ONE IN THE FIELD IS NOT A WARRIOR: Working with Nanobody Polyclonals

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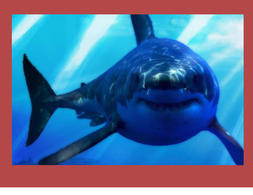

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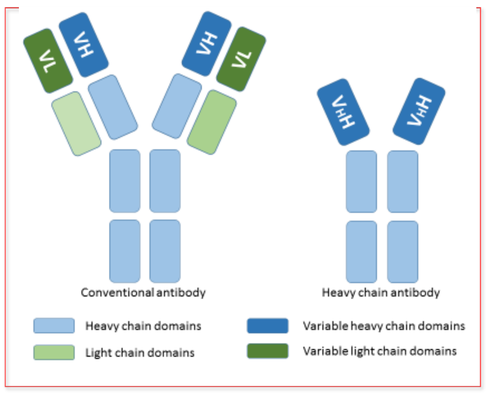
Introduction

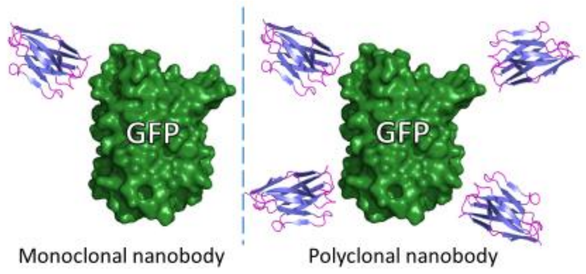


Antibodies (Abs) are proteins produced by immune system to capture antigen epitopes. There are two types of antibodies: conventional and heavy chain Abs (HCAb). Conventional antibodies consist of heavy and light chains and interact with antigens via both variable domains. HCAb is a type of antibody comprising only of heavy-chain domains. They were found in 1990's in the family *Camilidiae* and cartilaginous fishes. The VHH domain on the HCAb is referred to as the *nanobody,*

Nanobodieshave some significant advantages compared with other types, as seen in Table 1.

Table 1

ANTIBODY	NANOBODY
Will denaturate at higher temperature and pH	Resistant to heat and pH
Large in size	Small in size
Has two HC and two LC	Has variable HC domain
Complex protein	Simple protein
Hydrophobic	Not hydrophobic



Antibodies can be monoclonal, where they are specific only for one epitope on an antigen and polyclonal, where there are mixtures of antibodies that can bind an antigen on many different epitopes. Our question is, what is the best way to capture an antigen using nanobodies?

Hypothesis

Different nanobody combinations coupled with magnetic beads will perform better in affinity capture experiments than a single nanobody.

Aims

The overall aim is to produce mixtures of individual nanobodies and compare their efficiency in affinity capture experiments to monoclonal antibodies.

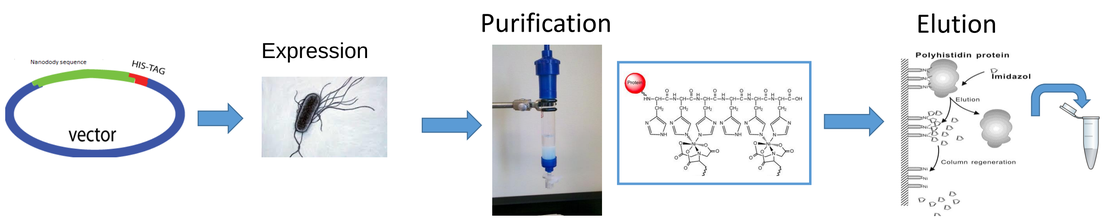
Specific Aims:

1. **Make nanobodies**
2. **Conjugate nanobodies to the beads in two different methods.**
3. **Perform affinity capture experiments**
4. **Compare the results**

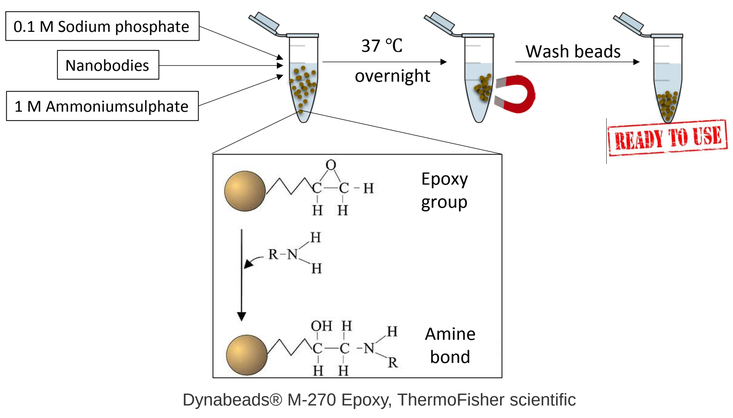
Methods

Nanobody purifications

Nanobodies were extracted from *E.coli* cells. Periplasmic lysate was loaded onto a column Ni^{2+}NTA agarose resin in order to purify nanobodies tagged with HIS_6.



