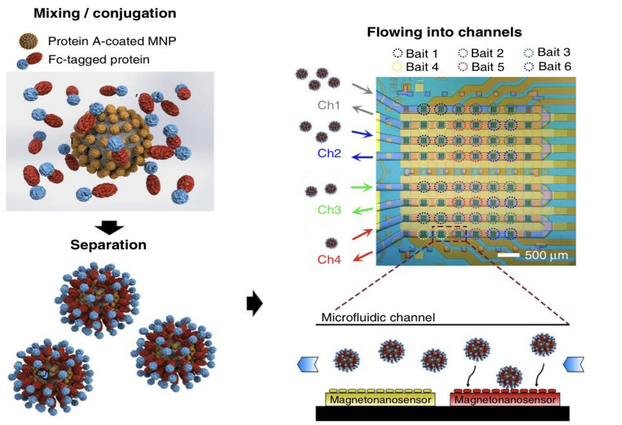
Overview & Introduction

The paper proposes conspicuous labeled biosensor, magneto-nanosensor platform which can detect the protein-protein interactions(PPIs) between the immune checkpoint receptor, programmed cell death protein 1 (PD-1 (CD279)) and their ligands PD-L1(B7-H1 or CD274), PD-L2(B7-DC or CD273). Moreover, interestingly, it detected the binding between PD-1 and B7-1(CD80), and the binding between PD-L1 and PD-L2. Immune checkpoint is the regulator of the human immune system which involves immune-inhibitory pathways. Cancer cell hinders the immune response by activating T cell’s immune checkpoint receptor. One example is PPI between PD-L1 locating on cancer cell’s surface and PD-1 on T-cell. The solution of this phenomenon for immunotherapy is to blockade the combination by the immune checkpoint inhibitor, which needs critical 2 elements: the identification of all interacting partners, and the measurement of binding affinities between the domains. This sensor, MNS measures the dissociation constant of the PPI and characterize the binding affinity.

Additionally, MNS has lots of advantages of identifying PPIs over previous sensors, such as SPR. First, its predominant mode of interaction is bivalent mode, so it needs less reagent to measure the micromolar interactions. Second, MNS detects the multiplexing (1 prey protein/ several bait protein) which reduces the amount of consumed reagents, time taken, and the errors that may occur within multiple measurements. Third, it is not affected by pH and salinity, so it allows PPI research to be held in various conditions. Fourth, it can implement multiple test about various concentration of prey simultaneously. Lastly, it can detect the low affinity binding utilizing the multivalent(mostly bivalent) ligand-receptor bindings .

This magneto-nanosensor is much more improved version than the previously developed MNS(ref.18). The previous sensor, which is based on the natural diffusion of MNPs from bulk to reaction area, had two significant problems: (1) the binding signals being coupled with diffusion rate, and (2) relying heavily on a two-compartment model (bulk and the reaction zone) to estimate kinetic parameter mathematically. However, this MNS solved the former problem by making intentional continuous flow using microfluidic chip and solved the latter by reducing the mathematical model with simple Langmuir absorption isotherm.

This paper’s magnetonanosensor platform is based on magnetonanosensor chip integrated with microfluidic chip. (1) First, prey need to be pre-conjugated with A-tagged MNPs(micro nanoparticles) in microfluidic chip. (2) After, under the magnetic fields, unbound Fc-tagged proteins are washed away and prey-MNPs are eluted with PBS(phosphate-buffered saline). (3) MNPs are diluted to be 100%, 75%, 50%, and 25% of the eluted concentrations respectively. (4) Because the bait is fc-tagged too, a blocking step is implemented on channels using soluble A. (5) A flow made by a multichannel syringe pump makes each diluted prey-MNP complexes to flow into four microfluidic channels. (6) Measure the concentration of the complexes in solution by measuring the absorbance of light which wavelength is 400~600nm. (7) Blocking step is held on Fc-tagged baits using soluble protein A. (8) Bait and the prey-MNP complex multivalent binds and magneto-nanosensors produce signals using GMR to identify the amount of bindings on each sensor. (8) Using the Simple Langmuir Isotherm, can get the dissociation constant, the affinity of PPI.



