UNIVERSITY OF CALIFORNIA SAN DIEGO

Synthesis of Tag-Pollutants Conjugates for Synthetically Evolved Receptors Screening

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Jianbo Huang

Committee in charge:

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Professor Eric Schmelz, Co-Chair
Professor Gensheng Feng

2018

The Thesis of Jianbo Huang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

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I would also like to thank Professor Conor Caffrey, Professor Geoffrey Chang, Steven Rees and Dominic Mcgrosso who cooperate with me to do all the carry out all the works. I truly appreciate that you helped me open up my horizon to the biological research and taught me how to be a researcher.

In the end, I would like to especially thank my parents for mentally and financially support me for the past 4 years. For every time not giving up on me when I disappointed you; for every moment you worked hard just to pay my tuition and for every moment you spent praying for me, I could express my feeling with words. I just hope I can make you proud with all the work I have done for the past years.

This thesis uses unpublished material coauthored by Prof. Geoffrey Chang.

ABSTRACT OF THE THESIS

Synthesis of Tag-Pollutants Conjugates for Synthetically Evolved Receptors Screening

by

Jianbo Huang

Master of Science in Biology

University of California San Diego, 2018

Professor Dionicio Siegel, Chair
Professor Eric Schmelz, Co-Chair

Fast on-site detection of organic pollutants using biosensors is developing rapidly. To select Nanobodies which have binding specificity to varies of pollutants to make the biosensors, the pollutants and tag conjugates was synthesized in lab and applied to cell culture. Our results demonstrated that the recombination of the naïve nanobody culture for generations does produces the nanobodies possessing high binding affinity towards organic pollutants.

Chapter1: Introduction

Organic pollutant molecules found in soil, water, and food can be harmful to both environment and health. Effectively detecting these organic molecules is imperative to controlling and curbing the environmental pollution. Current techniques used for the analysis of pollutants like High Pressure Liquid Chromatography **(HPLC)**, Mass Spectrometry **(MS)**, Enzyme-Linked Immunosorbent Assay **(ELISA)** are performed in the lab, making them less ideal for field condition investigations. As a result, there is a rising needs for rapid, cost-effective, portable analytical methods used to detect these molecules. For example, biosensor has grown in recent years in response to demands for the on-site measurements of pollutants. However, no single method with high affinity and specificity for small molecule pollutants has yet been developed. Most biosensors still cannot fully compete with conventional lab-based analytical methods mentioned earlier. In cooperate with Geoffrey Chang's Lab in Skaggs School of Pharmacy and Pharmaceutical Science, we will access a new and powerful platform to produce *Synthetically Evolved Biosensors (SEBs)* shown in figure 1. By producing a protein scaffold that incorporates both *Synthetically Evolved Receptors (SERs)* and optical transducer will enable the biosensor SEBs to glow as it binds to pollutants. As the SEBs could be spotted on a piece of paper, the color change of the paper will show the existence of certain pollutants.

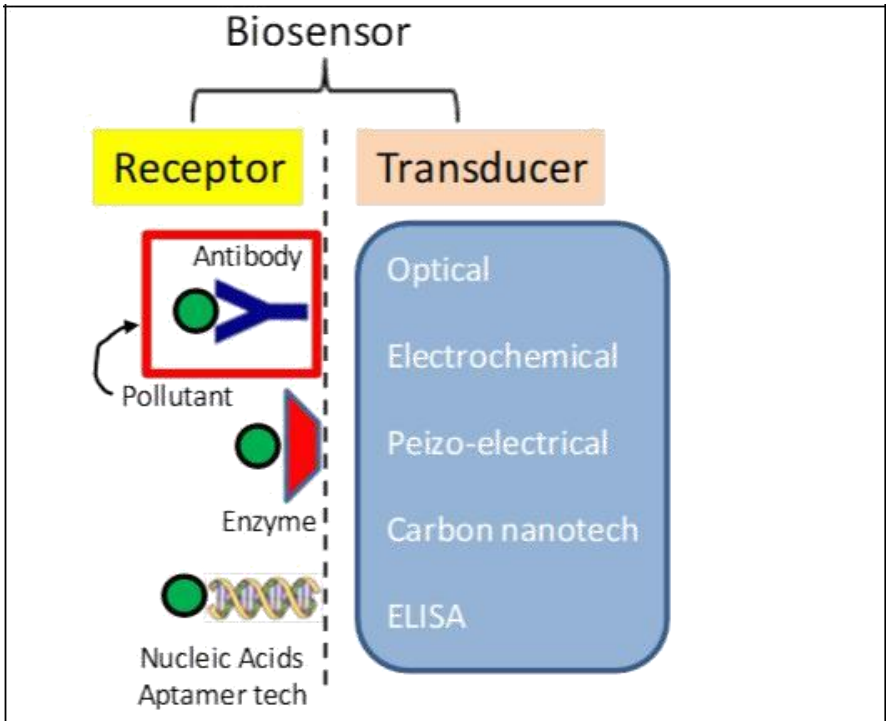


Figure 1: Synthetically Evolved Biosensors (SEBs)

The binding components of This *Synthetically Evolved Receptors -nanobodies (Nbs)* are commonly used due to its small size comparing to normal antibody and its specificity of binding to small molecules. In order to select the Nanobodies having binding specificity to target molecules, screening process requires the synthesis of toxicants & tag conjugates.

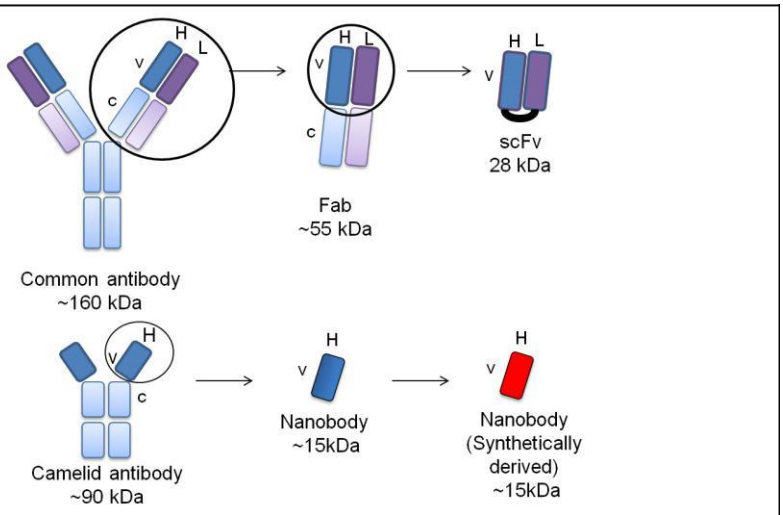


Figure 2: Synthetically Evolved Receptors (SERs) compared to common antibodies and their products.

1.1 SELECTION OF POLLUTANTS

All the compounds being synthesized were substances that are most commonly found at facilities on the National Priorities List (NPL) and which are determined to pose the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure at these NPL sites.

1.1.1 Polybrominated diphenyl ether

Lower-brominated (1-4 bromine atoms per molecule) diphenyl ethers have been known to affect hormone levels in thyroid gland. Studies have linked them to reproductive and neurological risks at certain concentration(Birnbaum, L. S., & Staskal, D. F. 2004). The existence of PBDE is quite common in domestic environment because of their prevalence in common foods. Also, because they are efficiently bioaccumulate and resistant to degradation process, people are easily exposed to PBDEs through food ingestion or inhalation.

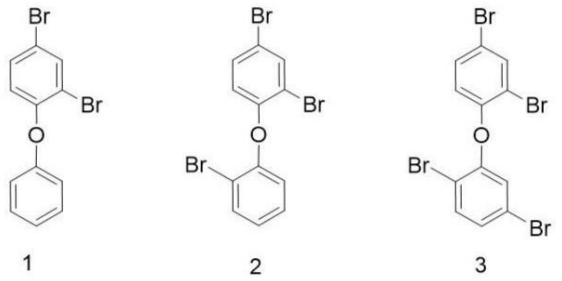


Figure 3: Chemical structure of polybrominated diphenyl ether synthesized in By Siegel Lab and used for nanobody sorting.

1.1.2 Phenanthrene

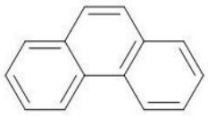


Figure 4: Chemical structure of phenanthrene

Phenanthrene is polycyclic aromatic hydrocarbon that contributes to both air and soil pollution. According to study, phenanthrene can induce cardiac hypertrophy[i] and is considered genotoxic (Huang, L & et als. 2016). The atmospheric Phenanthrene has shown to impair plant growth as well as biomass partitioning. (dos Santos Morais & et als, 2014).

1.1.3 Triclosan

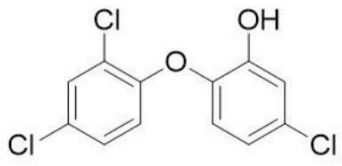


Figure 5: Chemical structure of triclosan.

Triclosan (sometimes abbreviated as TCS) is an antibacterial and antifungal agent found in some consumer products that was suspected to disrupt hormonal development. Triclosan is toxic to aquatic bacteria at levels found in the environment. It is highly toxic to various types of algae and has the potential to affect the structure of algal communities (Dorine Desalme & et als. 2011).

