# Attempting to Discover the Secrets of Nanobodies

## Laboratory of Protein Biochemistry & Structural Biology

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## Introduction

Our immune system protects us from disease-causing substances. One of the most important parts of this great biological network is antibodies, which are produced by B-lymphocytes. Conventional antibodies have been used in laboratory practice and medicine, but they have some disadvantages. For example, difficulties with production of antibodies using biotechnology and immunogenecity. How to avoid them?

An alternative to the conventional antibody is a **nanobody**. Actually, it is the variable domain of heavy chain antibodies which was discovered in the blood sera of members of the family Camelidae and class Chondrichthyes.

Why are we researching the properties of nanobodies?

The most evident advantage of a nanobody is its **molecular weight**. They are ~15 kDa but still can recognize their antigens with **great affinity**. Also they haven't any post-translation modifications. This fact provides us with a great opportunity to produce nanobodies using standard lab techniques such as production in bacterial cells.

Other advantages of nanobodies lead from their structure.

Variable domains of HCAb are easier to fold and stay soluble because of more hydrophilic surface residues. There aren't any mismatch connects of different domains which are common in variable domains of conventional antibodies. Also they can recognize **hidden epitopes** because of their longer, flexible CDR3 loop that can form finger-like extensions and reach cavities on target antigens inaccessible to conventional antibodies.

This technology has a great potential in biomedicine: it can help us in treating diseases like parasitic or autoimmune diseases through targeted drug delivery. It's also useful in the laboratory, because it can make methods like Western-blot and ELISA cheaper and easier.

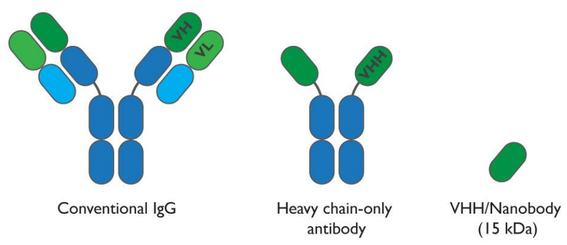


**Fig 1.** *Comparison between conventional antibodies and heavy-chain only antibodies showing the 15kDa nanobody (VHH) fragment. Nanobodies do not have an Fc domain which binds to cell receptors and complement protein, which In this way mediates different physiological effects like a strong immune response [1]*

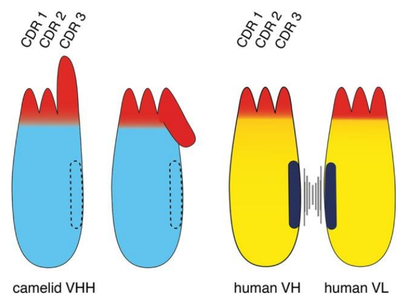


**Fig 2.** *Comparison between variable domains of heavy-chain only antibodies and conventional antibodies.*

## Aim 1: How structural changes affect function of nanobodies?

We used **Affinity Capture** (AC) assay to investigate whether mutated nanobodies interact stronger or weaker with its antigen in **comparison** to un-mutated (wild-type) nanobody **LaG19**.

LaG19 is a nanobody which interacts with the protein green fluorescent protein (GFP). Molecular weight of LaG19 is 15.528 kDa and its' $K_D$ is 24.6 nM [3].

We have mutant LaG19 nanobodies (m3, m4, m5), which were made using site-directed mutagenesis. Single point mutations were made in CDR1, CDR2 or CDR3 domains. Mutants were provided to us by Natalia Ketaren. It is known that affinity of LaG19 (wild-type nanobody) is the lowest compared to the mutants, and affinity of m3 mutant is the highest.

Below is the affinity of wildtype and mutant nanobodies:

**LaG19 < m4, m5 < m3**

We would like to test whether mutant nanobodies perform the same, better or worse at capturing target protein complexes as wild-type nanobody. We know from Hakhverdyan et al (2015)[1] the conditions which optimally capture the three complexes: Tcb2, Arp2 and Ent2. Therefore we tried to replicate these results. We used transgenic yeast which Tcb2, Arp2 and Ent2 genes were fused with GFP gene. Our affinity capture involved conjugating nanobodies to Dynabeads and then following an amended version of the protocol as described in [2].