Method of Detection of the Magneto-nanosensor

Mixing/ Conjugation

- *MNP*
  MNPs consist of multiple superparamagnetic cores embedded in a matrix of dextran, with a hydrodynamic diameter of 46+-13nm. (number-weighted mvy- dynamic light scattering). Also, they have a reported zeta potential of 211 mV (ref. 38) so, these particles are superparamagnetic and colloidally stable, do not aggregate or precipitate during the reaction. The unbound MNP tags contribute negligible signal in the absence of binding because only tags within ~150 nm of the sensor surface are detected.

- *Interaction between A-coated MNPs & Fc-tagged prey*
  MNPs are coated with protein A and prey protein is tagged with Fc-region of human IgG1. Protein A and Fc-region occurs PPI, so when A-coated MNPs and Fc-tagged prey are mixed, they are conjugated in such order: MNP-A-Fc-Prey. In these interactions, it is assumed that the bivalent interaction is the main contribution of binding because if it is monovalent or trivalent, substantial discrepancy is yielded.

Separation

- *Elution*
  After the conjugation, the mixture is added to a column placed under a strong magnetic field (a magnetic separation unit 130-042-602), and the MNPs are trapped inside the column while the unbound proteins are washed away. When the external magnetic field is removed and the MNPs are eluted with PBS.

- *Dilution*
  MNPs are diluted with PBS to be 100%(undiluted), 75%, 50%, and 25% of the eluted concentrations respectively. This made the sensor to available the multiple test over various concentration simultaneously.

Blocking step & Flowing into channels

- *Channels*
  There are four channels and the lower channel, and the concentration MNPs are flowed(ch1->2->3->4 : 100->75->50->25%). Each channel has 8x8 sensors (8 for in and out path) where 6 different baits and negative control(prey itself) is imbedded. Each sensor is dimensioned 100x100 micrometer.

- *Concentration of the MNPs*
  Measurement of the concentration of complexes before binding by measuring the absorbance of the light which wavelength is between 400~600nm. Here, researcher used 425nm wave. Absorbance and the concentration of the complex is linear (graph b).

- *Blocking step*
  Since the bait proteins are also Fc-tagged, blocking step using soluble protein A is needed to avoid undesirable bindings.

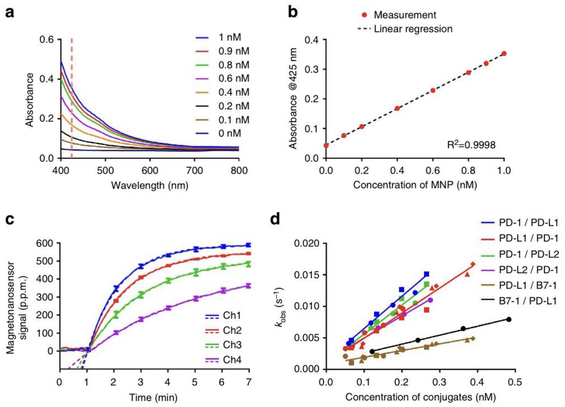
- *Flow and bindings*
  By syringe pumps individually connected to each channel, MNP complexes flow into the channel and as MNPs flow, prey protein and bait protein interacts. Because there are multiple baits in single sensor, many prey proteins conjugate on single sensor and bivalent interaction is predominant.

Detection- Number of bindings

- *GMR nanosensor*
  Giant magneto resistive nanosensor. GMR is a quantaum mechanical magnetoresistance effect in which the electrical resistivity of a magnetic material changes greatly when it has an external magnetic field as compared with the absence of an external magnetic field (Faraday’s law). This sensor used the spin valve GMR which is composed of 2 layers: alternating ferromagnetic free top layer and magnetic fixed bottom layer. The GMR sensor detect the change of orientation of top layer’s magnetization(Θ), which means the change of stray field by the magnetic tag, to infer the number of captured analytes. (when the antibody(target)-MNP complexes are captured, the change in magnetic field causes the change of top layer’s magnetization orientation. And, it causes change in the electrical resistance → allows the monitoring and quantifying of kinetics of binding). The GMR biosensor array can simultaneously monitor hundreds to thousands of sensors at sensitivities of 1 pg /mL or below and dynamic ranges of 6 log or more