1.2 SELECTION OF TAGS

Commonly, the screening process requires using of dye upon the binding of nanobodies and targets. In our experiment, we synthesize the Pollutants-tag conjugate to treat the culture

immediately which will enable the binding target to have special traits upon binding. The screening involves both fluorescence separation and magnetic field separation to ensure the accurate and thorough detection of target nanobodies. 4-nitrobenzofurazan and Biotin were chosen to be the ideal tags.

1.2.1 4-nitrobenzofurazan (NBD)

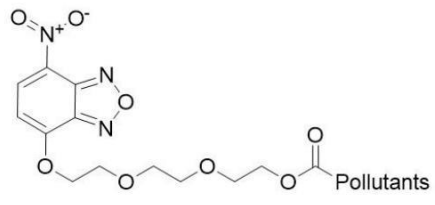


Figure 6: chemical structure of NBD-pollutant conjugate. The triethylene glycol chain was added to conjugate to increase its water solubility.

The 4-nitrobenzofurazan (NBD) fluorophore is a relatively small fluorescent probe with wavelengths well suited to in vitro biological systems. Furthermore, NBD is solvatochromic, exhibiting very low fluorescent activity in polar and protic environments but much increased fluorescence in hydrophobic environments. Suggesting that NBD fluorescent tag may allow the binding molecule to possess different fluorescence which differentiate it from the nonbinding background (Morris, S., Rowe, M., Holtzman, S., & Meinhold, A. 1993). In our experiment, the greenness of the conjugate will increase as the nanobody bind to the NBD-Pollutant conjugates. NBD has been used previously for labeling Free fatty acid receptor (Elisabeth Christiansen & et als. 2016) and small amine containing molecules (Gerardo Turcatti & et als. 1995).

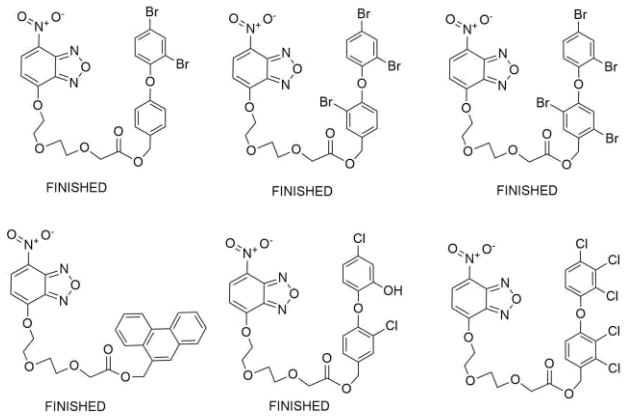


Figure 7: Chemical structure of NBD-pollutants conjugate

1.2.2 Biotin

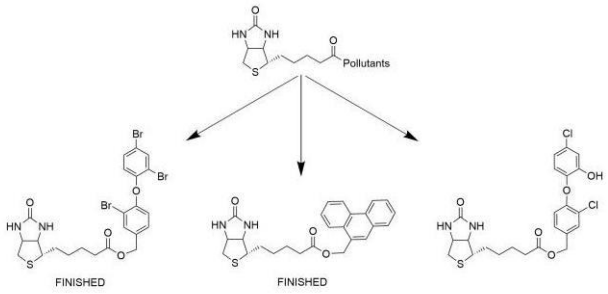


Figure 8: Chemical structure of the biotin pollutants conjugates.

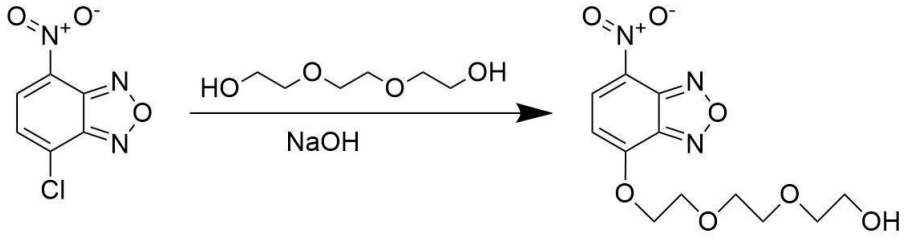
To make sure the most thorough screening of the Nbs, Geoffrey lab introduced a different binder----biotin to be conjugated to pollutants. Biotin is a common tag used for protein marking. Biotinylation with proteins and nucleic acids have been widely utilized to elucidate DNA sequence (Morris, S & et als. 1993), purify and detect tagged protein. In our experiment, we perform the biotinylation at terminal Carbonyl. With small molecule binding to Biotin, the biotin-pollutants conjugate can be used in affinity chromatography together with a column that has streptavidin bound to it, which is the natural ligand for biotin. So far, the tribrominated diphenyl ether, phenanthrene and triclosan all showed pretty good result in the NBD bonded fluorescence cell sorting, so those molecules were synthesized to bond to Biotin to afford magnetic sorting.

Chapter2: Experiment

2.1 SYNTHETIC CHEMISTRY PROCEDURES

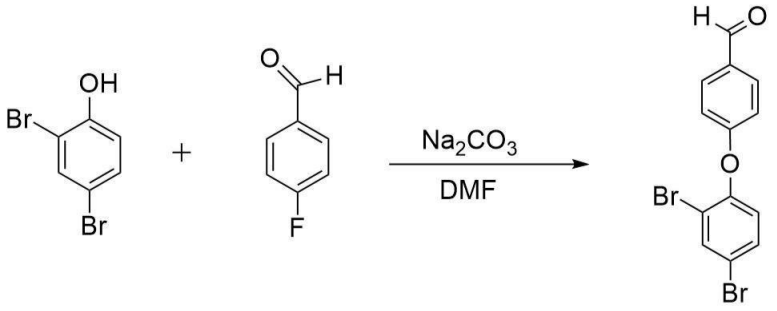
The synthesis of the tag-pollutant conjugates includes 2 large process: 1) the synthesis of the pollutants. Some of the pollutants can be purchased through market while some of the pollutants have to be synthesized in lab. 2) Biotin is directly purchased through market while NBD-triethylene glycol conjugate has to be synthesized in lab. The detailed procedure is provided in the following sections.

2.1.1



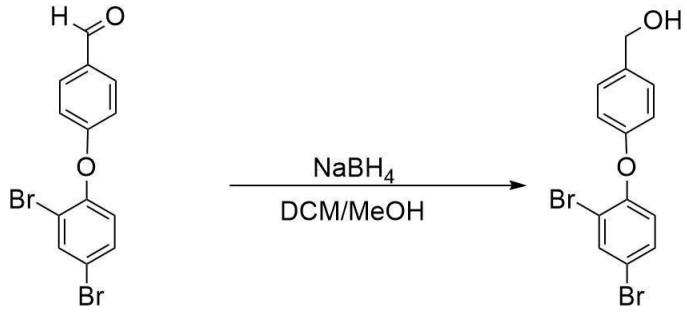
NBD-Cl (1.0 g, 5.0 mmol) was suspended in Triethylene glycol (10 ml) and treated with a solution of NaOH (0.4 g, 10 mmol) in triethylene glycol (20 ml) at room temperature. The reaction mixture was stirred for 1 h and then acidified with 5 M HCl (20 ml). The resultant aqueous layer was extracted with EtOAc (3 x 30 ml). The organic layers were dried over Na2SO4, filtered, concentrated and dried in vacuo. Flash chromatography (pure DCM to 1 :99 MeOH : DCM) afforded the final compound as an orange oil (1.33 g, 85 percent).

2.1.2



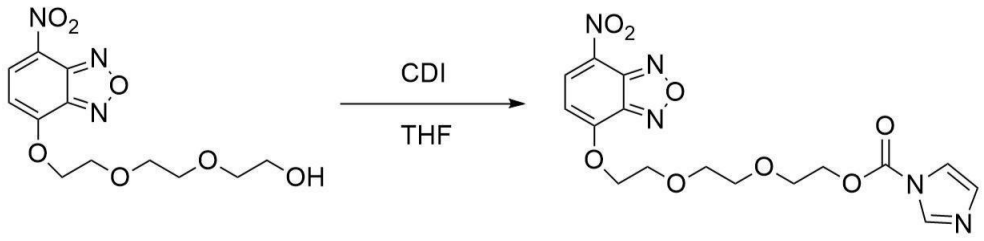
2,4-dibromophenol (1.241g, 4.93mmol) and 3-bromo-4-fluorobenzaldehyde (1g, 4.93mmol) was dissolved in 7 ml DMF (0.7M) with 0.679g (6.4mmol) Na_2CO_3 suspended in the solution. The mixture was heated to 170 ºC overnight. The reaction was diluted in 40 ml EtOAc and washed with Brine (25ml * 3). The organic layer was dried over Sodium Sulfite and concentrated to afford column Chromatography (10% EtOAC to 20% EtOAc in Hexane). 1.885g final compound was obtained (88% yield) with Rf = 0.6 in 20% EtOAc Hexane solution.