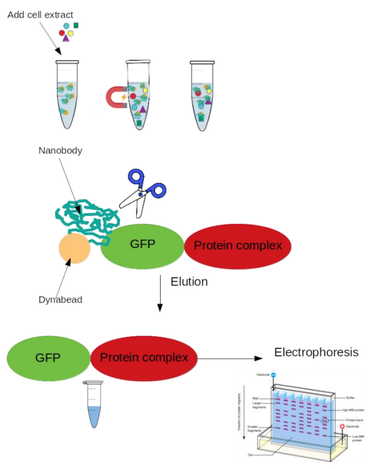


***Fig. 3.*** *Experimental scheme of the Affinity capture experiments used in Aim 1.*

### *Isolating Tcb2 complex*

***Extraction buffer: 40 mM Tris pH 8, 150 mM NaCl, 250 mM trisodium citrate, 5 mM CHAPS***

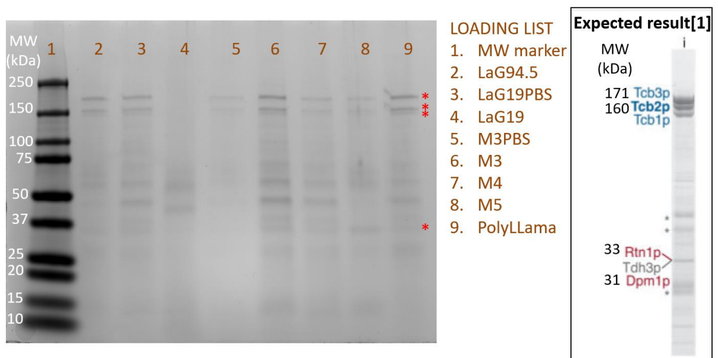


***Fig. 4.*** *SDS-PAGE of the affinity isolation of Tcb2 complex using different nanobodies*

### *Isolating Arp2 complex*

***Extraction buffer: 40 mM Tris pH 8, 150 mM NaCl, 250 mM trisodium citrate, 10 mM CHAPS***

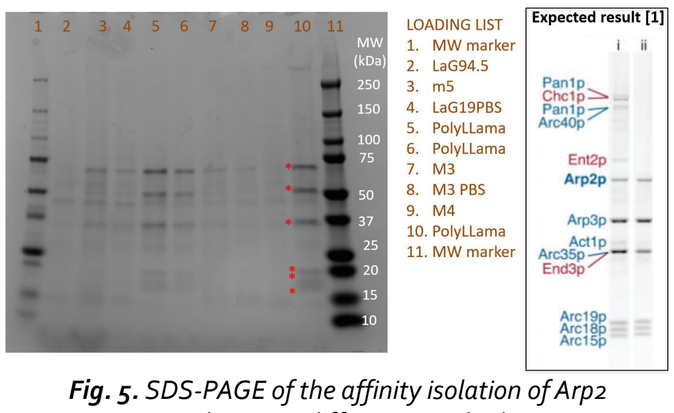


***Fig. 5.*** *SDS-PAGE of the affinity isolation of Arp2 complex using different nanobodies*

### RESULTS

**Tcb2 complex purification:**

- Nanobodies LaG19, M3, M4 & M5 were able to purify the complex (**Fig. 4**).
- M3 showed more non-specific binding.

**Arp2 complex purification:**

- LaG19, M3 & M5 were able to purify the complex (**Fig. 5**).
- M3 showed non-specific binding.

### DISCUSSION

- **The affinity capture experiments need to be repeated to help us determine the validity of the results.**
- **Nanobodies can purify complexes as well as the polyclonal, even though it recognizes only one epitope!**

## Aim 2: What does a perfect nanobody look like?

Protein crystallization is very popular, because it makes us able to see the 3D structure of macromolecules at atomic resolution. X-ray beam reflected from protein crystal can show us the location of atoms inside the molecule. The problem is to get good protein crystals to perform such experiments. Process of crystallization can take a very long time like months or even years. It is caused by the rearrangement of protein molecules in solution to form an ordered lattice through the evaporation of solvent in the crystallization drop. So, we require very specific circumstances to create protein crystals big enough for X-ray diffraction experiments to determine a 3D structure of macromolecule [5].

The purpose of this aim was to determine conditions in which LaG94.5 can create crystals in the presence and absence of its antigen (GFP). We chose this nanobody because (1) it is stable over a variety of different environmental conditions like temperature, pH or buffer conditions which give us a big flexibility of experiments to do with and (2) we wanted to know ***how*** the strongest affinity nanobody for GFP interacts with its antigen.

