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UNIVERSITY OF CALIFORNIA,
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Engineering Nanobody-Modified Nanocarrier Proteins for HER2 positive Breast Cancer targeted therapeutics

THESIS

submitted in partial satisfaction of the requirements.
for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Xiaohe Ma

Thesis Committee:

Associate Professor Jered Haun, Chair

Associate Professor Michelle Digman

Assistant Professor Fangyuan Ding

2024

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DEDICATION

To

My Family and Friends

For their support and love

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ACKNOWLEDGEMENTS

I would like to express my profound gratitude to Professor Jered Haun for his tremendous patience and extensive support. His expertise and insights have not only enriched my two years of graduate studies but have also been immensely valuable to my personal and professional development.

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Finally, a special thank you to all my lab colleagues, particularly Ailin Zhang, who went above and beyond to assist and support me when I needed it most, helping steer my experiments to successful completion.

ABSTRACT OF THE THESIS

Designing Nanobody-Modified Nanocarrier Proteins for HER2 positive Breast Cancer targeted therapeutics

By

Xiaohe Ma
Master of Science
University of California, Irvine, 2024
Professor Jered Haun, Chair

The development of nanobody research holds the potential to revolutionize targeted therapies. This study focuses on nanoparticles functionalized with recombinant Anti-HER2 nanobodies to specifically bind to cancer cells that overexpress HER2 receptors. Utilizing the high affinity and specificity of nanobodies, these nanoparticles are optimized to serve as precise tools for recognizing and adhering to cancer cells. This method not only facilitates the direct delivery of drugs to tumor sites but also minimizes damage to healthy tissues. This method involves constructing recombinant proteins with the 2Rb17c HER2 nanobody in the pRS-4420 vector and expressing the proteins in Saccharomyces cerevisiae. The recombinant nanobody proteins were successfully conjugated to nanoparticles. This study demonstrates the potential for future applications in targeting HER2-positive cells, offering promising prospects for enhanced binding efficacy in targeted therapies.

INTRODUCTION

Breast cancer become the second most common cancer, affecting millions of women worldwide. [1] Based on the immunohistochemical expression of hormone receptors, human epidermal growth factor receptor positive (HER2+) cancer as one of the most common subtypes of breast cancer, accounts for approximately 15-30% and serves as a biomarker. [2] The overexpression of HER2 amplifies loci on tumor cells, which enhances the signaling of tyrosine kinase receptors. This amplification leads to increased cell proliferation and reduced apoptosis. Consequently, HER2 overexpression is linked to the identification of metastatic or recurrent breast cancer in 50% to 80% of cases, thereby improving diagnostic accuracy. [3] The development of targeted therapy for HER2-positive cancers reached a pivotal milestone with advent of trastuzumab, the first monoclonal antibody specifically designed to target HER2. This approach significantly differs from traditional cancer treatments such as chemotherapy, radiation, and surgery, which are broadly cytotoxic, by providing a more focused method that minimizes the side effects. [4]

Monoclonal antibodies are highly effective in identifying tumor-associated antigens, yet they present certain functional constraints, including limited penetration into solid tumors and minimal interaction with the immune system. [5] Single-chain antibodies (scFv) encompass the entire antigen-binding site, composed of the variable heavy (VH) and variable light (VL) chains of an antibody. [6] The compact structure of ScFv enables their synthesis in basic prokaryotic organisms. Although these antibody fragments maintain the same antigen affinity as traditional full antibodies, they result in fewer immunogenic responses and enhanced penetration to tumor site. [7]

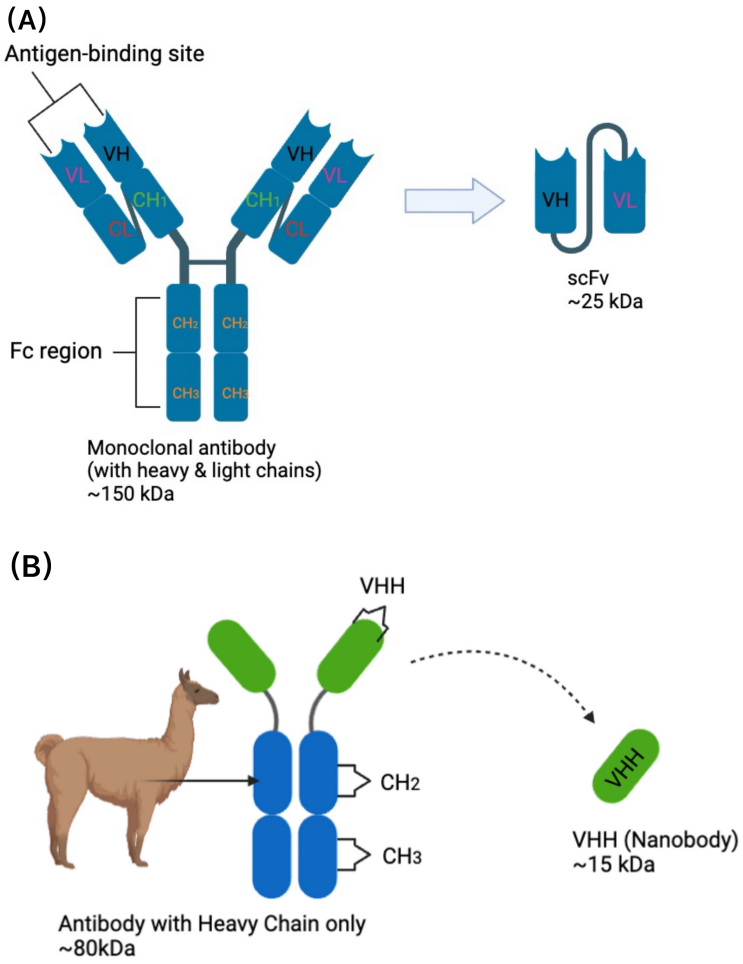

Figure 1. (A) Conventional monoclonal antibody to a single-chain variable fragment (scFv). On the left, the monoclonal antibody is shown with its heavy and light chains, highlighting the antigen-binding sites composed of variable heavy (VH) and variable light (VL) regions. The right side of the figure illustrates the scFv, where the VH and VL regions are linked by a flexible linker, reducing the molecular weight from approximately 150 kDa to about 25 kDa. **(B) Camelid Antibodies to Nanobodies.** The derivation of a nanobody from a heavy-chain-only antibody in camelids is illustrated. The VHH segment detaches to form an isolated nanobody, reducing in size from approximately 80 kDa in the full antibody to about 15 kDa as a standalone fragment.

Despite the advancements in single-chain antibodies (scFv), which enhance tumor site penetration due to their smaller size, the development of nanobodies—derived from heavy-chain only antibodies (HCAbs) in Camelidae—offers a more refined solution.[8] These nanobodies not only overcome challenges such as stability under physiological conditions but also provide the benefit of even smaller size for improved penetration. [9]

In camelids, antibodies composed solely of heavy polypeptide chains, which lack light chains, remain functional in antigen binding.[10] Nanobody typically consists of a single heavy-chain variable domain (VHH) and remain a full capacity for specific antigen binding.[11] The complementarity-determining regions (CDRs) in nanobodies are modified compared to those in human antibodies, contributing to the enhanced solubility and stability of nanobodies.[12] Nanobodies have three CDRs, the CDR3 region plays a crucial role in determining both the specificity and affinity for antigen compared to CDR1 and CDR2.[13] The length of CDR3 in nanobodies is longer than in regular antibodies, which provides a greater degree of flexibility to cope with diverse and recessed epitopes on antigen.[14][15]

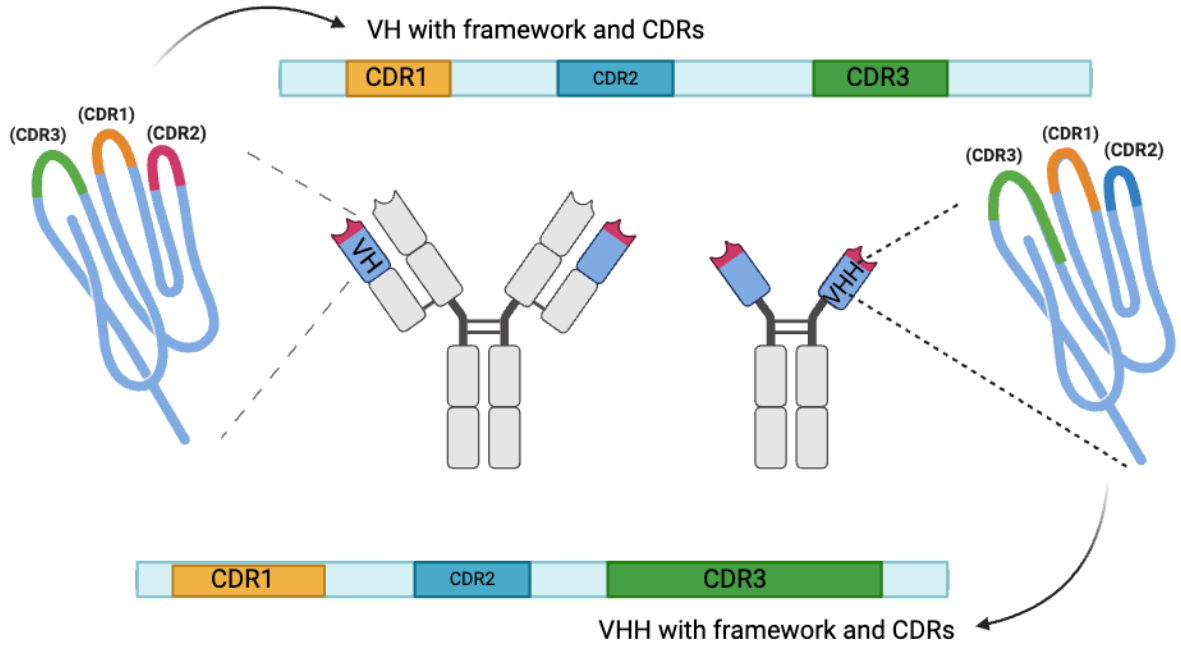

Figure 2. Structure of Framework and CDRs for VH in Conventional Antibodies and VHH Derived from Camelids.
VHH includes CDR1, CDR2 and an extended CDR3, which enhancing binding capability.