2.1.3



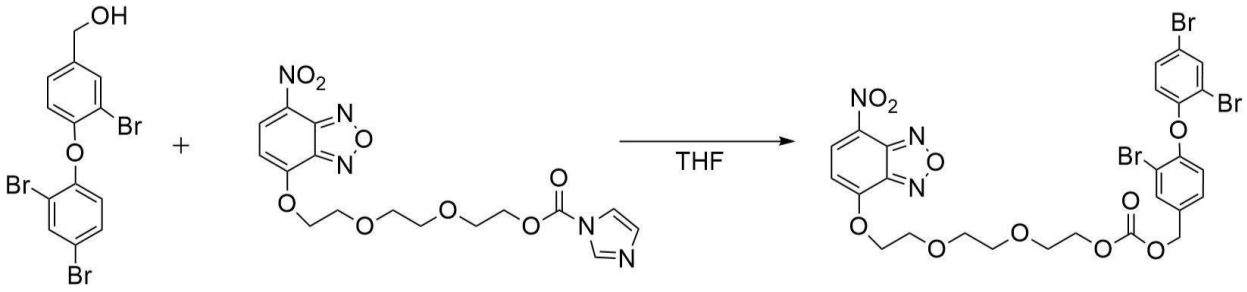
3-bromo-4-(2,4-dibromophenoxy)benzaldehyde(790mg, 1.816mmol) and Sodium Borohydride (82mg, 2.18mmol) are dissolved in 4ml 1:1 DCM/MeOH. The reaction was stirred for 3h and concentrated. The crude was flushed through column chromatography(EtOAC : Hexane).

2.1.4



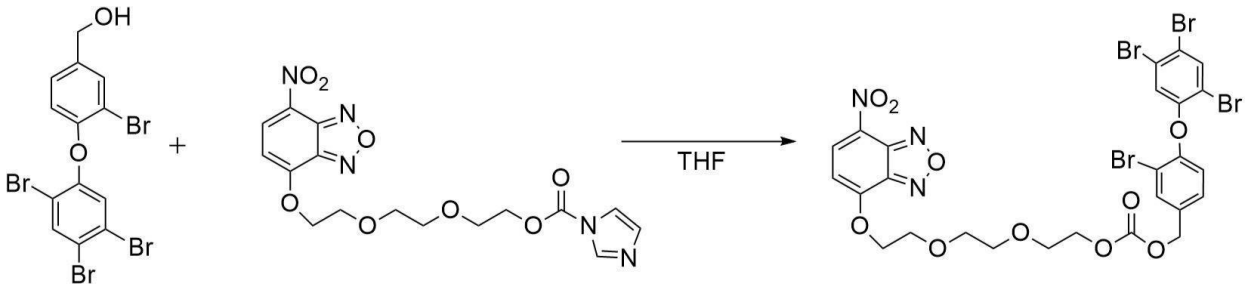
NBD-TEG conjugate was dissolved with 1.3 equiv. of carbonyl diimidazole in 0.1M THF and stirred at room temperature for 5 hours. The reaction mixture was concentrated and afforded after column chromatography (DCM to 1.5 : 98.5 MeOH : DCM) the NBD-Imidazole conjugate.

2.1.5



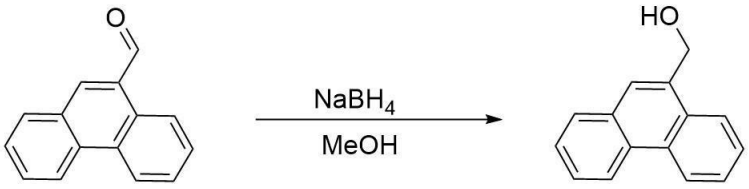
NBD-Triethylene glycol conjugate is mixed with Carbonyl diimidazole in 0.1M THF for 2 hours. Then 1 equivalence of 3-bromo-4-(2,4-dibromophenoxy) benzalcohol was added to the reaction. Let reaction stirred overnight, concentrated and purified through column chromatography(DCM/MeOH).

2.1.6



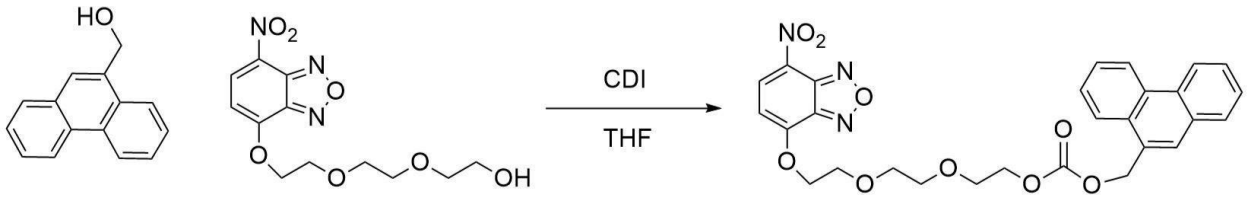
NBD-Triethylene glycol conjugate is mixed with Carbonyldiimidazole in 0.1M THF for 2hours. Then 1 equivalence of 2,5-dibromo-4-(2,4-dibromophenoxy)benzaldehyde was added to the reaction. Let reaction stirred overnight, concentrated and purified through column chromatography(DCM/MeOH).

2.1.7



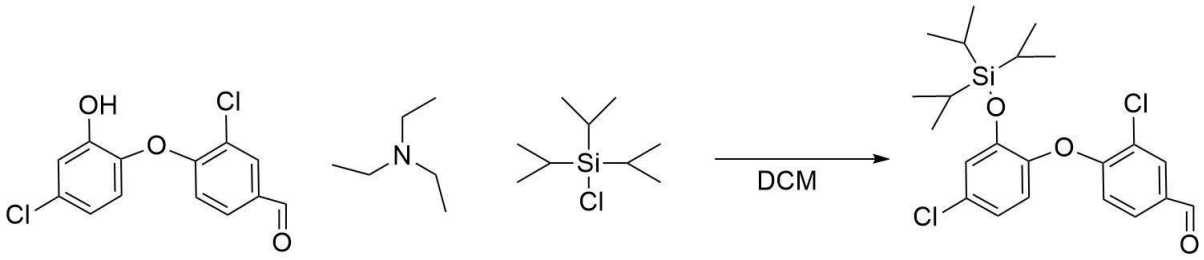
9-phenanthrenecarboxaldehyde is suspended in MeOH. 1.4 equi of $NaBH_4$ was added to the mixture and stirred for 2h. Then Acetone was added to react with excess of $NaBH_4$. The crude reaction was concentrated and flushed through the column chromatography to afford 9-phenanthrenecarboxalcohol.

2.1.8



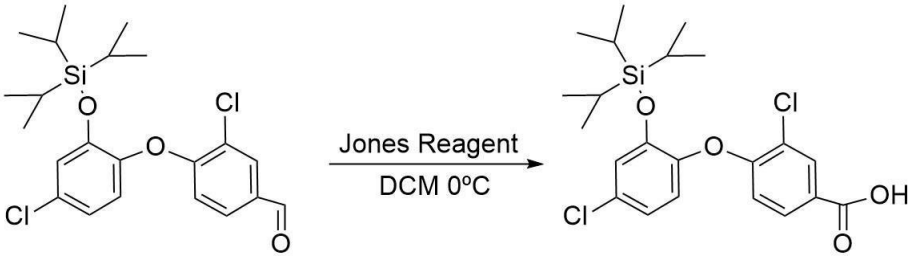
NBD-TEG conjugate was mixed with CDI in THF for 3h to get the mixture 1. At meantime 9-phenanthrenecarboxalcohol was dissolved in THF and adjust pH to 3 using NaOH. Then mixture 1 was added to the 9-phenanthrenecarboxalcohol solution and stirred overnight. The crude reaction was concentrated and purified through column chromatography(DCM/MeOH).

2.1.9



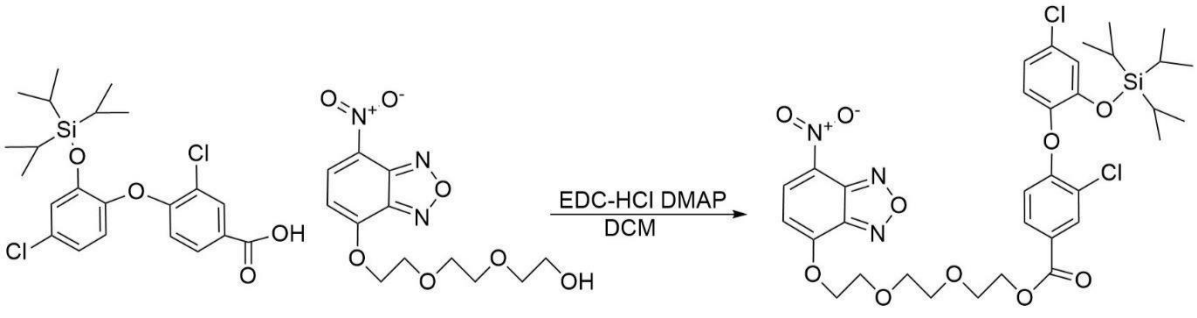
3-chloro-4-(4-chloro-2-hydroxyphenoxy)benzaldehyde is dissolved along with 1 equivalence of Triethylamine and Tips-Cl in DCM and stirred for 2h. The crude reaction was concentrated and directly used for next step.