Dynabeads conjugation with nanobodies:



Dynabeads® M-270 Epoxy, ThermoFisher scientific

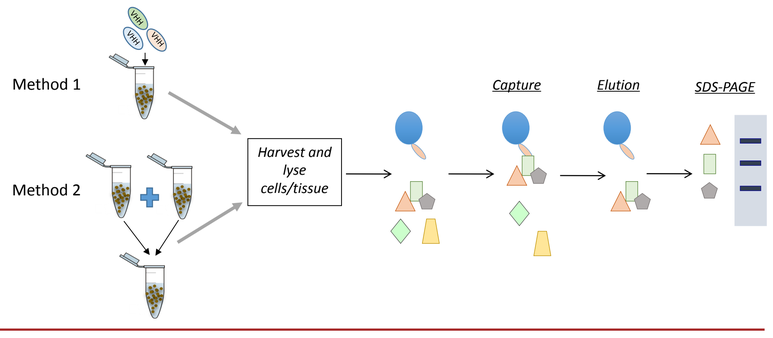
Chemistry of Conjugation
Dynabeads are paramagnetic beads that carry an epoxy group. These epoxy groups covalently bind to amine groups on the proteins.

Affinity capture experiments:

Two different approaches were performed:

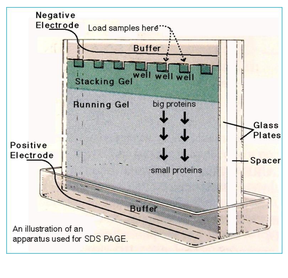
Method 1: mixing different nanobodies, then conjugating mixture to beads

Method 2: mixing different beads conjugated with single nanobodies



SDS-PAGE Gel electrophoresis

In order to separate proteins according to their molecular weight we used SDS-PAGE. SDS is a detergent which denatures and impart negative charge to protein molecules. An electric field is applied across the gel, causing the negatively charged proteins to migrate across the gel away from the negative electrode



We used several types of gels:
- 4-20% The Criterion™ TGX™ precast gel (prestained)
- 7.5% Criterion™ TGX™ Precast Gel
- Polyacriamide gel we made – 12% AA pH=8.8 for resolving gel, 6% AA pH=6.8 for stacking gel.

Results

Purification of Nanobodies

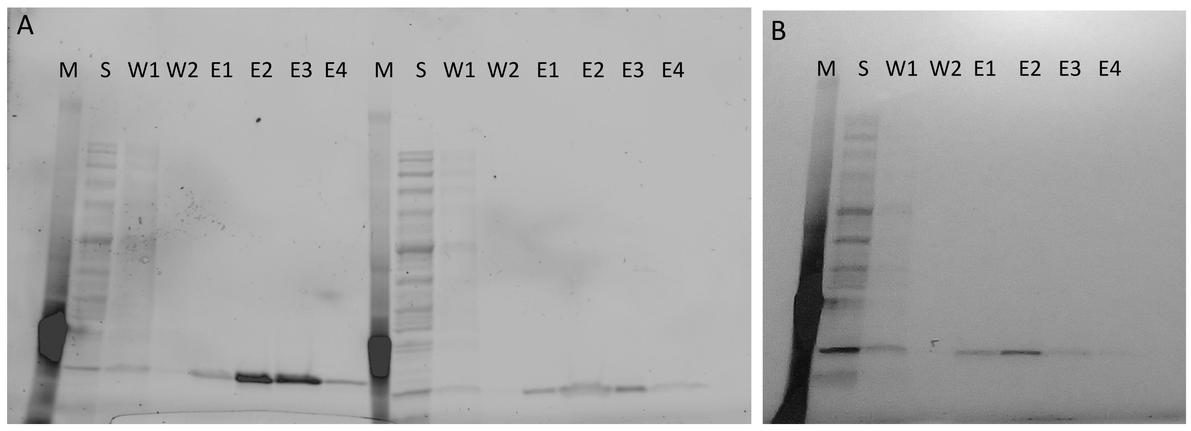
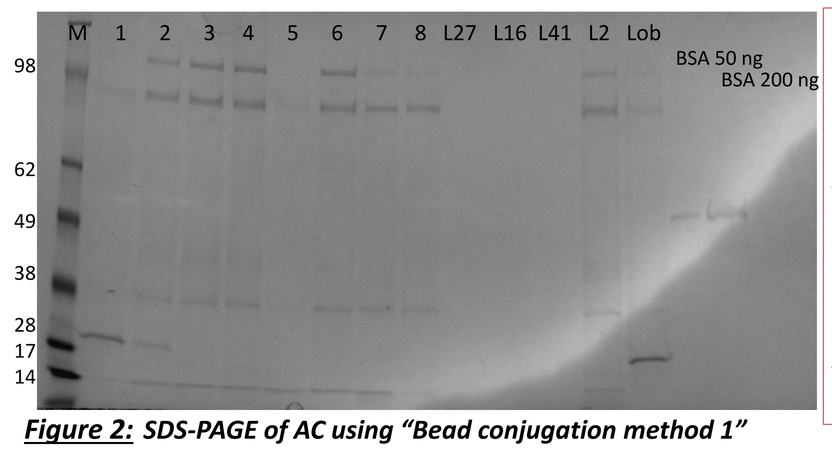