Identify- Affinity of the PPIs

- *Simple Langmuir Isotherm*



Formula 1.

$$\Theta_M = \frac{K_{ad}[M]}{1+K_{ad}[M]} \qquad \Gamma_M = \Gamma_M^{max}\frac{K_{ad}[M]}{1+K_{ad}[M]}$$

Formula 2.

$$n = n_{max}\left[\frac{1-e^{-k_{on}(C_0-n_{max}A/V)t}}{1-\frac{n_{max}A}{C_0V}e^{-k_{on}(C_0-n_{max}A/V)t}}\right]$$

Formula 3.

$$\theta = \frac{C}{C+K_D^{bi}}\left(1-e^{-\left(k_{on}^{bi}\times C+k_{off}^{bi}\right)t}\right)$$

Formula 4.

$$Y = A\times\left(1-e^{-k_{obs}\times(t-t_0)}\right)$$

Calculation Methods

Mathematical model: The Langmuir Isotherm

- *The Langmuir Isotherm*
  The Langmuir Isotherm describes a progressively increasing surface occupancy as a function of pressure up until the entire surface area is coated with a single layer of molecules and no further adsorption can occur in solid-gas system or solid-solution system(in this paper: latter). This describes the maximum amount of gas or solution that shale can hold according to a pressure, at a specified temperature. In this paper: the concentration gap occuring the pressure is important. Langmuir Isotherm is based on the idea of equilibrium depicts that the rate of association and the rate of dissociation is the same.

- *Formula 1*
  Θ is a fraction of surface sites bounded by the molecules . Kad is a constant which is Ka/Kd= rate constant of association/ rate constant of dissociation. [M] means the mole concentration of the bulk(before bounding). In second isotherm, which explains the same as the first, r is the number of molecules bounded.Original Langmuir isotherm model assumes that there is a negligible depletion of reactants(bait) and that the concentration of reactants near the surface is the same as that in the bulk. Which can’t be satisfied.

Previous Simple Langmuir Isotherm (ref18)

- *Formula 2*
  This formula assumes that koff is zero because antibody–antigen dissociation is typically negligible on the 400–1,000 s timescales of our experiments. (1)n is the surface concentration of bounded MNP complexes over the sensor(2D). (2)n max is the maximum moles of surface-bound complexes per area(limited by the maximum concentration of closepacked MNPs) (2D). (3)C0 is the bulk concentration of MNP complexes(3D). (4)V is the entire are and A is the occupied area of the sensor. The quantity of nA/V is equivalent to the bulk concentration in the reaction zone that would be consumed to produce the surface concentration n, according to mass conservation(3D). (5)C0-n(A/V) means the depletion of MNP complexes in reaction zone and might represent the magnitude of the flow, pressure. (6)t is a time. This simple model is only available when C0V>>nmaxA so the flow is fast. (because if C0 is too small, n converge to zero).

Paper’s Simple Langmuir Isotherm

- *Formula 3*
  The author reduced the variables from the previous isotherm and made Formula 3. Θ means coverage of the bait proteins bound to the complexes. C is a concentration of the complexes in solution. K are the constants of the bivalent interactions. Kd=koff/kon, koff=rate of dissociation and, kon=rate of association. (the monovalent interaction’s KD is a square root of bivalent’s). T means time. Θ is gotten from the GMR and C is gotten from absorbance measurement. Knowing all the parameters, we can get Kd and Ka mathematically with association and dissociation curve.(Paper only showed association curve). Consequently, able to estimate the binding affinity. This formula is available when the flow rate is sufficiently fast to reach the reaction-limited regime.

- *Formula 4, Graph c.*
  Formula 4 is a simple exponential function to fit the binding curves base on non-linear regression. Variables are: A, amplitude and Kobs, observed binding rate excluding the time shift t0. Kobs= kbi on*C + kbi off refer to the formula 3. This fitting curve matches well with the binding curve as you can see in the graph c.