2.1.10



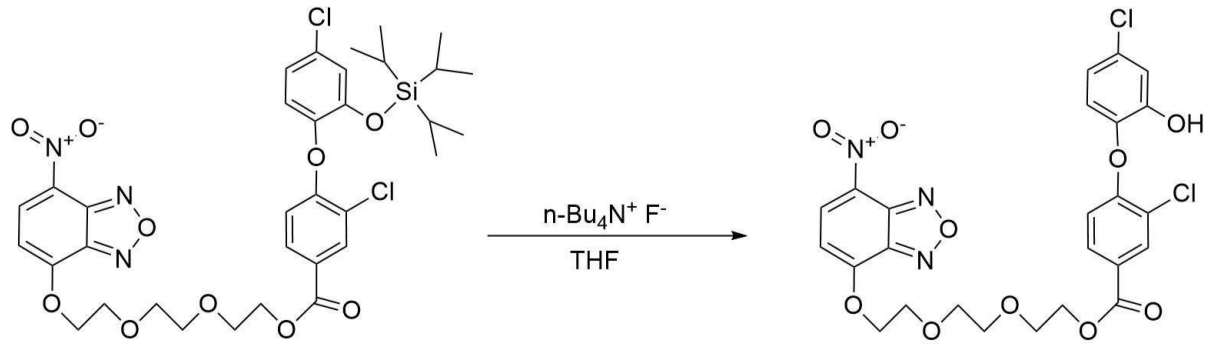
Tips-protected 3-chloro-4-(4-chloro-2-hydroxyphenoxy)benzaldehyde is dissolved in DCM and stirred in ice bath for 20mins. Then the 1.1equivalence of Jones reagent was added dropwisely into the reaction. Let the reaction run for 1h, then the reaction was diluted with EtOAC, quenched sodium thiosulfate and neutralized with $NaHCO_3$. The aqueous layer was extracted with EtOAC for 3 more times. The organic layer was dried over Na_2SO_4 and concentrated to afford column chromatography.

2.1.11



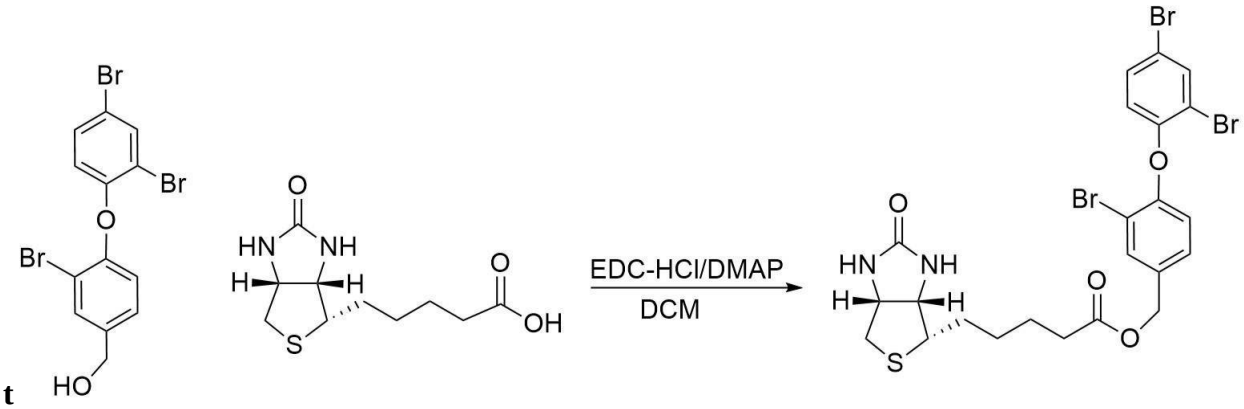
Tips-protected 3-chloro-4-(4-chloro-2-hydroxyphenoxy)benzoic acid, along with 1.3 equivalence of EDC-HCl & DMAP was dissolved in DCM and stirred for 10mins. After that, 1 equivalence of NBD-TEG conjugate was dissolved in DCM and added into the stirred mixture. After 10hours, the crude reaction was concentrated and purified by column chromatography.

2.1.12



Tips-protected NBD-triclosan conjugate was dissolved in THF and cooled to 0 degrees in ice bath. n-butyllithium was added slowly. Let the reaction run overnight. Crude reaction was concentrated and purified through column chromatography(DCM/MeOH).

2.1.13



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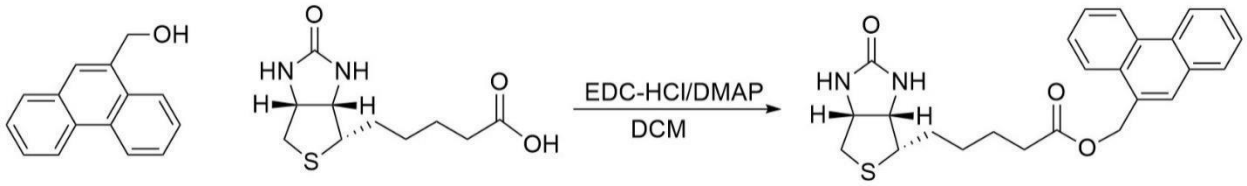
Biotin, along with 1.3 equivalence of EDC-HCl & DMAP, were dissolved in DCM and stirred for 10mins. After that, 1 equivalence of tribrominated diphenyl ether was dissolved in DCM and added into the stirred mixture. After 10hours, the crude reaction was concentrated and purified by column chromatography.

2.1.14



Biotin, along with 1.3 equivalence of EDC-HCl & DMAP, were dissolved in DCM and stirred for 10mins. After that, 1 equivalence afford 9-phenanthrenecarboxalcohol of dissolved in DCM and added into the stirred mixture. After 10hours, the crude reaction was concentrated and purified by column chromatography.

2.2 BIOLOGY EXPERIMENT DESIGN

The biological test of the compound is carried out by Geoffrey Chang's Lab in Skaggs School of Pharmacy and Pharmaceutical Science. The Nanobodies library is expressed on the bacterial cell surface and the selection of proper nanobodies (Nbs) start with treating the Nbs library with fluorescently conjugated pollutants. Nb binders were screened using 2 methods: 1) FACS Cell Sorter sort the cell based on fluorescence. 2) Biotin-Pollutants bonded Nbs were purified using magnetic beads. Initial Nb binders were usually rare and Geoffrey Chang's lab in UCSD utilized 2 method to propagate the targets. (1) The binders can be collected and plated on LB-agar with antibiotic. (2) The binders were pooled collectively together in liquid culture to form sub-library of antigen specific Nbs with corresponding DNA sequences that can readily undergo Genetic Algorithm INspired (GAIN) recombination to produce additional variants. The new generation of Nbs is re-grown as a population and binding checked again by adding fluorescently (magnetically) labeled pollutants. The further selection was achieved by lowering the concentration of conjugates and repeat the previous steps. The binding affinities could be checked by performing flow cytometry using cell analyzer which will give the standard binding curves. Once the Nbs having strong binding affinity to Pollutants were selected, the binding structure will be elucidated by crystal structure.

Chapter 3: Result

3.1 Proton NMR (H-NMR)

3.1.1 Tribrominated Diphenyl Ether – NBD Conjugate

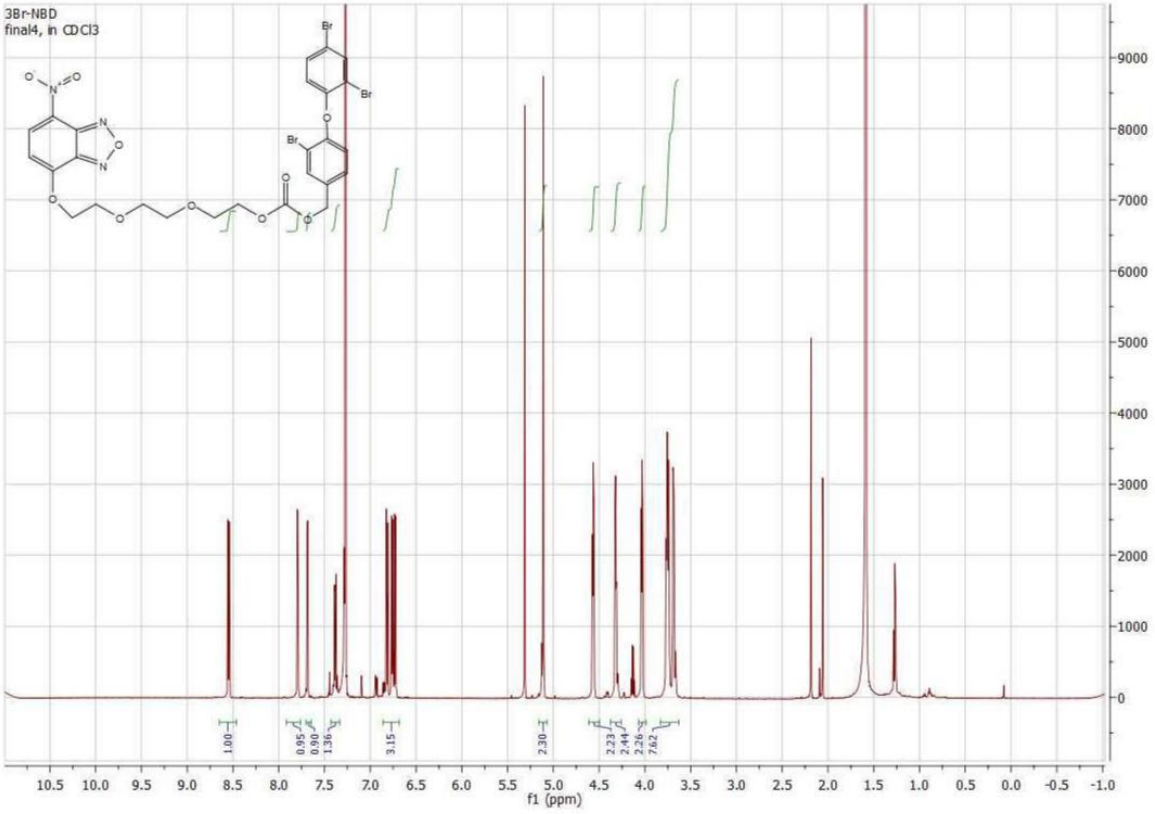


Figure 9: H-NMR of Tribrominated Diphenyl Ether – NBD Conjugate.