Nanotechnology plays a crucial role in enhancing drug delivery and imaging for cancer therapy and detection. It reduces degradation during systemic delivery and accurately targets drugs to the correct sites. Active targeting of nanocarriers enhances the efficacy of tumor targeting by using ligands such as antibodies, nanobodies, or other cancer-specific antigens.[16][17] The specific properties of multivalent binding in nanocarriers enable them to bind with multiple ligands, targeting receptors on the cell surface. This capability enhances the precision and effectiveness of interactions by increasing the avidity of the nanocarriers.[18][19] The increased number of receptors on tumor cells provides a significant platform for targeting ligands, as exemplified by HER2 nanobodies (Nbs) that target HER2 receptors.[20]

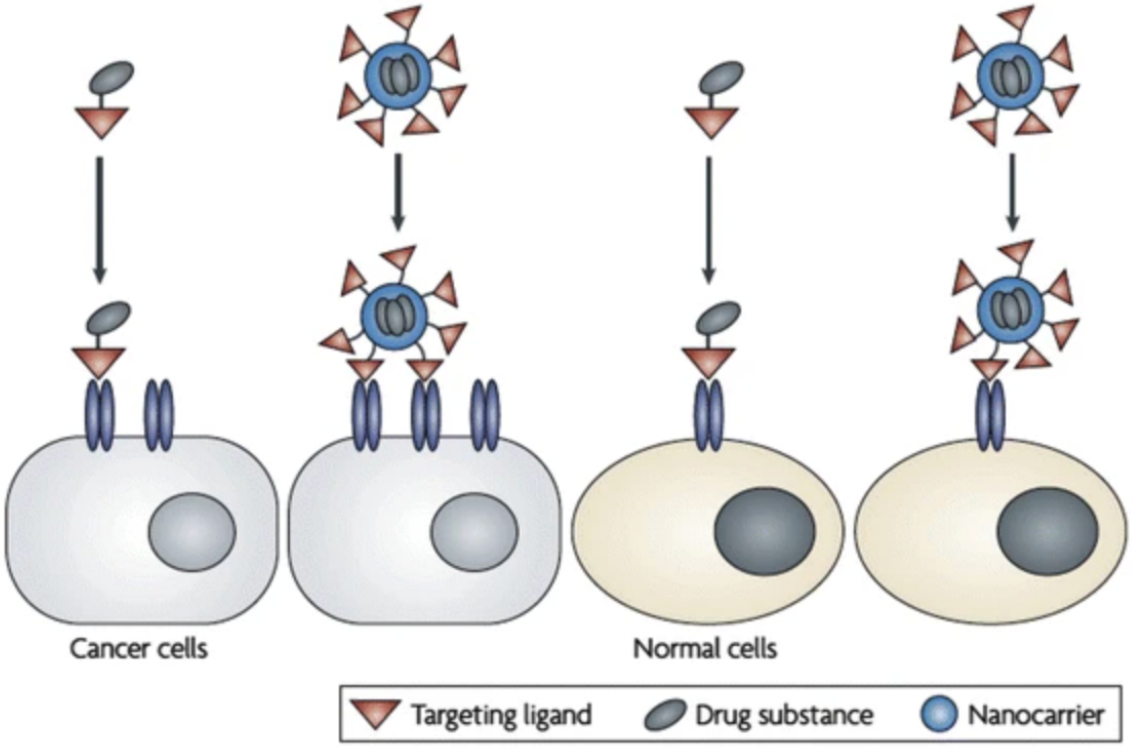

Figure 3. selective targeting mechanism of nanoparticles designed for cancer therapy.[21]
The nanoparticles are decorated with multiple targeting ligands that enable strong multivalent binding to cancer cells, which typically exhibit a high density of specific receptors. This targeted binding allows for the efficient delivery of the drug substance carried by the nanoparticles specifically to cancer cells while minimizing the interaction with normal cells, which have a lower density of the corresponding receptors.

In the previous study, selecting single-chain variable fragments (scFvs) with different binding characteristics could influence the adhesive behavior of nanoparticles under fluid flow. Also, increasing the valency and size of the adhesion molecules could directly enhance the rate of nanoparticle attachment without the need to alter bond kinetics. Thus, the prior findings production of existing pRS (4420) expression vectors offered a strategic direction for designing nanobodies with optimized adhesive properties.[18]

This study focuses on developing nanoparticles functionalized with recombinant Anti-HER2 Nanobodies to bind specifically to cancer cells that overexpress HER2 receptors. Leveraging the high affinity and specificity of nanobodies, these nanoparticles are engineered as precision tools for the accurate recognition and adherence to cancer cells. This approach not only facilitates the targeted delivery of therapeutic agents directly to cancer cells but also minimizes damage to healthy tissues.

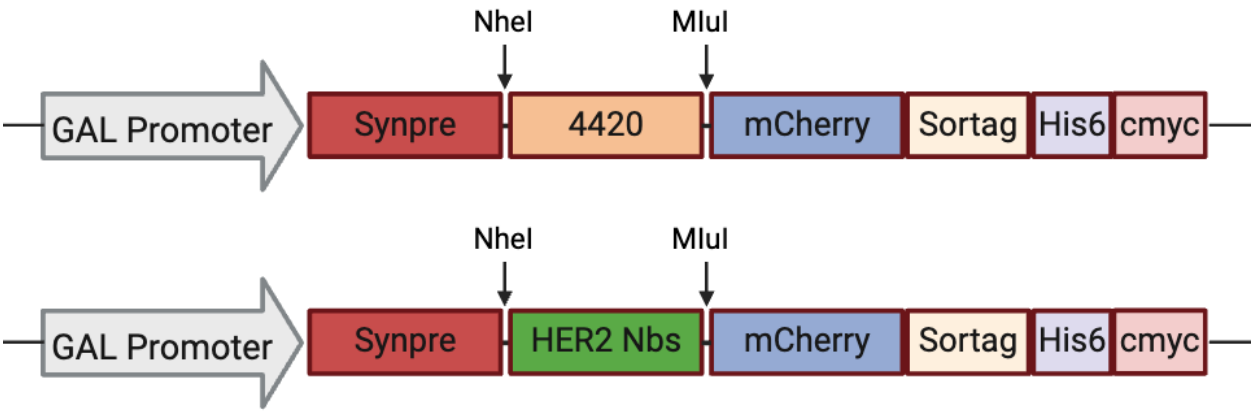

Figure 4. General Construct of pRs (4420) modified with HER2 Nbs.

Additionally, by synthesizing our knowledge of scFv binding characteristics with nanoparticle attachment behavior, we aim to enhance the targeting and treatment of cancer cells. The introduction of recombinant anti-HER2 nanobodies and the utilization of MSNs are expected to improve the delivery efficiency of therapeutic drugs, offering a more precise and effective strategy for cancer treatment. This innovative method represents a significant advancement in cancer therapy, pushing towards more personalized and targeted treatment options.

Chapter 1: Construction of recombinant proteins with 2Rb17c in pRs-4420 vector

1.1 Introduction

In prior research, the engineering of fluorescent proteins aimed to enhance their binding affinity by augmenting their molecular dimensions. Extending this research, we have developed a series of nanobody-based proteins that enable precise targeting of the well-established biomarker, HER2.

Within the scope of this chapter, a HER2-specific nanobodies (Nbs) 2Rb17c, has been cloned into the pRS-4420-mcherry-sortA-his6-cymy plasmid derived from preceding studies. The 4420 regions has been substituted with the strategically designed antibody and nanobody proteins. This includes the insertion of the Nb sequence between the NheI and MluI restriction sites in the pRS-4420-mcherry-sortA construct.

1.2 Materials and Methods

1.2.1 Reagents, Materials and Kits

All reagents and materials for Gene cloning and propagation are posted in Appendix A.

1.2.2 Plasmid Design

Based on the previous pRs-4420-mcherry-sortag-his6-cmyc plasmid, the Nbs sequence has replaced the 4420 segments.

Primers (listed below) have been designed to link the inserted gBlock to the appropriate restriction enzyme site.

Figure 5. Construct of 2Rb17c nanobody with pRs-based plasmid

Primers	sequence
Enzymatic_F	5'ATCAGTACGCCATGAGACGC 3'
Enzymatic_R	5' AGATCGAATGCATGGACGCCT 3'
2Rb17c_F	5'GCTAGCGAAGTTCAGCTGCAG 3'
2Rb17c_R	5'ACCTCCACCACGCGTAGAA 3'

Table1. Primers used for PCR cloning.