Graphical Analysis on pH, Salinity

pH and Salinity

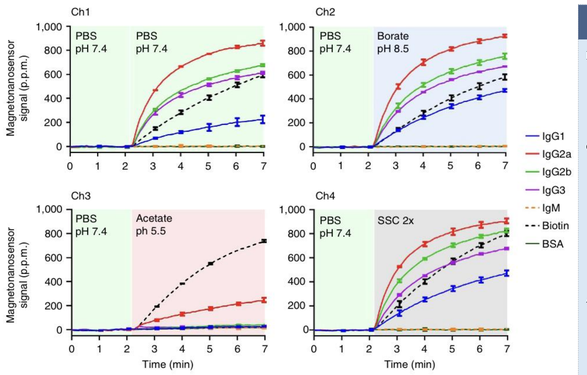
- pH and salinity has no effect on the magnetonanosensor signals.

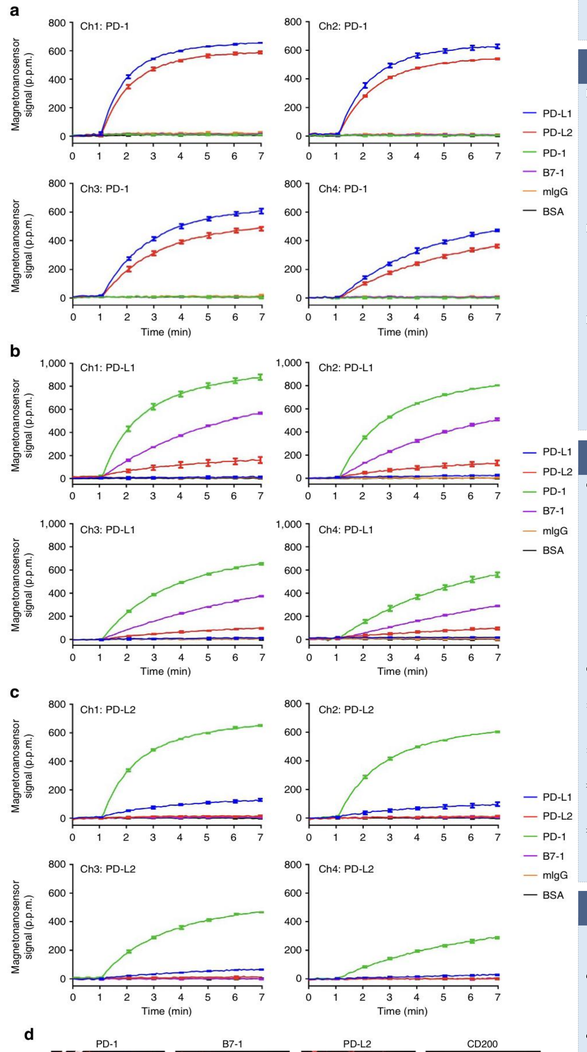
Experiment & Observation

- They used the solutions of the same amounts of protein A-coated and streptavidin-coated MNPs. Each solution was flowed into different channels where each murine IgG isotypes, IgM, BSA, and biotinylated BSA were immobilized. This means that the murine IgG isotypes, IgM, BSA, and biotinylated BSA were acting as the bait proteins.
- At first, the channels had a pH of 7.4 with phosphate-buffered saline.
  - In the control channel (Channel 1), the pH and salinity remained constant. The magnetonanosensor detected increasing signal for all bait proteins except IgM and BSA, which means that the MNPs only bound to IgG1, IgG2a, IgG2b, IgG3, and Biotin.
  - In Channel 2, the pH was increased from 7.4 to 8.5 by adding borate, which means that the solution got more basic. The graph shows relatively unaffected binding signal for IgG2a, IgG2b, IgG3, and Biotin, and an increased binding signal for IgG1. Both IgM and BSA didn’t have any interaction with the MNPs.
  - In Channel 3, the pH was decreased from 7.4 to 5.5 by adding acetate, which means that the solution got more acidic. The graph shows that all bait proteins except Biotin and IgG2a didn’t interact with the MNPs. While the binding signal for Biotin remained relatively constant, the binding signal of IgG2a decreased significantly. This is a well-known phenomenon.
  - In Channel 4, the salinity increased by introducing SSC 2X to the channel. All bait proteins except IgG1 showed no change in their binding signals. The binding signal of IgG1 increased slightly as compared to that in Channel 1.