3.1.2 Tetrabrominated Diphenyl Ether – NBD Conjugate

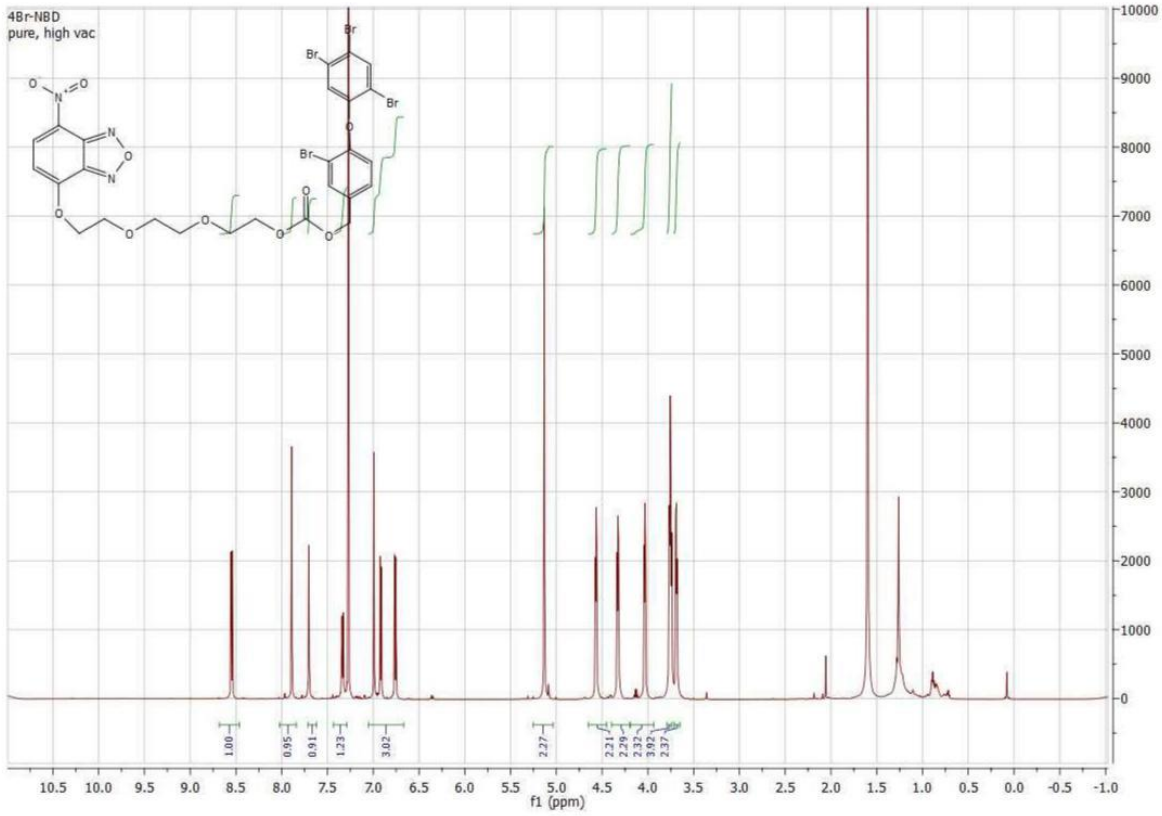


Figure 10: H-NMR of Tetrabrominated Diphenyl Ether – NBD Conjugate.

3.1.3 Phenanthrene – NBD Conjugate

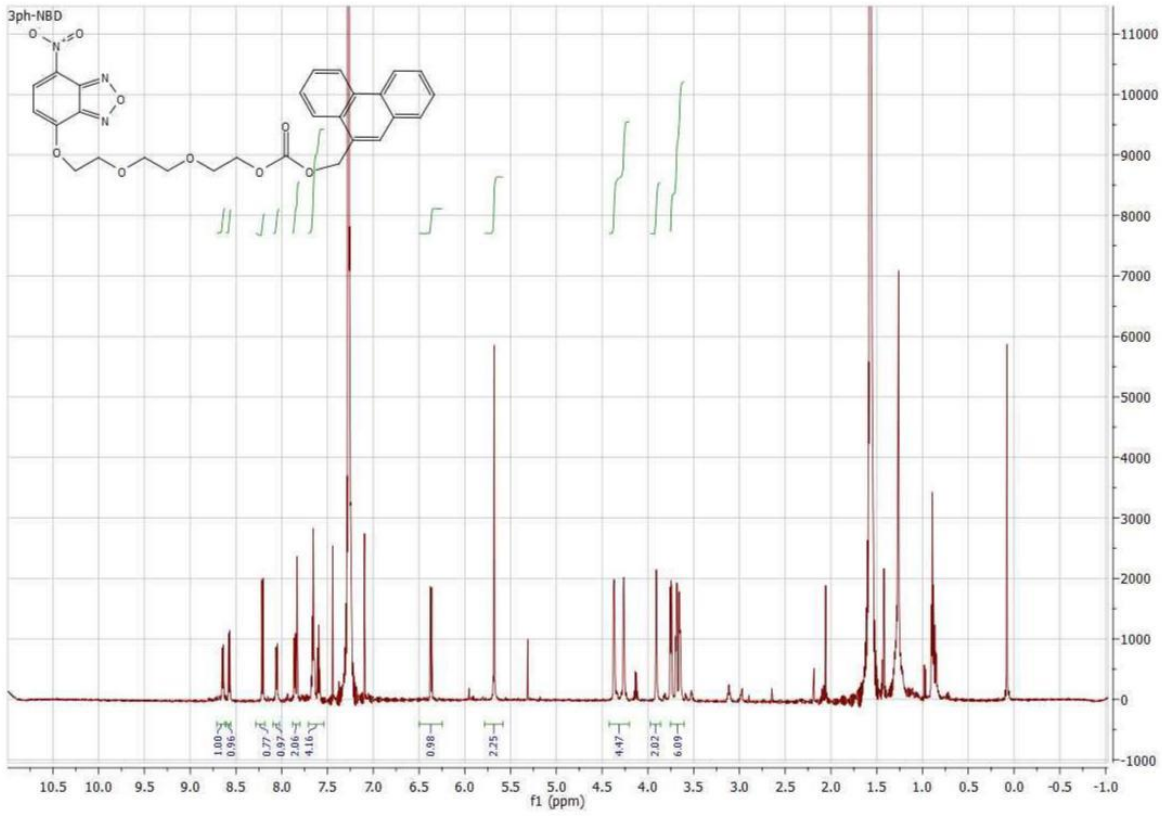


Figure 11: H-NMR of Phenanthrene – NBD Conjugate.