Completed sequence of 2Rb17c gBlock:

5'ATCAGTACGCCATGAGACGCGCTAGCGAAGTTCAGCTGCAGGAATCTGGTGGTGGTCTGGTTC
AGCCGGGTGGTTCTCTGCGTCTGTCTTGCGCGGCGTCTGGTTTCATCTTCTCTAACGACGCGATG
ACCTGGGTTCGTCAGGCGCCGGGTAAAGGTCTGGAATGGGTTTCTTCTATCAACTGGTCTGGTAC
CCACACCAACTACGCGGACTCTGTTAAAGGTCGTTTCACCATCTCTCGTGACAACGCGAAACGTA
CCCTGTACCTGCAGATGAACTCTCTGAAAGACGAAGACACCGCGCTGTACTACTGCGTTACCGGT
TACGGTGTTACCAAAACCCCGACCGGTCAGGGTACCCAGGTTACCGTTTCTTCTACGCGTAGGCG
TCCATGCATTCGATCT 3'

1.2.3 Construction of plasmid pRs-2Rb17c-mcherry-sortag-his6-cmyc

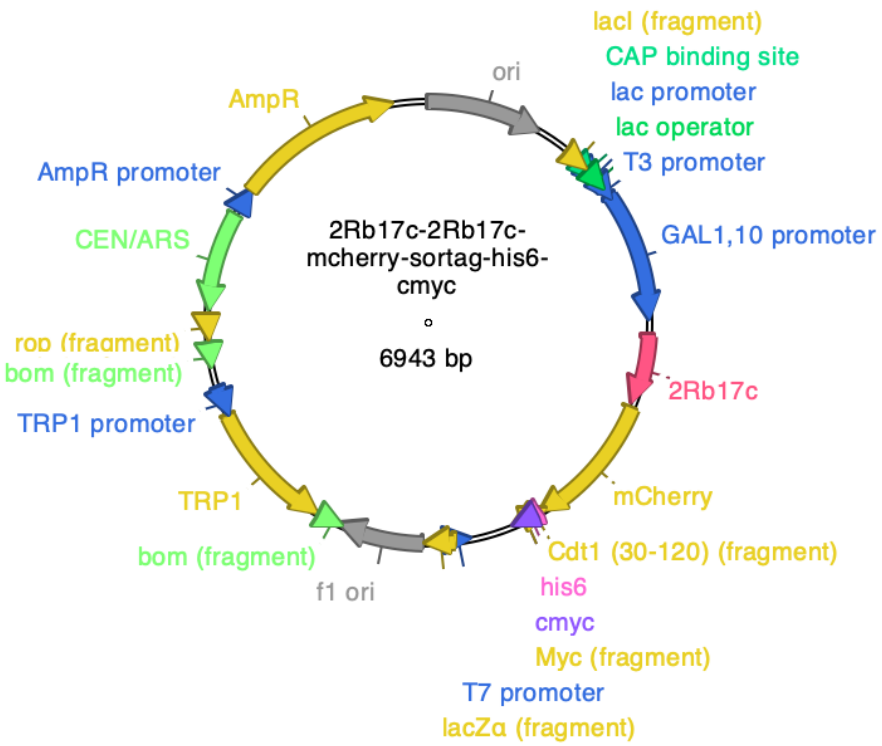

Figure 6. Plasmid map of pRs-based 2Rb17c Nb. pRs-2Rb17c-mcherry-sortag-his6-cmyc constructed by GAL1,10 promoter (blue), 2Rb17c Nb (red), mcherry(yellow), his6 tag (pink), and cmyc(purple). 2Rb17c Nb inserted into plasmid by restriction enzyme site NheI and MIu1.

In this study, the polymerase chain reaction (PCR) was employed to replace the 4420 regions with 2Rb17c nanobodies (Nbs). Double-stranded DNA (dsDNA) templates for the nanobody proteins were procured as gBlocks from Integrated DNA Technologies (IDT). These gBlocks were combined with deoxynucleotide triphosphates (dNTPs), Vent DNA polymerase, and both sense and antisense primers to introduce the restriction enzyme sites NheI and MluI. Following the PCR, both the pRs-4420-based plasmid and the PCR products were digested to generate flanking ends with NheI and MluI for ligation. Gel electrophoresis was utilized to confirm the successful double digestion of both the plasmid

and the PCR products. Subsequently, the desired fragments were extracted using the Qiagen Gel Extraction Kit.

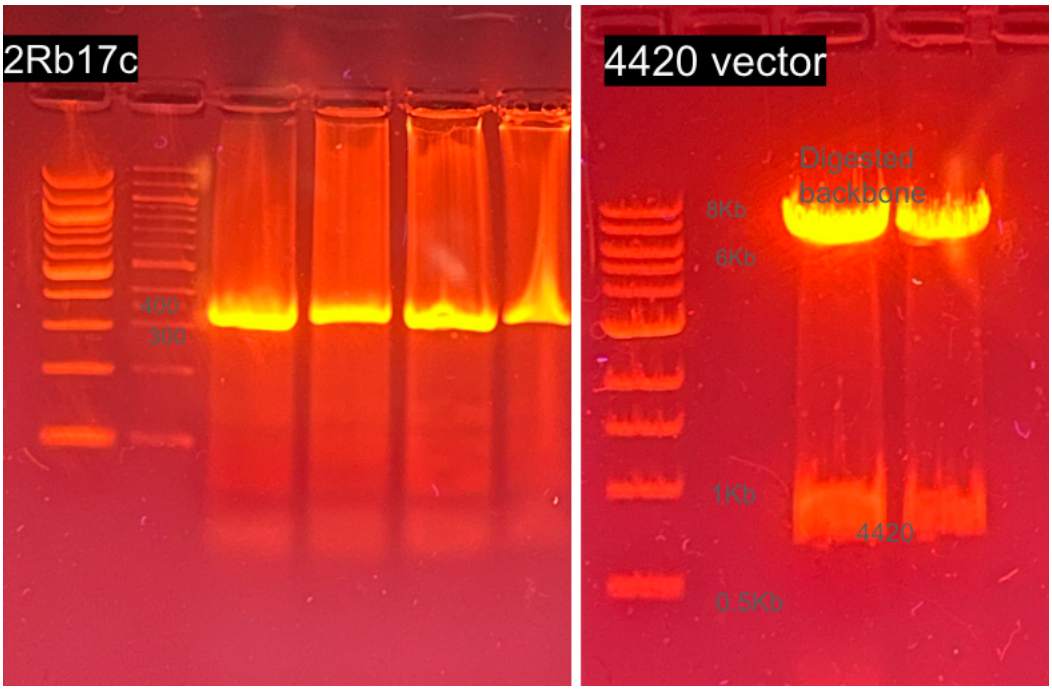

Figure 7. Gel electrophoresis of 2Rb17c (left) and 4420 (right).
2Rb17c's expected size is 357, which the amplified product aligns around 350bp band of the ladder. Digested Vector' expected size is 6592bp and expected digested 4420's expected size is 765bp. the Amplified digested vector aligns between 8Kb and 6Kb band of the ladder, Amplified 4420aligns is between 1Kb and 0.5Kb band of the ladder.

The digested plasmid was ligated to the PCR products of gBlocks 2Rb17c, 2Rs15d, and ML39 using T4 ligase at a 3:1 molar ratio of PCR product to plasmid. This ligation was conducted at room temperature overnight to produce the recombinant plasmid pRs-(2Rb17c)-mCherry-Sortag-His6-cMyc.

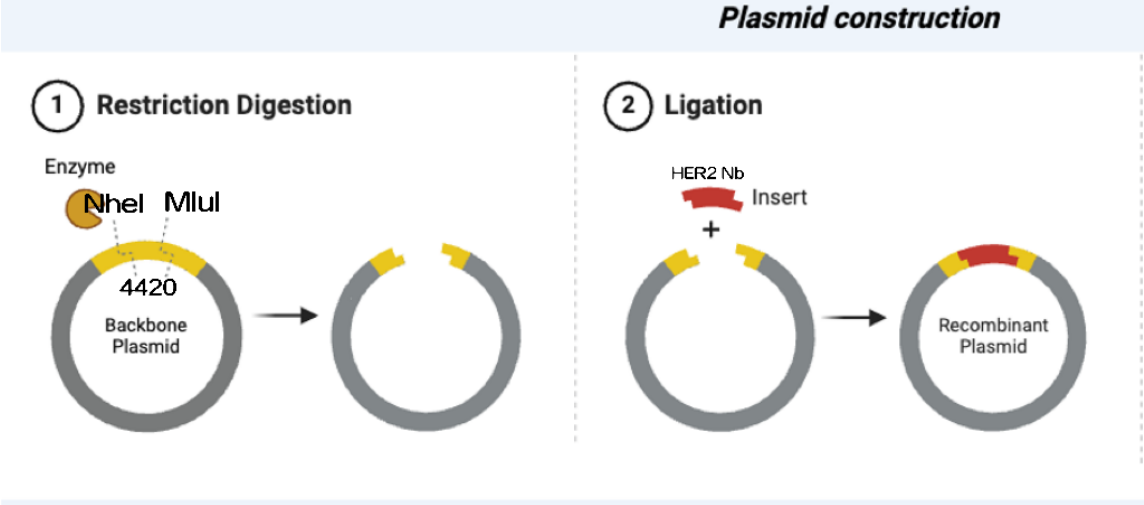

Figure 8. Process of plasmid construction.
The backbone plasmid is cut using specific restriction enzymes, NheI and MluI, at designated sites, creating linear fragments from the circular DNA. The original plasmid backbone, marked at position 4420, is shown being cleaved to prepare for the insertion of new genetic material. In the second step, the cut plasmid backbone is joined with the insert, which in this case is a HER2 nanobody (Nb).

1.2.4 Transformation of pRs-(2Rb17c)-mCherry-Sortag-His6-cMyc

The plasmid was transformed into DH5 alpha E. coli. First, the E. coli cells were thawed on ice for 30 minutes before the transformation. Then, 2 µL of the extracted plasmid DNA was added to 50 µL of DH5 alpha E. coli on ice, followed by incubation for 30 minutes to ensure effective plasmid uptake by the cells. After that, the cells were subjected to a heat shock at 42°C for 20 seconds and then immediately transferred back to ice for a few minutes. Next, 950 µL of LB medium was added to the mixture, and it was incubated at 37°C with shaking at 225 RPM for one hour. Finally, the mixture was diluted and plated onto LB-ampicillin agar plates for selection and incubated overnight.

1.2.5 Verification of plasmid sequence

To confirm the insertion of the nanobody (Nb) sequences, we performed colony PCR on six selected colonies using specially designed Nb primers. Each colony was picked using an inoculating loop and resuspended in 50 µL of ultrapure water. We then added 5 µL of

the resuspended colony to a mixture containing dNTPs, Vent polymerase, and the designed primers. The amplified sequences were analyzed on a 2% agarose gel to verify the correct Nb insertion, as indicated by the presence of a positive band.