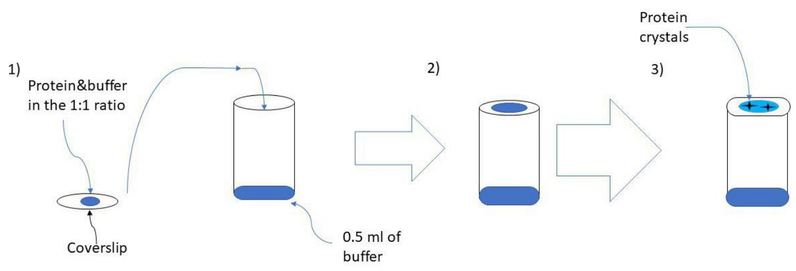


***Fig. 6.*** *Scheme of hanging drop vapor diffusion technique*

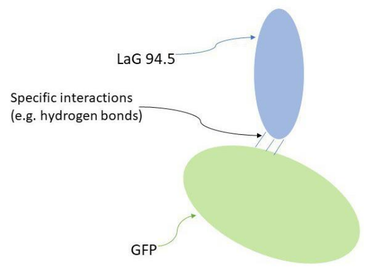


***Fig. 7.*** *Simple scheme of binding of GFP by LaG94.5 nanobody*

In this experiment we used hanging drop vapor diffusion technique. This technique consists of putting a small amount of very concentrated protein solution with crystallization buffer on a coverslip that is then placed above a well with the same buffer . We refer to this as the crystallization chamber. Water enclosed in the hanging drop, will slowly move out to try to create an equilibrium with the buffer in the well below [4]. In our experiment we used Molecular Dimension 3D structure screen "MD1-13".

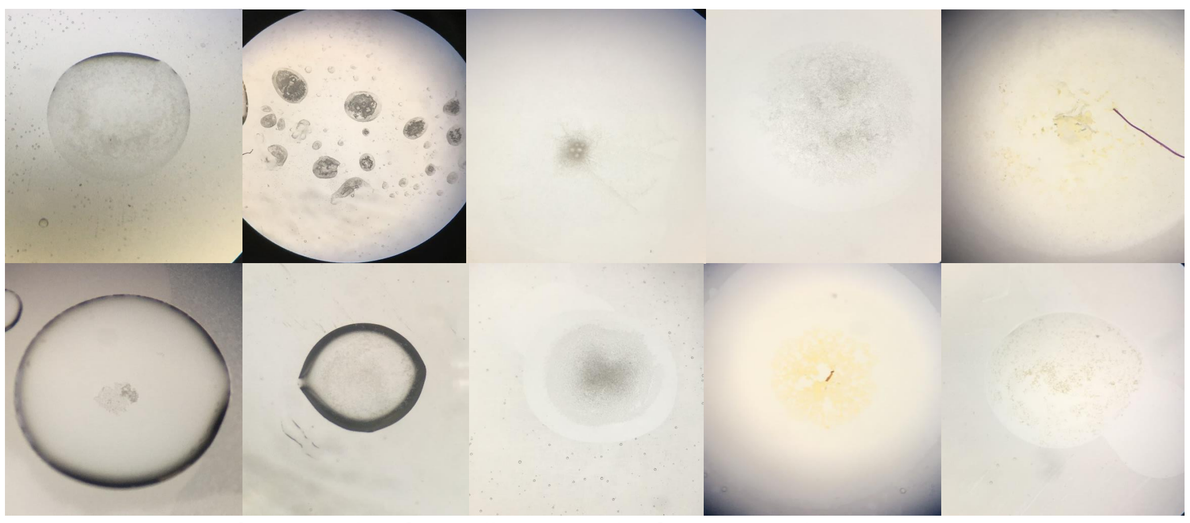

***Fig. 8.*** *Results of crystallization trials of LaG94.5 and LaG94.5+GFP.*

### RESULTS & DISCUSSION

**The complex of LaG94.5+GFP showed more non-amorphous precipitation, which is generally a precursor of crystal growth. This could be due to the more stable interaction formed between the nanobody and GFP in addition to the natural dimerization of GFP at high concentrations creating stable interaction surfaces, promoting crystallization of the complex. The next step will be to replicate the conditions that produced non-amorphous precipitation and microcrystals and make more specific screens around these conditions.**

**References:**

1. Hakhverdyan et al. Rapid, Optimized Interactomic Screening. *Nat. Methods*. (2015). 12(6):553-560
2. Hong Sh et al. In Vivo Model for Testing Effect of Hypoxia on Tumor Metastasis. *J Vis Exp.* (2016). Dec 9(118)
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5. Alexander McPherson. Introduction to protein crystallization. *ScienceDirect*. (2004). Vol. 34(3): 254-265