3.1.4 Triclosan – NBD Conjugate

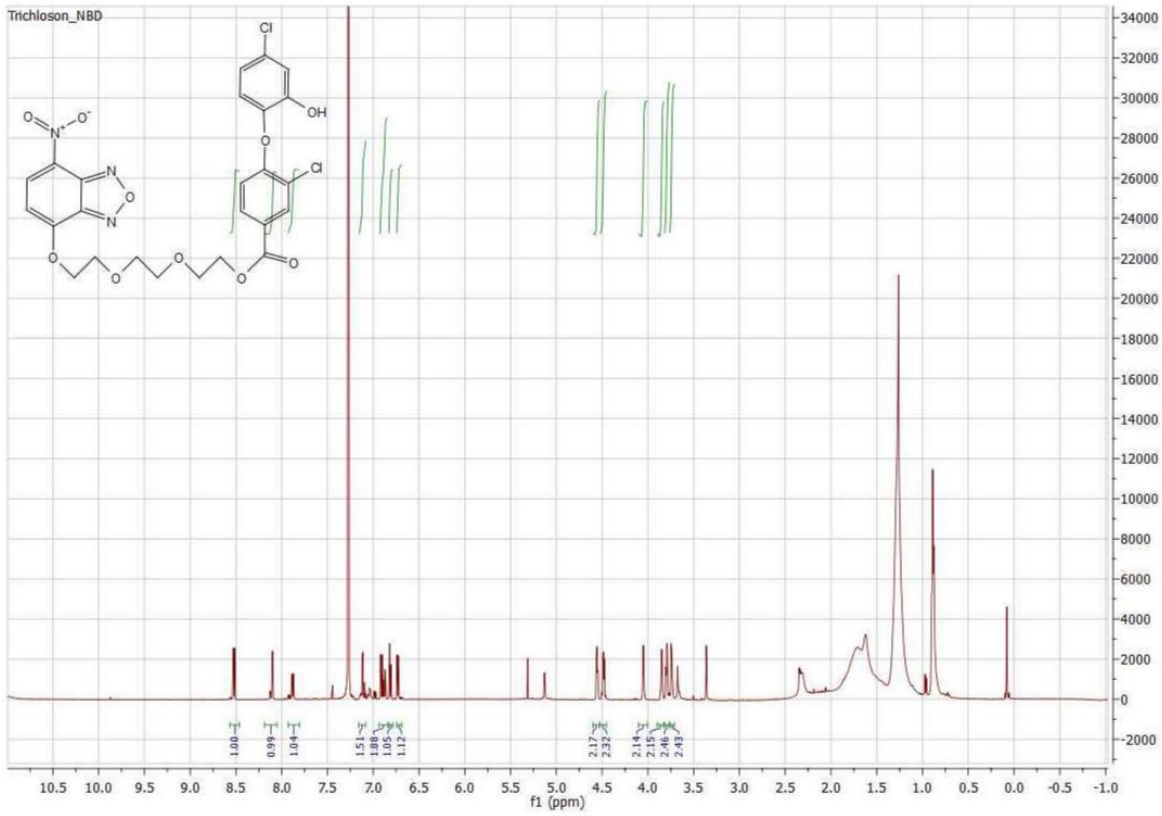


Figure 12: H-NMR of Triclosan-NBD Conjugate.

3.1.5 Tribrominated Diphenyl Ether – Biotin Conjugate

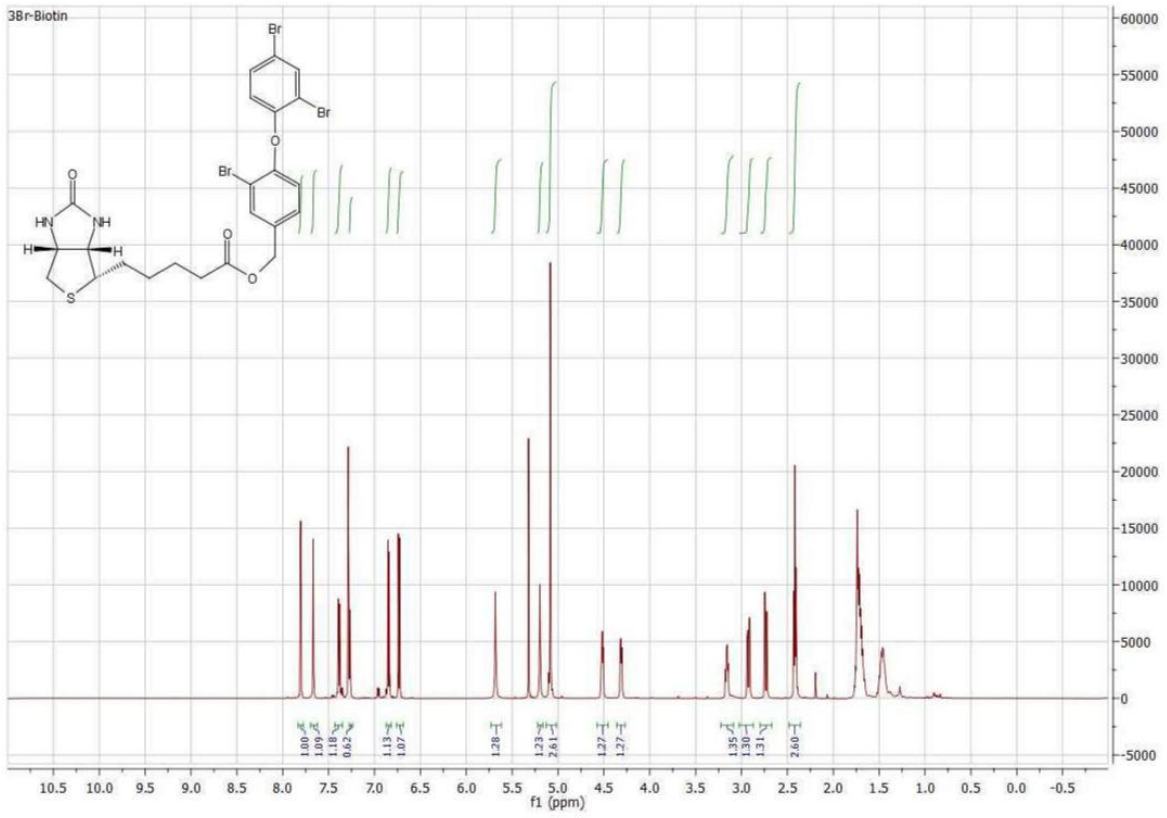


Figure 13: H-NMR of Tribrominated Diphenyl Ether – Biotin Conjugate.

3.1.6 Phenanthrene – Biotin Conjugate

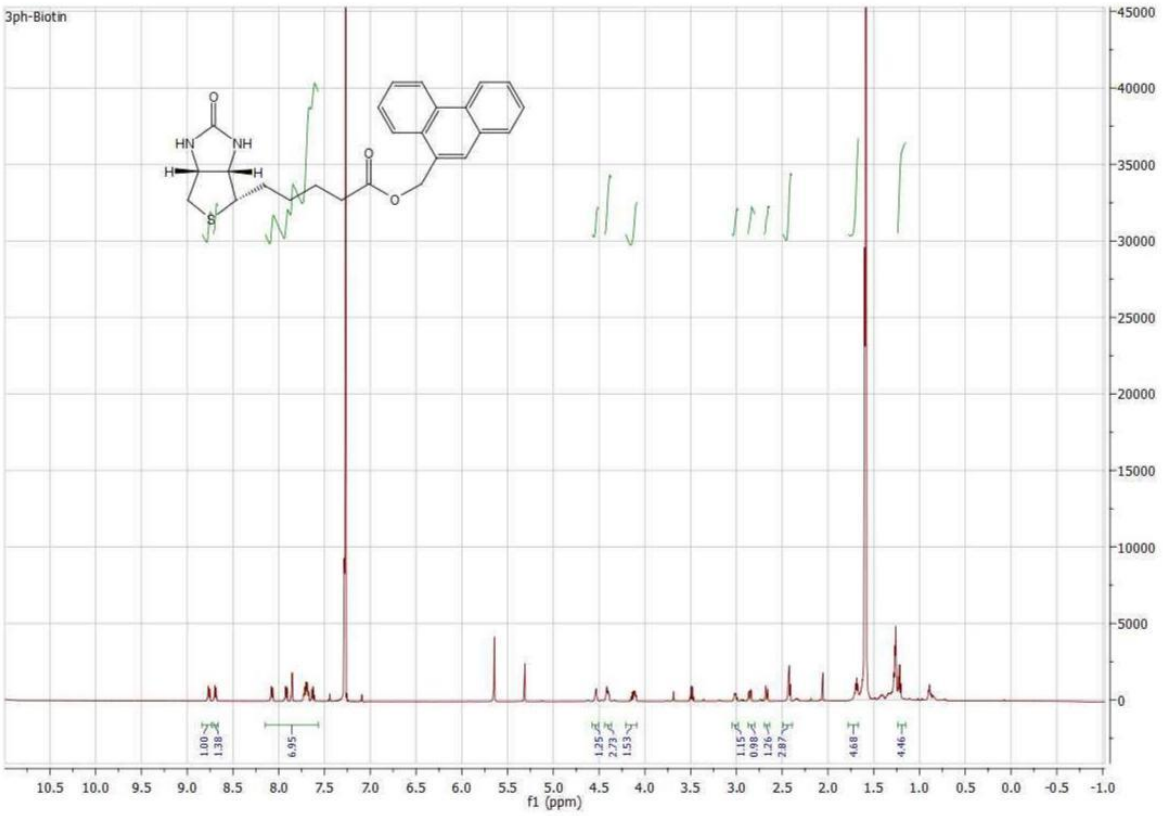


Figure 14: H-NMR of Phenanthrene – Biotin Conjugate.

3.2 FLUORESCENCE CELL SORTING RESULT

So far, the fluorescence cell sorting against triclosan, phenanthrene and tribrominated diphenyl ether have been completed. The figures regarding triclosan sorting have been made by Geoffrey Lab and will be presented in the following section.

3.2.1 Fluorescent Cell Sorting against Control Group

Graph shown below is the Control group. For the control, minor noise in system around proper bacterial size. No events on scale on green sort. The graph on the left shows the brightness of the clusters. The more clusters shown on the left, the closer the bonding between conjugates and the nanobodies on the cell.