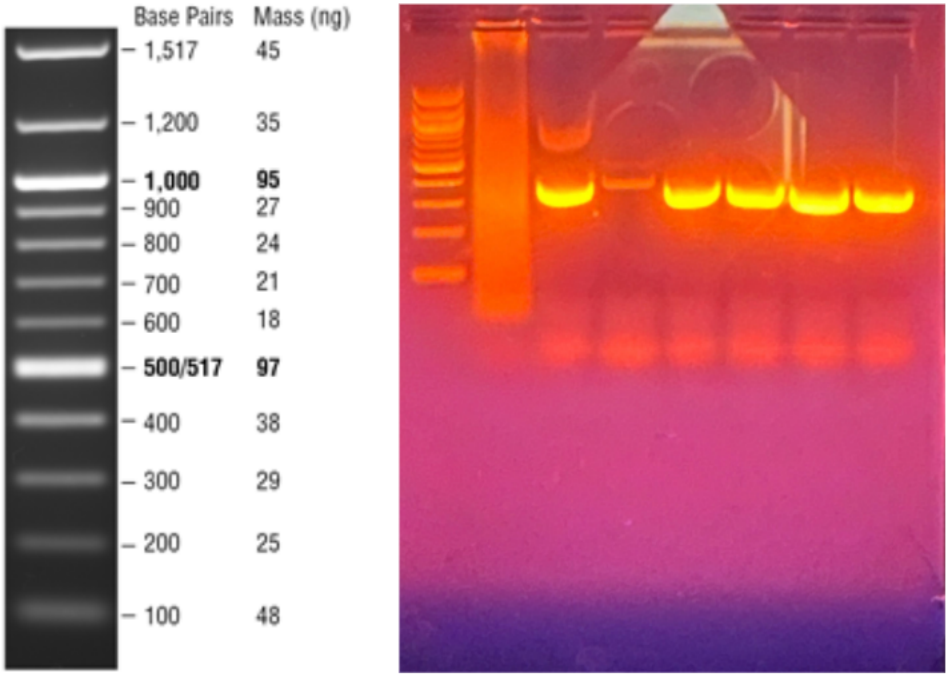

Figure 9. Gel electrophoresis for colony PCR.
To verify the insertion of the nanobody, 2Rb17c forward and reverse primers were used for PCR amplification. The expected size of the amplified 2Rb17c product is 372 base pairs (bp). When compared with a ladder reference, the observed bands align correctly, falling within the 300-400 bp range, confirming the successful insertion.

After identifying positive samples, the E. coli suspensions were re-streaked onto fresh LB-ampicillin media and incubated overnight to increase the biomass for DNA sequencing. The DNA sequencing was subsequently performed and confirmed by Primodium Labs.

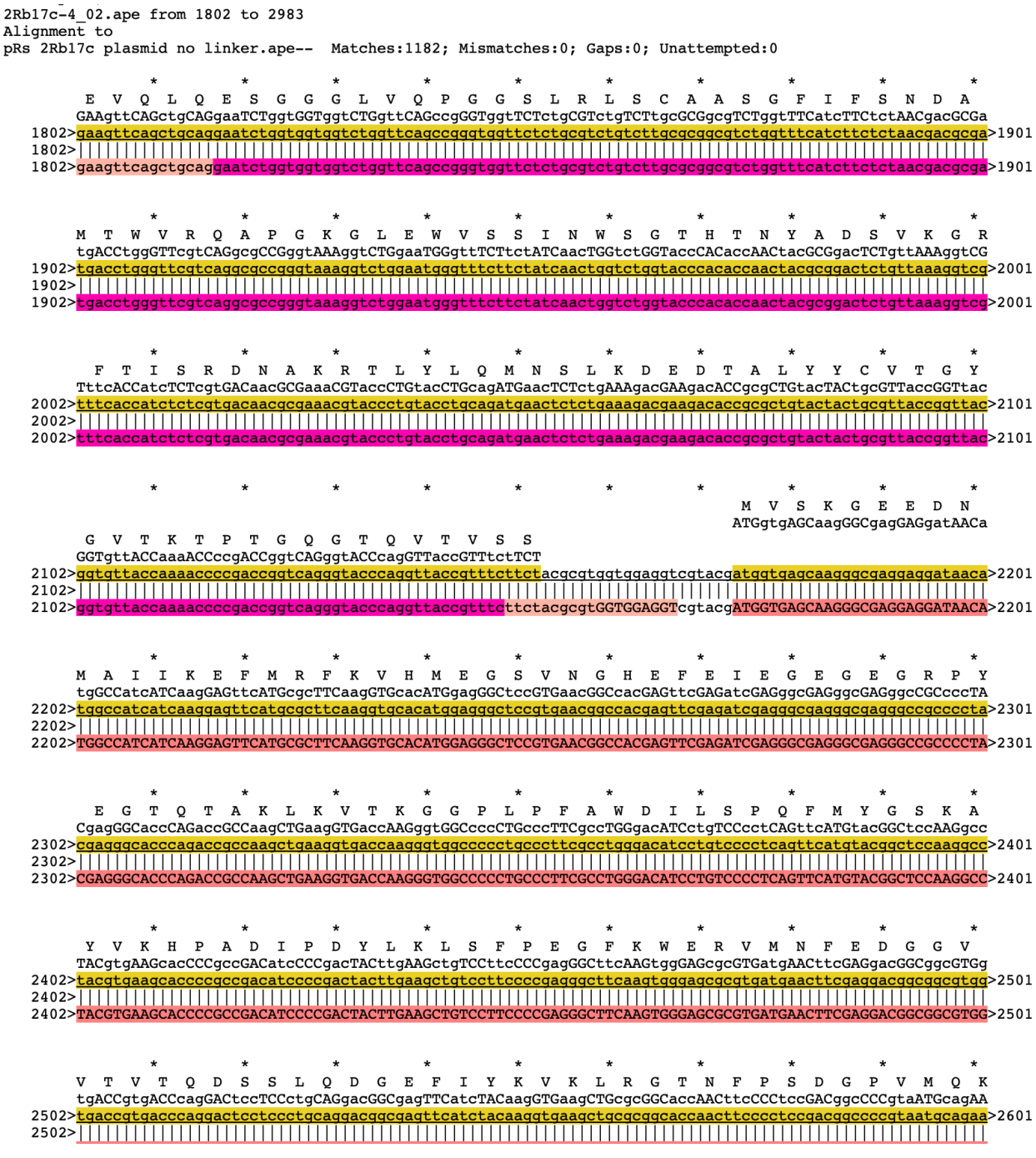

Figure 10. Genewiz Sequencing Result of pRS-2Rb17c-mcherry-sortag-His6-cmyc.
Sequencing result is aligned with a reference pRS-2Rb17c-mcherry-sortag-His6-cmyc.

Chapter 2. Expression of Recombinant Nanobody proteins in Saccharomyces Cerevisiae

2.1 Introduction

In chapter 2, HER2 nanobody 2Rb17c was successfully cloned in to pRs-4420 based vectors. Yeast expression system is used in this chapter to the production of recombinant proteins, to express the proteins that are difficult to produce in E. coli. Protein expression in yeast can include post-translational modifications such as glycosylation, phosphorylation, and acetylation. This capability makes yeast well-suited for producing proteins that require these modifications to function properly. Also, protein expression in yeast is also safer than in bacterial systems because yeast does not produce endotoxins.

In this chapter, Saccharomyces Cerevisiae strand BJ5464 use galactose inducible promotor (GAL 10) gene located in the pRS plasmid, which allows controlled expression of inserted protein based on the presence of galactose. Thus, utilizing the BJ5464 expression system to express pRs-2Rb17c-mcherry-sortag-his6-cmyc. The size of produced protein is verified by Western blotting.

2.2 Material and Method

2.2.1 Reagents, Materials and Kits

All reagents and materials Yeast culture and Protein production are posted in Appendix B.

2.2.2 Strains and Media

Saccharomyces cerevisiae BJ5464 was used for pRs-2Rb17c Nanobody protein production.

Medias and plates are used in BJ5464 culture are listed in the following table:

Media Name:	Recipes：
YPD Media (1L)	10g yeast extract, 20g peptone, 20g dextrose
SDCAA Media (1L)	20 g dextrose, 6.7 g Yeast N 2 Base w/o Amino Acids, 5 g Casamino Acids (-ADE, -URA, -TRP), 5.4 g Na2HPO 4, 7.46 g NaH2PO4, and Ultrapure water up to 1L
SDCAA+Ura Media (1L)	20g dextrose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, 20mg Uracil, add Ultrapure Water up to 1L
SGCAA Media (1L)	20 g galactose, 6.7 g Yeast N 2 Base w/o Amino Acids, 5 g Casamino Acids (-ADE, -URA, -TRP), 5.4 g Na2HPO 4, 7.46 g NaH2PO4, and Ultrapure water up to 1L
SGCAA+Ura+0.1%BSA Media (1L)	20g galactose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g NaH2PO4 monobasic, and Ultrapure Water up to 1L
SDCAA+Ura plates	5.4g Na2HPO4 Dibasic, 8.56g Na2HPO4 monobasic, 182g sorbitol, 15g Agar in 900 mL Ultrapure Water ** Autoclave.

Table 2. Recipes of Media and Plates used in Yeast culture and Protein purification.

Buffers used in Protein purification through Ni-NTA kits:

NPI-10: 50mM NaH2PO4, 30mM NaCl, 10mM Imidazole, pH 8

NPI-20: 50mM NaH2PO4, 30mM NaCl, 20mM Imidazole, pH 8

NPI-500: 50mM NaH2PO4, 30mM NaCl, 500mM Imidazole, pH 8

2.2.3 Yeast Culture and Recombinant protein production

After successfully transforming the plasmid pRS-2Rb17c-mcherry-sortag-his6-cmyc into DH5 alpha E. coli, it was then transferred to yeast strain BJ5464 using the Lithium Acetate method, as outlined by Gietz RD and RA Woods (refer to Protocol in Appendix B). During the transformation, PEG 3500, LiAc, and plasmid DNA were layered on top of the yeast cell pellet, and the mixture was vigorously vortexed to ensure thorough mixing and resuspension of the cells. The cells were incubated at 42°C for 1 hour, then cultured on an SD-CAA plate at 30°C with shaking at 225 RPM for 1 day. Subsequently, the cells were transferred to SD-CAA+Ura media and incubated in a shaking incubator at 30°C, 225 RPM for an additional day. After this culture inoculation, protein expression was induced by transferring the cells to SG-CAA+Ura+0.1% BSA media and incubating at 30°C with shaking at 225 RPM for 2 days.