Analysis

- The consistent binding signal of the non-binding negative controls IgM and BSA indicates the insensitivity of the magnetonanosensor to the changes in pH.
- The graph of Channel 4 shows no significant change in the binding signals, which also implies the insensitivity of the magnetonanosensor to the changes in salinity.





Analysis on the Prey Protein/Bait Protein Interaction

Background

- The graph shows the ability of the nanosensor to detect micromolar affinity interactions by using human PD-1 and its ligands (PD-L1 and PD-L2). The four graphs for each letter represents different concentrations of the prey protein with 100(undiluted), 75, 50, and 25% that are then flowed into Channel 1, Channel 2, Channel 3, and Channel 4 as shown in Figure 1, respectively.
- Negative controls are murine IgG, BSA, and prey itself. B7-1 is a newly discovered receptor.

Observation

- Graph (a) represents the binding signal detected by the magnetonanosensor in which PD-1 was flowing in the channel as the prey protein. It shows that PD-1 was binding to both PD-L1 and PD-L2 with similar binding affinity, which are the known ligands of PD-1. PD-1, that were placed inside the channel as bait proteins. PD-1 did not interact with B7-1, IgG, and BSA.
- Graph (b) represents the binding signal detected by the magnetonanosensor in which PD-L1 was flowing in the channel as the prey protein. It shows that PD-L1 was binding with PD-1 with the highest signal. The binding signal of PD-L1/B7-1 is higher than that of PD-L1/PD-L2.
- Graph (c) represents the binding signal detected by the magnetonanosensor in which PD-L2 was flowing in the channel as the prey protein. It shows that PD-L2 was binding with PD-1 mostly, with a slight interaction with PD-L1.
- As the time passed, the graphs converged to a certain value.

Analysis

- Generally, as the concentration of the prey protein decreased, the binding signal decreased overall.
- The graph converging to a certain value represents the completion of the binding between the prey protein and the bait protein, which means that the bait protein has all bound to the prey protein and thus is maxed out in its binding regions.
- The interaction between PD-1 and its ligands was expected.
- The fact that any of the prey proteins interacted with the negative control receptors represent the specificity and accuracy of the magnetonanosensor since it only detected the binding interactions that are expected to occur.
- The PD-L1/PD-L2 interaction shown in graph (c) was not an expected interaction, so a further study is needed to confirm the mechanism behind the PD-L1/PD-L2 interaction.

PD-L1/PD-L2 and PD-L1/B7-1 Interaction

PD-L1 and PD-L2 Interaction

- The unexpected interaction between PD-L1 and PD-L2 has been observed.
  - The PD-L1/PD-L2 interaction showed a dose response, which is the trend in which the signal increases as time passes.
- One possible explanation for this phenomenon, which is that the binding affinities are exceptionally low so that it wasn’t detected before, turned out to be false. The dissociation constants of the monovalent interaction between PD-L1 and PD-L2 came out to be 10.7 uM when PD-L1 flowed and 9.1 uM when PD-L2 flowed, which is not an exceptionally low value.
- The interaction is believed to be human-specific since it was only observed when using human ligands, but wasn’t observed when using the murine ligands.

PD-L1 and B7-1 Interaction

- The interaction between PD-L1 and B7-1 was observed when PD-L1 was flowing as the prey protein and B7-1 was immobilized as the bait protein.
- This is a known interaction, but it had a relatively low-signal response, which is consistent with the research that the PD-L1/B7-1 interaction was difficult to detect using cell surface detection method.

Image Analysis

- Going from left to right, the pictures represent immobilized PD-1, B7-1, PD-L2, and CD200 as bait proteins in different channels as PD-L1 flowed. The flowing PD-L1 had protein coatings in which the first row had anti-hIgG coated MNPs and the second row had streptavidin-coated MNPs.
- The image shows the red spots for all PD-1, B7-1, and PD-L2 pictures, which represents the binding interaction between the prey protein and the bait protein.
- CD200 is the negative control.