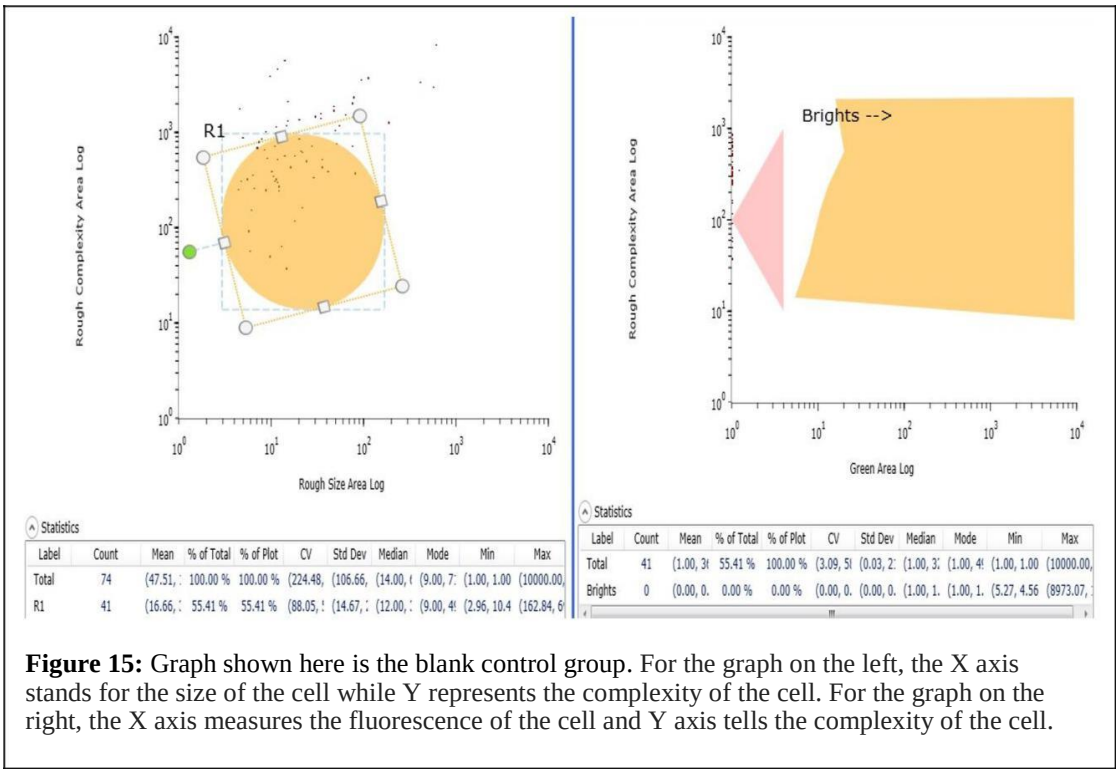


Figure 15: Graph shown here is the blank control group. For the graph on the left, the X axis stands for the size of the cell while Y represents the complexity of the cell. For the graph on the right, the X axis measures the fluorescence of the cell and Y axis tells the complexity of the cell.

3.2.2 Triclosan-NBD Sorting Against Naïve Library

The blank library was sorted against 1uM Triclosan-NBD conjugate. No events clearly away from the pack to indicate much brighter than everyone else. The population resides on brighter edge was collected and put into recombination to give generation 1.

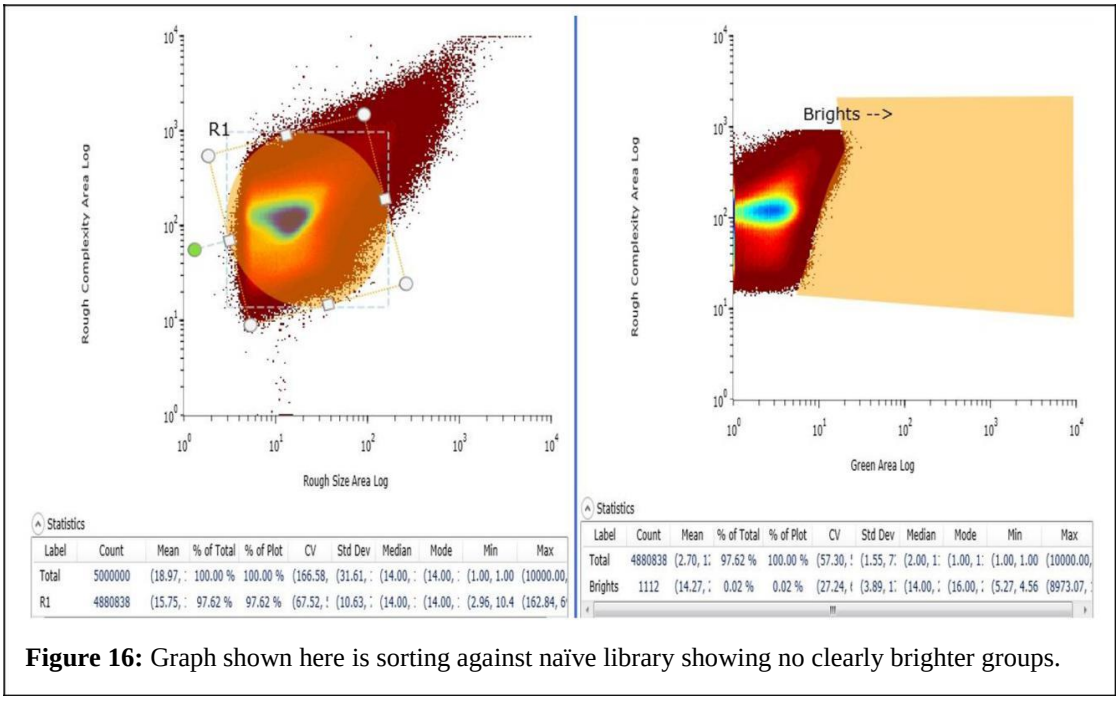


Figure 16: Graph shown here is sorting against naïve library showing no clearly brighter groups.

3.2.3 Triclosan-NBD Sorting Against Generation 1.

Generation 1 was treated with 1um Triclosan-NBD conjugate and sorted at 500nM. More events were observed breaking away from the edge comparing to the blank library. Then the events reside on and over the bright edge were collected and put straight into the recombination to give generation 2.

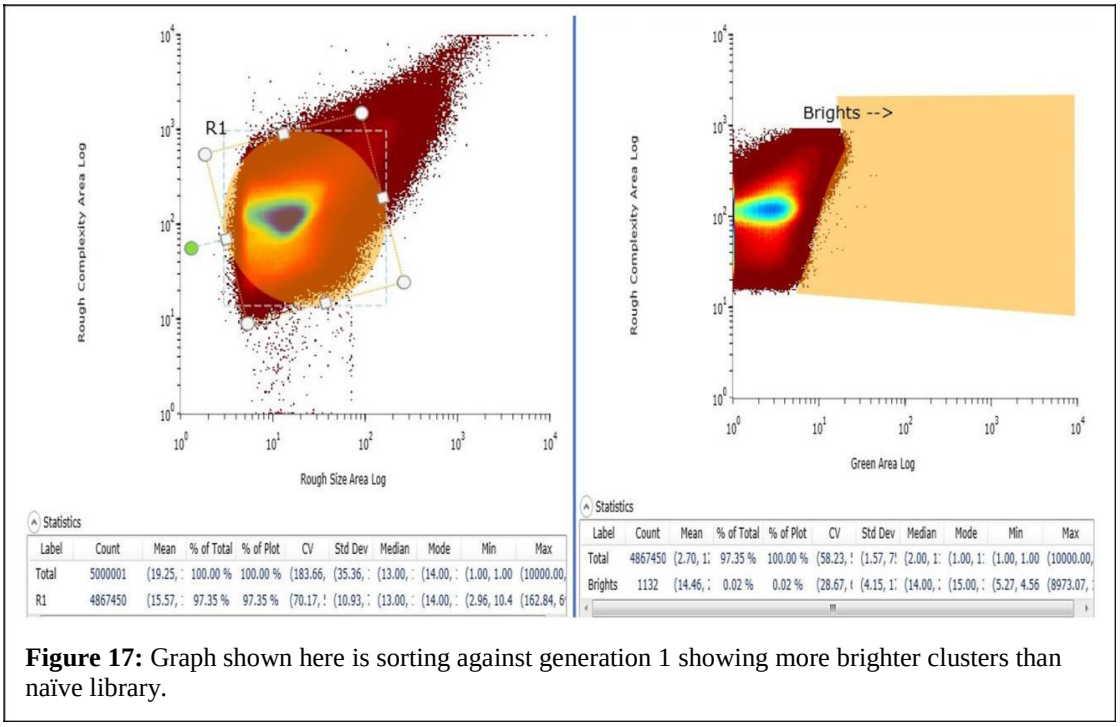


Figure 17: Graph shown here is sorting against generation 1 showing more brighter clusters than naïve library.

3.2.4 Triclosan-NBD Sorting Against Generation 2

Generation 2 was sorted against 50nM triclosanNBD. As the concentration of the ligand goes down, s pretty clear population that is much greener than the rest was observed(<0.1% of the sort was on the edge and brighter). Population 2 was screened to get a small population of 117 positive events which were then subjected to another round of recombination to get generation 3.