2.2.4 Yeast Purification

After inducing protein expression in yeast using SG-CAA+Ura+0.1% BSA media, the liquid culture was centrifuged at 3000 RPM for 5 minutes to collect the supernatant, containing the proteins. The supernatant was then processed through a 15 mL Amicon 10 kDa MWCO centrifuge filter to isolate proteins with molecular weights above 10 kDa. Following this, the proteins were purified using the Qiagen Ni-NTA purification protocol. The size and integrity of the purified proteins were verified by SDS-PAGE and Western blotting.

2.2.5 Protein size verification

The protocol for SDS-PAGE and Western blotting is detailed in Appendix C. SDS-PAGE, which does not specifically target any protein, displays all proteins present in the gel.

For Western blotting, non-specific binding of antibodies to the membrane is blocked using clear milk. The membrane is then incubated with a primary antibody that binds to the target proteins. Any unbound antibodies are washed away using PBST buffer. A secondary antibody, conjugated to a fluorescent tag, is then added. The size of the protein can be detected without the need for imaging devices. The results of the SDS-PAGE and Western blot are illustrated in the figure below.

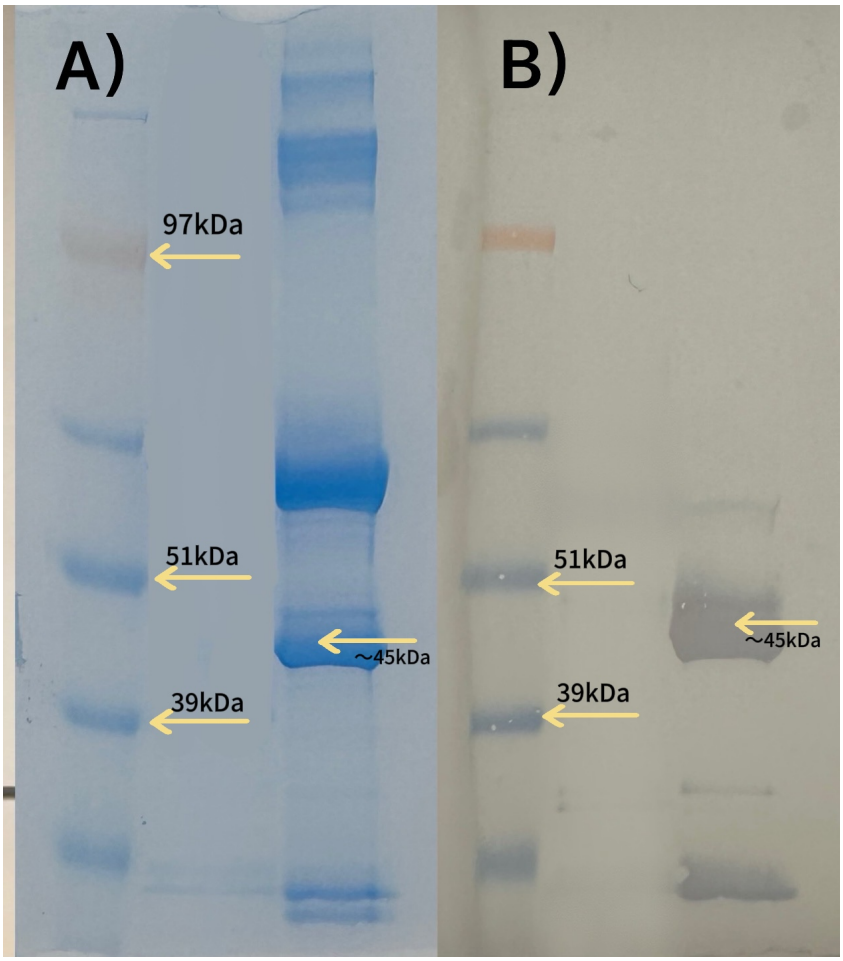

Figure 11. SDS-PAGE and Western Blot of pRS-2Rb17c-mcherry-sortag-his6-cmyc nanobody.
A) shows an SDS-PAGE gel, the first column is the ladder, and second column is of pRS-2Rb17c-mcherry-sortag-his6-cmyc nanobody. All the protein bands are stained blue, and protein distribution and estimate size are visualized. B) the first column also a ladder, the second column shows the band corresponding to proteins that have been successfully transferred onto a membrane and detected with antibodies specific to the target protein. The molecular weights of the detected bands can be inferred by comparing their positions with the size standard in panel A, which is between 51kDa and 39kDa.

The correct size of specific protein production through BJ5464 is 45.52 kDa for pRS-2Rb17c-mcherry-sortag-his6-cmyc. Thus, the experimental results obtained from SDS-PAGE and Western blotting provide reliable and consistent data regarding the protein of interest. The SDS-PAGE confirmed the presence of proteins within the expected molecular weight range, and the corresponding Western blot specifically identified and validated the target protein. The accurate detection of the protein's size on Western blot, matching the expected molecular weight, confirms the protein's identity and integrity, suggesting that it has not undergone significant degradation or unexpected post-translational modifications. These results not only verify the effectiveness of the antibodies used but also demonstrate the overall validity of the experimental protocols followed. This consistency is crucial for advancing our understanding of the protein's role and functionality within its biological context, supporting further investigative and application-specific studies.

Chapter 3. Conjugation of Recombinant Nanobody Proteins with Particles

3.1 Introduction

In the previous two chapters, we successfully produced and purified pRS-2Rb17c-mcherry-sortag-his6-cmyc proteins. This conjugation plays a pivotal role in enhancing diagnostics and therapeutics by facilitating targeted delivery. The recombinant nanobody proteins, when conjugated with particles or nanoparticles, impart additional imaging and targeting properties to the nanoparticles. The process involves bioconjugation techniques that utilize crosslinking agents to form stable covalent bonds, securely attaching proteins to the particles.

In this chapter, we build on the processes described in Chapter 2, using the purified proteins and modifying them with GGG PEG-TCO and Sortase buffer to create covalent binding sites. This sortase-mediated ligation is employed to produce site-specifically modified proteins, facilitating the attachment of GGG PEG-TCO, a cleavable linker utilized in the synthesis of antibody-drug conjugates. Subsequently, these modified proteins are conjugated to 100 nm particles using EDC-NHS chemistry.

The biofunctionalization of nanoparticles is achieved by utilizing carboxylic acid as the surface group for cross-linking to the primary amines of the nanobody proteins. The EDC-NHS coupling reaction forms amide bonds between the carboxyl and amine groups on the surface of the particles, enhancing the stability of active intermediates through NHS.[24]

The synthesis of Tz- and TCO-functionalized polypeptides, polypeptoids, and polypeptide-block-polypeptoids utilizes reactions between 1,2,4,5-tetrazines (Tz) and trans-cyclooctenes (TCO).[25] These reactions are instrumental in the final stages of conjugating nanoparticles with nanobody proteins. To evaluate the effectiveness of these particle conjugations, we measure their intensity and Zeta potential, which provide insights into the stability and functionality of the conjugates.

3.2 Materials and Methods

3.2.1 Chemical Reagents

All materials and reagents used in particle conjugation are posted in Appendix D.

3.2.2 Protein preparation

Proteins are modified with GGG PEG4-TCO in Sortase buffer, which facilitates the creation of a PEG-TCO binding site for covalent attachment to the nanoparticles. Growth with shaking in dark for 3 days.

3.2.3 Particle preparation

Covalent conjugation of antibodies or nanobodies to nanoparticles is achieved using carbodiimide activation chemistry (EDC/NHS) to form amide bonds. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) serves as a crosslinker to activate carboxyl groups on the particle surface, while N-Hydroxysuccinimide (NHS) stabilizes the reactive amine, enhancing the efficiency of EDC. This combination effectively binds the amine groups on the nanobody.

3.2.3 Particle conjugation with protein

Add the prepared particles and proteins for conjugation, ensuring to wash them three times with PBS both before and after the conjugation process. Measure the number, intensity, and zeta potential of the protein-nanoparticle conjugates and compare these values with those of the bare nanoparticles.

3.2.4 Measurement of number, intensity, and zeta potential

To minimize random errors, the number, intensity, and zeta potential of the particles should be measured multiple times. The number of particle conjugates assists in determining the efficiency of the conjugation reaction. Intensity is useful for assessing the quality and stability of the conjugates, particularly the stability of fluorescent markers, which indicate functional integrity and successful conjugation. Zeta potential is critical for assessing the surface charge of the particles, providing insight into the modifications in surface chemistry resulting from the conjugation process.