Conclusion & Questions

Conclusion

- Functional implications + future uses
  - The developed magnetonanosensor(MNS) shows many promising features, including the utilization of bivalent interactions, high-throughput kinetic assays, minimization of the risks of experimental variations, insensitiveness to pH and salinity, and more.
  - The finding of the PD-L1/PD-L2 interaction can be implemented in studying the Reed-Sternberg cells in Hodgkin’s lymphomas, which is the disease where both PD-L1 and PD-L2 are commonly targeted for chromosome 9p24.1 amplification.
  - The further understanding of the PD-L1/PD-L2 interaction and therefore understanding the PD-1-PD-L1/PD-L2 inhibitory checkpoint may open another possibility for developing immunotherapy.
- Improvements + further development
  - The usage of the finding of the PD-L1/PD-L2 interaction is yet to be determined, and it requires further research. Also, a research to confirm the orientation of the potential physiological interactions, such as: cis- or trans- orientation?, is needed. Need to figure out weather the PD-L1/PD-L2 interaction is competitive or synergistic in the process of PD-1 binding and functional stimulation.
  - In the range of ~256 sensors, several prey proteins or bait proteins in various concentrations might be detected simultaneously in user-wanting forms. Also, the data point, which represents the accuracy only depends on the number of sensors or channels. So this MNS should be developed in the respect of increasing the sensors.

Questions-Answers

- What exactly is the benefit of the bivalent interactions?

> *Bivalent interactions enhance the binding affinity.*

- We believe that the accuracy might depend on the order of arrangement of the Baits because, if the position of the Bait is further behind, there could be less binding since the Prey has already bound to the Baits that are positioned closer to the entrance of the channel.

> *It is solved by the flow, enough amount of MNPs, and the duplicate positioning of each baits.*

- Is dilution a process used just for this experiment or is it an essential process of this sensor? Why?

> *It is necessary for a efficient sensing. Also this sensor is aiming to be used as the PPI affinity detecting than diseased cell detecting yet.*

- Mathematical model - why is the exponential model is used? Just to match with shape of the graph?
- What is the determinant that this sensor can detect the low affinity bindings?

> *Bivalent binding*

- The fast flow is essential so that diffusion of MNPs does not happen and the binding rate becomes maximum.
- In original Langmuir Isotherm, KD= Ka/Kd but here, KD= Kd/Ka. Is there any reason of this?

Definitions

- **Affinity**

Strength of a single interaction of an antibody that is determined by net force of weak interactions between single antibody binding site and its epitope (portion of the antigen that is capable of stimulating an immune response by binding to a specific antigen receptor on the cell surface).

- **Kinetic assay**

Enzyme-based assay that measures the amount of substrate present by correlation of the rate of reaction with the known dependence of the rate on substrate concentration.

- **Multivalent ligands-receptor interaction**

Many biological ligands are composed of clustered epitopes so, when ligands-receptor interaction happens, epitopes create internal diffusion and it increases the functional affinity. Multivalent ligand comprises of multiple copies of ligands conjugated on scaffold, in this paper: MNPs. Bivalent binding is predominant in the case of PPIs.

- **Labeled/ Label-free biosensor**

Labeled biosensor is a biosensor that incorporates sensitively detectable label, which allows the complex to be determined sensitively by detecting the label. Label-free biosensor is a biosensor that is designed that the antigen-antibody complex is directly determined by measuring the physical changes induced by the formation of the complex.

- **Protein-protein interaction (PPI)**

Physical contacts of high specificity established between two or more protein molecules as a result of biochemical events steered by interactions. This is involved in various diseases, which is why targeting PPIs is a well-known pathway to treat diseases and develop new drugs.

- **Surface plasmon resonance (SPR)**

Standard label-free method of monitoring protein binding interactions which operates by measuring changes in the refractive index of a thin film when unlabelled solute molecules bind to the surface.