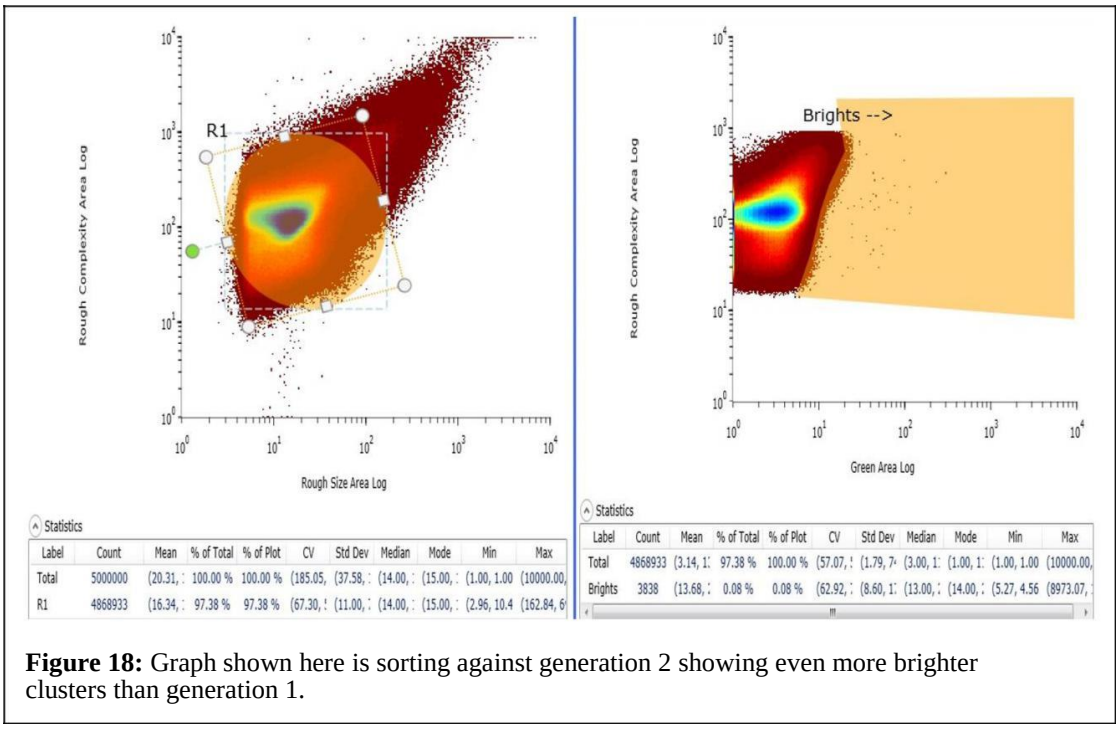


Figure 18: Graph shown here is sorting against generation 2 showing even more brighter clusters than generation 1.

3.2.5 Triclosan-NBD Sorting Against Generation 3

Generation 3 was sorted against 50nM triclosan-NBD. As the concentration of the ligand goes down, s pretty clear population that is much greener than the rest was observed (<0.1% of the sort was on the edge and brighter). Population was screened to get a small population of 117 positive events which were then subjected to another round of recombination.

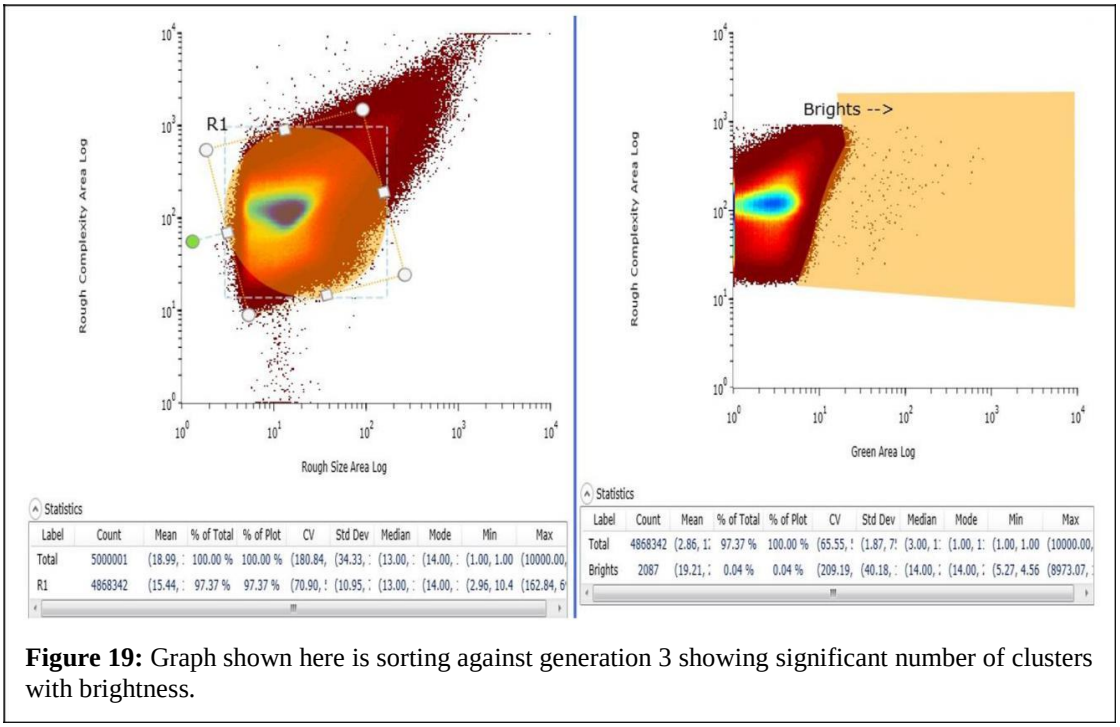


Figure 19: Graph shown here is sorting against generation 3 showing significant number of clusters with brightness.

Chapter 4: Discussion

4.1 Drug Mechanism of Action

The platform of conjugating fluorophore with small molecules for easier in vitro cell sorting has shown significant success in screening of Nbs. The same platform can also be utilized to achieve the in vitro visualization of the small molecules. For example, the other project that I worked on in cooperation with Winzeler Lab in School of medicine.

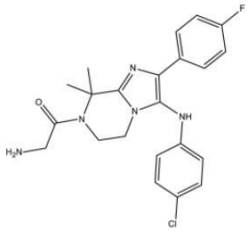


Figure 20: Chemical Structure of GNF179

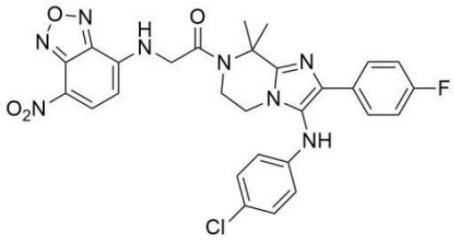


Figure 21: NBD-GNF conjugate.

The GNG-179 shown in Figure 20 is a drug developed by GNF that exhibit strong anti-malaria activity. The fluorescently tagged compound shown in Figure 21 enable researchers to identify the binding target. By taking the image of the compound working in parasite, we could possibly infer the mechanism of action and by tagging the potential target using fluorescent protein, we can see if the compound would colocalize thus validating the assumption.

4.2 Future Study on SERs

The gradually the increasing of clusters having stronger binding affinity to triclosan shown in figure 22 below which demonstrates that Genetic Algorithm INspired (GAIN) recombination did successfully produces variants that shows significant improvement in binding affinity. Once significant amount of nanobodies with decent binding affinity have been collected.

The control study with NBD-TEG will be performed to eliminate the nonspecific binding to pollutants. Then the nanobodies will be treated with pollutants themselves to form the crystal structure which will give more detail on the binding between pollutants and nanobodies.

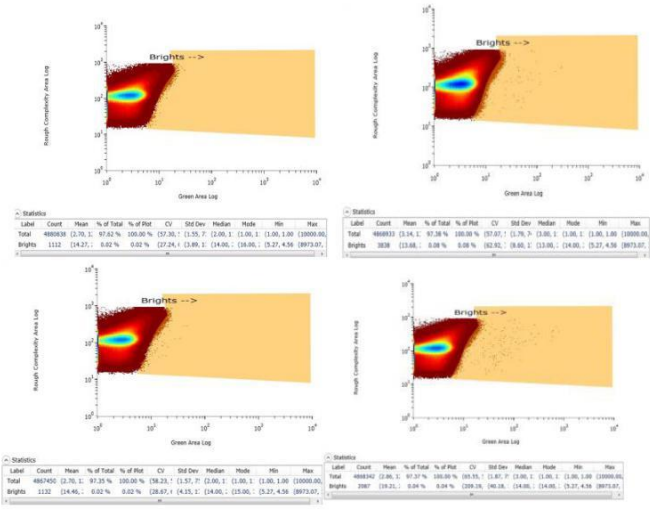


Figure 22: Increasing number of clusters with high binding affinity.

4.3 Biotin tagged pollutants

So far, the study regarding Biotin-pollutants conjugate has not started yet. Once the Biotin-pollutants conjugates have been mixed with Library for a while, the culture will be flushed through magnetic beads column. Due to the high binding affinity b/t streptavidin and biotin, the binded Nbs & conjugate was able to be separated from the rest. Then the Nbs with binding affinity will be recombinated to generate future generations which follow the similar concepts as the Fluorescence cell sorting.

This thesis uses unpublished material coauthored by Prof. Geoffrey Chang.

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