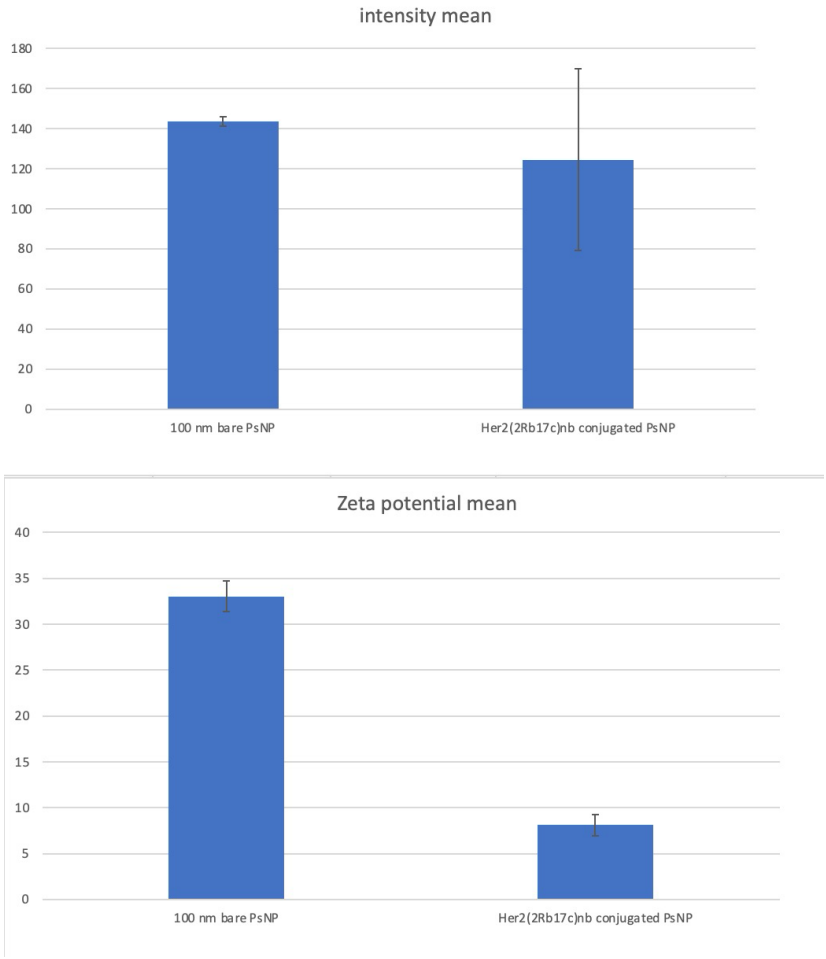

Figure 12. Data of the characteristics of 100nm bare polystyrene nanoparticles (PsNp) and HER2 (2Rb17c)Nb. This figure shows the mean of intensity and zeta potential for result with standard deviation bar.

The observed decrease in both intensity and zeta potential in the conjugated nanoparticles indicates that the conjugation process may alter the physical and chemical properties of the particles, potentially affecting their effectiveness in biological applications. While conjugation facilitates targeted delivery, it may also introduce challenges, such as reduced stability, that must be overcome to fully capitalize on the capabilities of these conjugated nanoparticles in targeted therapy and diagnostic applications.

Conclusion and Future Work

In summary, the advancements in the field of targeted cancer therapy through the development of nanobody-modified nanocarrier proteins in this thesis. Due to the high affinity and specificity of recombinant anti-HER2 nanobodies, this research demonstrates the potential in precisely targeting and binding to overexpression HER2 receptors on the tumor site. In this thesis, 2Rb17c nanobody has successfully cloned into pRS-4420 based plasmid, and expression the protein on yeast. After protein purification, protein has been successfully adhered on the nanoparticles. The significant contribution to the realm of cancer therapy, especially targeted therapy by innovating nanobody modified nanocarrier proteins. The utilization of Saccharomyces cerevisiae for protein production helps post-translational modifications. Furthermore, the conjugation between nanoparticles and proteins possibly enhances the stability and function for clinical use.

In future research, based on the successfully cloned 2Rb17c nanobody protein, we need to build a panel of HER2 nanobody targeting receptors. This panel will facilitate the characterization of nanobodies with the optimal affinity and specificity of binding HER2-positive cancer cells. Additionally, research will focus on the particle conjugation techniques to attach nanobody proteins and nanoparticles, ensuring the stable interactions with the target cancer cells. Finally, the binding testing between HER2-positive cancer cells and conjugated nanobody proteins-particles will demonstrate the precision of drug delivery systems and shows the efficacy of treatment to minimizing adverse effects on healthy cells.

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Appendix A: Gene Cloning and Propagation

Overall Objective: To insert a nanobody sequencing (2Rb17c) between MluI and NheI site in the PRS-4420-Bsiwi-mcherry-sortag-His6-cmyc.

Day 1: PCR and Double Digestion

Objective: To amplify 2Rb17c sequencing. To digest plasmid and PCR product to get flanking ends for ligation down the road.

PCR protocol:

1. Take the following reagents out of the -20C and thaw on ice:
   2Rb17c gBlock, Enzymatic_F (primer F) Enzymatic_R (primer R), Vent polymerase, Thermopol buffer, dNTPs

2. Add the following into 1 PCR tube:
   1uL 2Rb17c gBlock
   1uL Enzymatic_F (primer F)
   1uL Enzymatic_R (primer R)
   1uL Vent polymerase
   1uL dNTPs
   5uL Thermopol buffer
   40uL ultrapure water
   Total volume: 50 uL
   Run thermocycler protocol: ~1:35 hours

3. Take PCR out and rerun the same protocol but change the gBlock to this PCR product.

   1uL PCR product
   1uL Enzymatic_F (primer F)
   1uL Enzymatic_R (primer R)
   1uL Vent polymerase
   1uL dNTPs
   5uL Thermopol buffer
   40uL ultrapure water
   Total volume: 50 uL
   Run thermocycler protocol: ~1:35 hours

Double digestion protocol:

1. Take the following samples/reagents out of the -20C and thaw on ice:
rCutsmart buffer, MluI restriction enzyme, NheI restriction enzyme
Plasmid: PRS-4420-Bsiwi-mcherry-sortag-His6-cmyc

2. Double digest the PCR product and the plasmid:
    a. PCR product double digestion (tube 1)
    4 uL rCutsmart buffer
    2 uL MluI enzyme
    2 uL Nhel enzyme
    4 uL water
    28 uL PCR product
    Total volume: 40 uL
    b. Plasmid double digestion (tube 2)
    4 uL rCutsmart buffer
    2 uL MluI enzyme
    2 uL Nhel enzyme
    12 uL water
    20 uL vector
    Total volume: 40 uL

3. Incubate in 37C overnight

Day 2: Gel Electrophoresis, Gel extraction, and Ligation

Objective: To isolate and purify DNA of interest from agarose gel.

Gel Electrophoresis Protocol:

1. For Vector gel electrophoresis Pour 1% agarose gel. (0.5 g in 50 mL TAE buffer, 2.5 ul of EtBr).
2. For nanobody gel electrophoresis Pour 2% LMW agarose gel. (1g in 50 mL TAE buffer, 2.5 ul of EtBr).
3. Make DNA ladder: 1 uL 1kb ladder + 1uL sample dye + 8 uL water. Add 6x sample dye to samples.
4. Load DNA ladder and sample into gel. Run at 75V for 45-50 min.
5. Visualize gel under UV platform. Use razor to cut out the band.
6. Follow protocol in the Qiagen Gel Extraction Kit. Measure concentration using Nanodrop.

Ligation Protocol:

1. Take the following samples/reagents out of the -20C and thaw on ice:
    T4 ligase buffer, T4 ligase
2. Add the following into 1 PCR tube:
    4uL ligase buffer
    1uL T4 ligase
    9uL water
    1ul digested vector
    5ul digested nanobody 2Rb17c

3. ligation overnight at room temperature.

Day 3: Transformation

Objective: To transform DH5 alpha competent cells with our ligated plasmid.

Transformation Protocol:

1. Take out and thaw DH5alpha cells on ice. Prewarm 1L of LB media at 37C.
2. Add 2uL ligation mixture to the 50uL DH5alpha cells and incubate on ice for 30 min.
3. Heat shock at 41- 42C for 20 seconds. Place back on ice for 2 min.
4. Add 950 uL of prewarmed LB media. Shake at 225 RPM 37C for 1 hour.
5. Warm LB-Amp plate at 37C.
6. Spin cells at 3000xg for 3 mins to pellet cells. Aspirate media. Resuspend cell in 150 uL of LB media. Plate on LB-Amp plate. Incubate at 37C overnight.

Day 4: Colony PCR

Objective: To confirm successful plasmid transformation in DH5 alpha competent cells.

Transformation Protocol:

1. Observe plate. Count colonies.

2. Pick 3 to 4 colonies. Add each colony to 50 uL of water.

3. Take the following reagents out of the -20C and thaw on ice:
   2Rb17c_F (primer F), 2Rb17c_R (primer R), Vent polymerase, Thermopol buffer, dNTPs

4. Run colony PCR:
   5 uL colony suspension
   0.5 uL 2Rb17c_F
   0.5 uL 2Rb17c_R
   0.5 uL Vent polymerase
   0.5 uL dNTPs
   2.5 uL Thermopol buffer
   20 uL ultrapure water
   Total volume: 29.5 uL

Media recipes

LB Broth

Base media(1L): 25g LB powder, filter sterilize. Add Ampicillin 50ug/ml

LB plates

40g LB agar powder/L, autoclave, add ampicillin at 50ug/ml once the media cool down.

Appendix B: Yeast Culture and Protein Purification

Media/Plates Recipes

YPD media (1L)

Base media (for 1 L): 10 g yeast extract, 20 g peptone, 20 g dextrose Filter sterilize

YPD plates

Autoclave bacto-agar (15 g/L) in 90% of the final volume. Filter sterilize carbon source, yeast nitrogen base and amino acids in 10% of final volume and add to autoclaved mixture once it has cooled.

SDCAA + URA Media (1L)

20g dextrose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na_2HPO_4 dibasic, 7.46g NaH_2PO_4 monobasic, 20mg Uracil, add Ultrapure Water up to 1L

**Use Nalgene vacuum filter to sterilize

SDCAA + Ura Plates

5.4g Na_2HPO_4 Dibasic, 8.56g Na_2HPO_4 monobasic, 182g sorbitol, 15g Agar in 900 mL Ultrapure Water

** Autoclave. Place flask in plastic bin. Bring to autoclave room. Add enough water to the bin to ~0.5 inch in depth. Place in autoclave machine. Choose "Liquid 30" cycle.

20g dextrose, 6.7g yeast N2 base w/o Amino Acids, 5g Casamino Acid, 20 mg Uracil in 100mL Ultrapure Water

**Use Nalgene vacuum filter to sterilize

SGCAA Media (1L)

20g galactose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na_2HPO_4 dibasic, 7.46g

NaH_2PO_4 monobasic, add Ultrapure Water up to 1L

**Use Nalgene vacuum filter to sterilize

SGCAA + URA + 0.1% BSA Media (1L)

20g galactose, 6.7g Yeast N2 Base w/o Amino Acids, 5 g Casamino Acids, 5.4 g Na2HPO4 dibasic, 7.46g

NaH2PO4 monobasic, 20 mg Uracil, 1g BSA, add Ultrapure Water up to 1L

**Use Nalgene vacuum filter to sterilize

Yeast Culture

Yeast Growth

1. Streak a single colony on YPD or SD-CAA plates and incubate at 30ºC for 2 days.
2. Inoculate 3 ml liquid culture with a single colony and grow overnight (YPD) or for 1.5 days (SD-CAA) at 30ºC while shaking at 200-225 RPM.