Figure 1: **SDS-PAGE gel showing results of nanobody purification. Elutions 1-4 for each gel contained nanobody. Purification was performed sucessfully. Lane identifiers are as follows:**
M – molecular weight marker, S – cell extract, W – wash, E – eluted sample

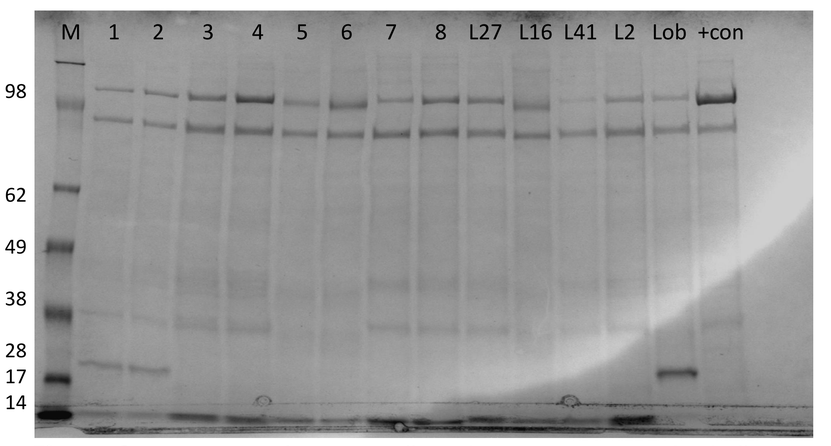
Affnity Capture Experiments

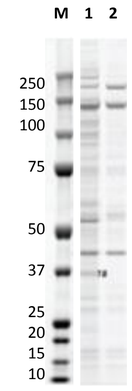
Pulling out Nup98-GFP complex using different nanobodies



Key:
M = molecular weight marker,
1 = LaG27 + LaG lobster,
2 = LaG41 + LaG lobster,
3 = LaG16 + LaG27 + LaG2,
4 = LaG16 + LaG41 + LaG2,
5 = LaG16 + LaG27,
6 = LaG16 + LaG41,
7 = LaG27 + LaG2,
8 = LaG41 + LaG2,
Lob = LaG lobster (LaG16 fused with LaG2)

Figure 2: ***SDS-PAGE of AC using "Bead conjugation method 1"***





Key:
1: 20 mM HEPES pH 7.4, 100 mM NaCl, 0.5% Triton-X100

2: 20 mM HEPES pH 7.4, 300 mM NaCl, 0.5% Triton-X100

Figure 3 ***SDS-PAGE of AC using "Bead conjugation method 2"***

Figure 4: Nup98-GFP control affinity capture experiments
(performed by Dr. John LaCava[2])

Our results indicate the following:
1) The affinity capture experiments performed with our purified nanobodies conjugated to beads, *did not work* Figure 2.
2) When we boil the conjugated beads that we made with nanobodies we made in lab, we do not see any nanobody (Figure 5 below).

Discussion

What might have gone wrong in our experiments:
- It is possible our protein degraded when it was stored in a buffer containing ~200 mM imidazole. It was stored in this buffer at 4 degC for ~30 hrs.
- The composition of PBS made in Spain is different to what is usually used.

Key for *Figure 5*:
1: MW marker
2: LaG16 (Student made)
3: LaG16 (control)
4: LaG27 (Student made)
5: LaG27 (control)
6: LaG41 (Student made)
7: LaG41 (control)

Figure 5: ***SDS-PAGE of boiled remaining beads.***

Future Directions

1. **Repeat experiments under optimal conditions, which include:**
   1. **Ideal equipment (cold room rotator, proper SDS-PAGE gel system…etc**
   2. **Ideal reagents (buffers)**
2. **Repeat experiments including a positive control every time.**

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Fridy P, C et al. A robust pipeline for rapid production of versatile nanobody repertoires. Nat Methods 11, 1253-1260 (2014).
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