Transformation (Lithium Acetate method from Gietz RD and RA Woods, Methods in Enzymology, 2002)

1. Inoculate 3 ml YPD with BJ5464 (transformation with pRS plasmid) or EBY100 (pCT plasmid) and grow overnight.
2. Heat single-stranded carrier DNA (2 mg/ml) in a boiling water bath for 5 min and then place on ice.
3. Spin yeast at 3000 RPM for 1 min, resuspend in 1 ml ultrapure water and transfer to a microcentrifuge tube. Spin again and discard the supernatant.
4. Layer the following on top of the pellet: 240 ul PEG 3500 (50% w/v), 36 ul LiAc (1.0 M), 50 ul carrier DNA (after vortexing) and 34 □l plasmid DNA (0.1 to 1 ug mixed in ultrapure water, typically use 2 ul). Vortex to mix and resuspend cells (pipet it necessary).
5. Incubate at 42ºC for 1 hr before centrifugation and aspiration of the transformation mix. Add 1 ml ultrapure water, resuspend and plate 100 ul on selective plate (SD-CAA).

Protein Induction and Purification

Small Scale production: culture inoculation and protein induction at 30mL

Day 1 – Culture Inoculation

Preparation work:

** Grab SDCAA + Ura liquid media and sample yeast SDCAA + Ura plate from the cold room. Spray the
bottle with 70% ethanol.

** Spray the surface you are going to work on with 70% ethanol. Wipe down with Kimwipe.

** Turn on the Bunsen burner.

1. Label a 50mL Falcon centrifuge tube for each sample.
2. Add 30mL of SDCAA + Ura liquid media to a 50mL Falcon centrifuge tube.
3. Remove the parafilm from the yeast SDCAA + Ura plate. Locate a round single colony on plate.
4. Take a 10uL pipette tip, scrape one colony. Drop the whole pipette tip into the tube.
5. Leave the cap loosely on the tube. DO NOT TIGHTEN THE CAP.
6. Incubate sample(s) in shaking incubator at 30C, 225 RPM for 24 hours.

Day 2 – Protein Induction

Preparation work:

** Grab SGCAA and SGCAA + Ura + 1% BSA liquid media from the cold room. Spray the bottles with 70%
ethanol.

** Spray the surface you are going to work on with 70% ethanol. Wipe down with Kimwipe.

** Turn on the Bunsen burner.

1. Retrieve the samples from the incubator.
2. Tighten the caps. Place into the centrifuge. Make sure the centrifuge is balanced.
3. Spin the samples at 3000 RPM for 5 minutes.
4. Discard the supernatant.
5. Wash the cell pellet by adding 10mL of SGCAA liquid media. Resuspend the cell pellet.
6. Spin the samples at 3000 RPM for 5 minutes.
7. Discard the supernatant.

8. Add 30mL of SGCAA + Ura + 1% BSA liquid media into the tubes. Resuspend the cell pellet.
9. Leave the cap loosely on the tube. DO NOT TIGHTEN THE CAP.
10. Incubate sample(s) in shaking incubator at 30C, 225 RPM for 48 hours.

Day 3 – Nothing needs to be done.

Day 4 – Concentrating Proteins and His-tag Purification

1. Retrieve the samples from the incubator.
2. Tighten the caps. Place into the centrifuge. Make sure the centrifuge is balanced.
3. Spin the samples at 3000 RPM for 5 minutes.
4. Add 15 mL of the supernatant to a labelled 15mL Amicon 10kDa MWCO centrifugal filter. You should use 1 Amicon filter per sample.
5. Take the samples to the centrifuge. Spin the samples at 4000xg (or max speed) for 15 minutes.
6. Discard the waste at the bottom of the Amicon centrifugal filter.
7. Repeat step 4 - 6 until all supernatant from each sample pass through the filter.
8. Add 5mL of NPI-10 buffer to each sample. Spin the samples at 4000xg (or max speed) for 15 minutes. Use the 200uL pipette to mix the sample in the top part of the filter well.
9. Protein Purification: Use the Qiagen Ni-NTA purification protocol to purify the samples. Use 1 spin column per sample. Briefly:

a. Retrieve the Ni-NTA spin column from the 4C fridge. One spin column per sample.
b. Add 600uL of NPI-10 to each spin column. Spin the columns uncapped at 890xg for 2 min using the mini-centrifuge. Discard the liquid waste.
c. Add 600uL of protein sample from step 8. Spin the column uncapped at 270xg for 5 min.
d. Get the liquid from the collection tube (bottom part of the spin column) and add it back up on the top. Repeat step c two more times.
e. Discard waste.
f. Add 600uL of NPI-20 to each spin column. Spin the columns uncapped at 890xg for 2 min. Discard the liquid waste.
g. Repeat step f.
h. Change the collection tube into a 1.5mL microfuge tube.

i. Add 300uL of NPI-500 to the spin column. Spin the columns uncapped at 890xg for 2 min.
j. Add the 300uL eluted protein in the microfuge tube into a 4mL Amicon 10kDa MWCO centrifugal filter.
k. Repeat step i and j.
10. Add 3mL of Tris-NaCl buffer to the Amicon centrifugal filter with the eluted protein.
11. Spin at 4000xg (or max speed) for 10 minutes.
12. Repeat steps 10 and 11 two more times.
13. Label microfuge tubes for protein storage.
14. Mix the sample at the top of the Amicon filter well. Take up the whole volume and transfer into
the labelled microfuge tube.
15. Use the Nanodrop to measure the protein concentration.
a. Open Nanodrop program.
b. Click "Protein A280" program.
c. Press "ok" when the pop-up window prompts you.
d. Add 2ul of Tris-NaCl buffer on the Nanodrop pedestal. Press "blank" on the screen.
e. Use a Kimwipe to gently wipe the 2ul droplet from the pedestal.
f. Add 2ul of your sample on the pedestal. Press "measure" to get the concentration.
16. Label the concentration on the side of the tube. Store at 4C.

Large Scale Production: culture inoculation and protein induction above 100mL
**Generally it is a very similar protocol compared to the small scale production. It will take an extra day.

Day 1 – Culture Inoculation
Preparation work:
** Grab SDCAA + Ura liquid media and sample yeast SDCAA + Ura plate from the cold room. Spray the
bottle with 70% ethanol.
** Spray the surface you are going to work on with 70% ethanol. Wipe down with Kimwipe.
** Turn on the Bunsen burner.

1. Label a 200mL glass or plastic flask for each sample using lab tape.
2. Add 50mL of SDCAA + Ura liquid media to each flask. DO NOT DISCARD THE FOIL FOR THE FLASK.
3. Remove the parafilm from the yeast SDCAA + Ura plate. Locate a round single colony on plate.
4. Take a 10uL pipette tip, scrape one colony. Drop the whole pipette tip into the flask. Cover flask with the foil.
5. Incubate sample(s) in shaking incubator at 30C, 225 RPM for 24 hours. MAKE SURE THAT THE FLASK STICKS FIRMLY ON THE TAPE IN THE INCUBATOR. If the flask does not stick to the tape, leave them in the incubator for a bit to warm up before pressing the flask down on the tape and does not move.

Day 2 – Continued Culture Inoculation

Preparation work:
** Grab SDCAA + Ura liquid media. Spray the bottle with 70% ethanol.
** Spray the surface you are going to work on with 70% ethanol. Wipe down with Kimwipe.
** Turn on the Bunsen burner.
1. Retrieve the samples from the incubator.
2. Label two 50mL Falcon centrifuge tubes for each sample.
3. Pour the 100mL liquid culture into the centrifuge tubes.
4. Place into the centrifuge. Make sure the centrifuge is balanced.
5. Spin the samples at 3000 RPM for 5 minutes.
6. Discard the liquid waste.
7. Grab and label one 500mL flask for each sample.
8. Use the 25mL serological pipette to add 30mL of SDCAA + Ura media to the 500mL flasks 6 times, for a total of 180 mL.
9. Add 20mL of SDCAA + Ura media into one of the centrifuge tubes. Resuspend the pellet. Take up the whole volume and add it to the second centrifuge tube with the same sample. Resuspend that pellet as well.
10. Add the combined cell suspension into the 180mL of media in the flask.

11. Incubate sample(s) in shaking incubator at 30C, 225 RPM for 24 hours. MAKE SURE THAT THE FLASK STICKS FIRMLY ON THE TAPE IN THE INCUBATOR. If the flask does not stick to the tape, leave them in the incubator for a bit to warm up before pressing the flask down on the tape and does not move.

Day 3 – Protein Induction

Preparation work:

** Grab SGCAA and SGCAA + Ura + 1% BSA liquid media from the cold room. Spray the bottles with 70% ethanol.

** Spray the surface you are going to work on with 70% ethanol. Wipe down with Kimwipe.

** Turn on the Bunsen burner.

1. Retrieve the samples from the incubator.
2. Label four 50mL Falcon centrifuge tubes for each sample.
3. Pour the 200mL liquid culture into the centrifuge tubes.
4. Place into the centrifuge. Make sure the centrifuge is balanced.
5. Spin the samples at 3000 RPM for 5 minutes.
6. Discard the supernatant.
7. For each sample, add 20mL of SGCAA liquid media to one of the centrifuge tubes. Resuspend the cell pellet. Take up the whole volume and add to the second centrifuge tube. Repeat until the cell suspension from the 4 tubes are all combined into one tube.
8. Spin the samples at 3000 RPM for 5 minutes.
9. Discard the supernatant.
10. Grab and label one 500mL flask for each sample.
11. Use the 25mL serological pipette to add 30mL of SGCAA + Ura + 1% BSA media to the 500mL flasks 6 times, for a total of 180 mL.
12. Add 20mL of SGCAA + Ura + 1% BSA media into the centrifuge tube. Resuspend the cell pellet.
13. Add cell suspension to the flask.
14. Incubate sample(s) in shaking incubator at 30C, 225 RPM for 24 hours. MAKE SURE THAT THE FLASK STICKS FIRMLY ON THE TAPE IN THE INCUBATOR. If the flask does not

stick to the tape, leave them in the incubator for a bit to warm up before pressing the flask down on the tape and does not move.

Day 4 – Nothing needs to be done.

Day 5 – Concentrating Proteins and His-tag Purification

1. Retrieve the samples from the incubator.
2. Label four 50mL Falcon centrifuge tubes for each sample.
3. Pour the 200mL liquid culture into the centrifuge tubes.
4. Place into the centrifuge. Make sure the centrifuge is balanced.
5. Spin the samples at 3000 RPM for 5 minutes.
6. Retrieve four 15mL Amicon 10kDa MWCO centrifugal filter per sample. Label them.
7. Add 15 mL of the supernatant to a 15mL Amicon 10kDa MWCO centrifugal filter.
8. Take the samples to the centrifuge. Spin the samples at 4000xg (or max speed) for 15 minutes.
9. Discard the waste at the bottom of the Amicon centrifugal filter.
10. Repeat step 7 - 9 until all supernatant from each sample pass through the filter.
11. Use the 200ul pipette to mix the sample in the top part of the filter well and combine the same sample into one filter.
12. Add 5mL of NPI-10 buffer to each sample. Spin the samples at 4000xg (or max speed) for 15 minutes.
13. Protein Purification: Use the Qiagen Ni-NTA purification protocol to purify the samples. You will want to use 4 spin columns for 1 sample. Briefly:

a. Retrieve the Ni-NTA spin column from the 4C fridge. One spin column per sample.
b. Add 600uL of NPI-10 to each spin column. Spin the columns uncapped at 890xg for 2 min using the mini-centrifuge. Discard the liquid waste.
c. Add 600uL of protein sample from step 8. Spin the column uncapped at 270xg for 5 min.
d. Get the liquid from the collection tube (bottom part of the spin column) and add it back up on the top. Repeat step c two more times.
e. Discard waste.
f. Add 600uL of NPI-20 to each spin column. Spin the columns uncapped at 890xg for 2 min. Discard the liquid waste.

g. Repeat step f.

h. Change the collection tube into a 1.5mL microfuge tube.

i. Add 300uL of NPI-500 to the spin column. Spin the columns uncapped at 890xg for 2 min.

j. Add the 300uL eluted protein in the microfuge tube into a 4mL Amicon 10kDa MWCO centrifugal filter.

k. Repeat step i and j.

14. Add 3mL of Tris-NaCl buffer to the Amicon centrifugal filter with the eluted protein.

15. Spin at 4000xg (or max speed) for 10 minutes.

16. Repeat steps 14 and 16 two more times.

17. Label microfuge tubes for protein storage.

18. Mix the sample at the top of the Amicon filter well. Take up the whole volume and transfer into the labelled microfuge tube.

19. Use the Nanodrop to measure the protein concentration.

a. Open Nanodrop program.

b. Click “Protein A280” program.

c. Press “ok” when the pop-up window prompts you.

d. Add 2ul of Tris-NaCl buffer on the Nanodrop pedestal. Press “blank” on the screen.

e. Use a Kimwipe to gently wipe the 2ul droplet from the pedestal.

f. Add 2ul of your sample on the pedestal. Press “measure” to get the concentration.

20. Label the concentration on the side of the tube. Store at 4C.

Cleanup:

It is important to clean up the waste we generated throughout the experiments.

For liquid waste, use a beaker to hold. After the experiments are done, move the beaker to the sink. Add enough bleach to reach a final concentration of 10%. Leave in sink for at least 10 min. Rinse out the beaker and dry.

Appendix C: SDS-PAGE and Western Blot

Day 1

A. Sample Preparation:

1. Preheat the dry heat block to 100C.
2. Take the 4x LDS Sample buffer out of the 4C fridge. Wait for the sample buffer to adjust to room temperature.

(If the sample buffer is not warmed up to room temperature, there will be precipitate in the buffer)

3. Take out new microfuge tubes and label them.
4. Add 10ng of protein into each tube. Add enough reducing agent (stock: 10x) and LDS sample buffer (stock: 4x). Raise the total volume to 40uL.

**Note: It is easier to mix the reducing agent and LDS sample buffer as a master mix. To make the master mix, add enough for N+1 samples. For example, if you are making 3 samples, make the master mix for 4 samples.

5. Mix the samples well. Put on the 100C heat block for 5 min.
6. Take the samples off the heat block. Centrifuge tubes in a mini-centrifuge machine.

**Clean Up: At this point, the protein should go back into the 4C fridge.

B. SDS-PAGE:

1. Dilute the MOPS running buffer (stock: 20x) to 1X for 400ml (1 chamber) or 800ml (2 chambers).
2. Get the 4-12% 10 wells gel from the 4C fridge. Open the plastic. Peel off the tape and remove the plastic comb in the gel.
3. Pour the running buffer into the chamber.
4. Clamp in the gel. Use a syringe to wash the wells.
5. Load 5uL of protein ladder. Load the total volume of each sample into each well.
6. Put the top of the gel box on. Run the gel at 180V for 60 min.

**Clean Up: All buffers and protein ladder should go back to the 4C fridge. The gel box should be rinsed thoroughly and placed on rack to dry. Graduated cylinder used to make running buffer should be rinsed as well. The plastic used to hold the gel should be thrown away.

C. Western blot:

1. After the SDS-PAGE stops running, dissemble the gel box. Use the tool to crack open the gel. Cut the top lanes and the bottom part off the gel.
2. Use the Invitrogen NC mini stacks to transfer the gel. Briefly, open the stack up. Take the top portion up from the stack to reveal the membrane. Place the gel on top of the membrane and use a roller to roll the gel in one direction to get rid of bubbles. Make sure to not rip the gel.
3. Run a piece of filter paper under DI water to wet it and place it on top of the gel. Roll again to get rid of bubbles.
4. Peel the square off the copper top layer and place the layer onto the stack. Roll again.
5. Lastly, put the absorption pad on the top of the stack, ensure that the electrodes touch both top and bottom. Close the cassette.
6. Turn on the iBlot 2 machine. Run "start last run".
7. During the transfer run, make blocking buffer from clear milk (stock: 10x). Dilute with water.
8. After transfer run, lift the stack to reveal the blot. The ladder should transfer to the blot. If the blot needs different primary antibodies probing, cut the blot.
9. Add 10mL blocking buffer to each tray. Place blot into each tray. Shake at room temperature for 1 hour.
10. During the hour, take the primary antibodies out from the -20C fridge and thaw on ice.
11. Discard blocking buffer into the sink. Dilute primary antibodies into 10mL of blocking buffer. anti-cmyc dilution factor: 1:1000, anti-His6 dilution factor: 1:5000
12. Shake in cold room overnight.

** Clean Up: The transfer stack can be tossed. Wipe down the iBlot 2 machine and turn it off.

Day 2

C. Western blot

1. Take the blots and shaker out of the cold room.
2. Discard the primary antibodies in blocking buffer.
3. Add enough PBST (~10mL) to the blot and shake for 15 min at room temperature.

4. Repeat step 3 2 more times.
5. Dilute secondary antibodies (1:1000) in 10mL PBST per tray. Shake for 1 hour at room temperature.
6. During the 1 hour, take the 1-Step TMB Blotting Ultra solution out of the 4C fridge. Aliquot ~5mL. Cover with foil and leave at room temperature.
7. After the hour incubation, discard the secondary antibodies in blocking buffer.
8. Wash blot with PBST (~10mL) 3 times, shaking for 15 min each at room temperature.
9. After the 3 washes, dry the blot with Kimwipe and dabbing the corners of the blot onto the Kimwipe. Place the blot onto the lid of the tray and pour the TMB solution onto the blot. Wait for it to develop (this step should take less than 5 min). Once you see the bands, dip the blot into water to stop the reaction.
10. Take a picture.

Appendix D: Particle Conjugation

Day1

Protein Preparation:

Material Preparation:

1. Remove the purified protein and GGG PEG-TCO from the 4°C refrigerator.
2. Take the Sortase buffer out of the -20°C freezer.

Protein Conjugation Setup:

3. Mix the following components to prepare the conjugation mixture:
   5.14ul GGG PEG-TCO
   12.25ul Sortase buffer
   145.94ul ultrapure water.
4. Incubate the mixture by shaking for 3 days in the dark to allow for adequate conjugation.

Day4

Nanoparticle Activation:

1. Prepare the following mixture in a clean tube:
   100ul nanoparticles (100uM concentration)
   28.76ul EPC
   5.18ul NHS
   10.9ul Tetrazine
   fill up to 1.5ml water
2. Shake the nanoparticle mixture for 1 day in the dark to promote activation.

Day 5

Particle conjugation with Proteins

1. Preparation: Particle Conjugation with Proteins
   30.82ul Particle
   947.5ul PBS
   21.68ul Protein conjugate

2. Transfer the mixture into a 15 mL Amicon 100 kDa MWCO centrifugal filter.
3. Fill the filter with PBS buffer up to 4 mL.
4. Centrifuge at 4000xg (or maximum speed) for 15 minutes to remove unbound components.
5. Incubate the sample for 3 hours with shaking in the dark to ensure thorough conjugation.
6. Wash the conjugated particles with 4 mL PBS buffer three times to ensure purity.
7. Label microfuge tubes appropriately for storage of the conjugated particles.
8. Thoroughly mix the sample in the Amicon filter to resuspend the conjugated particles.
9. Retrieve the entire volume and transfer it into the labeled microfuge tubes for storage or further analysis.