Next-Generation Antibodies

Production and characterisation of nanobodies against pmt1

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Abstract

- Nanobodies are a variable domain of Heavy Chain antibodies occurring naturally in *Camelidae*.
- VHH are 10 times smaller, more soluble and more stable than monoclonal antibodies.
- VHH can bind to unique epitopes, i.e. intracellular protein epitopes, and enzyme active sites.
- Nanobodies can be used to study Pmt1 methylation in fission yeast.
- To produce PMT1-specific VHH, Llamas are immunized with a PMT1 peptide linked to Ovalbumin.
- Panning was carried out against PMT1 fused to BSA to avoid getting anti-ovalbumin VHH.

1.Structure

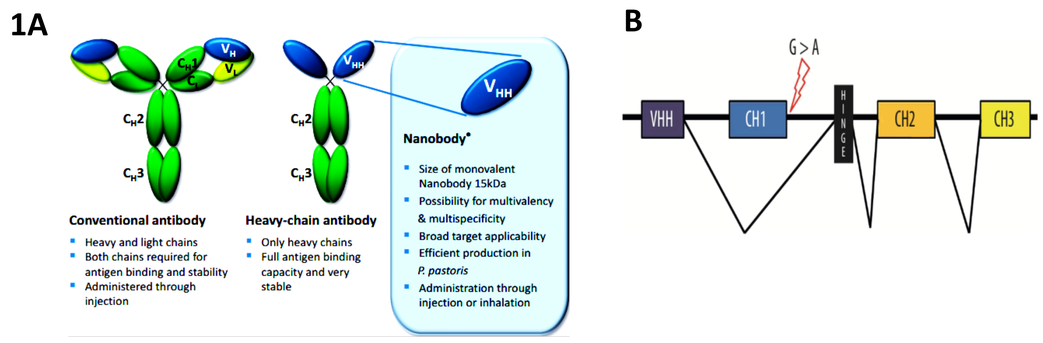
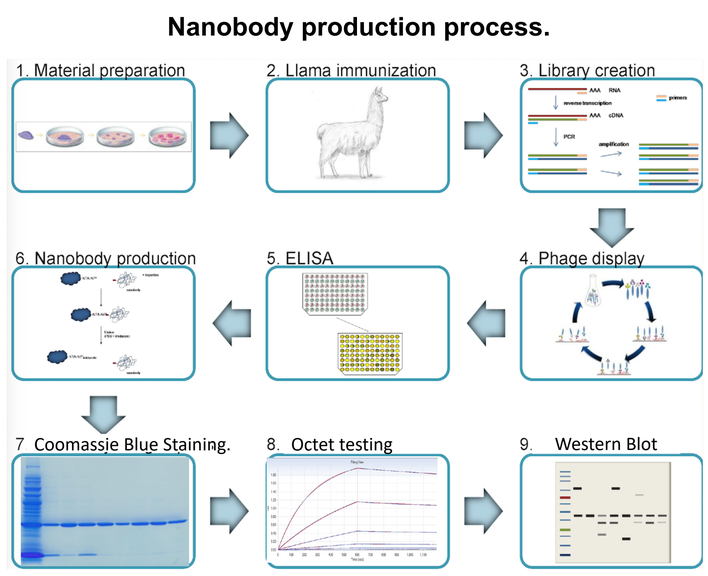


Figure 1. Structural comparison between conventional IgG antibody and a Heavy-chain antibody.
Both conventional and heavy-chain antibodies are found in Llama sera (50:50). Heavy-chain antibodies are devoid of a light chain and CH1 domain. **(B)** A single nucleotide polymorphism (G>A) at the 3' border between the CH1 exon and associated intron suggests a failure in the spliceosome recognition of the CH1 domain, causing its excision with surrounding introns. The missing CH1 domain enables this mutant to escape degradation by BiP, allowing its secretion into the blood sera.

2. Overview Of VHH Library Construction



1). Pmt1 is bound to Ovalbumin, increasing the pmt1 half-life and immunological response. **2)** The antigen is injected 3 times every 2 weeks to elicit an immunological response. A Llama blood sample is taken and lymphocytes are isolated. **3)** Total RNA is isolated from lymphocytes and a VHH cDNA library is prepared by transfection into bacteria. **4)** Three rounds of panning enrich pmt1-specific VHH. Each round of panning is plated on ampicillin-selective medium. **5)** Isolated colonies are picked and tested in ELISA. Positive colonies are sequenced and go under alignment.
6) A VHH sequence is chosen and expressed in bacteria.
7,8,9) VHH specificity is tested by ELISA, octet testing and Western blot.

3. Nanobody Library Expressed and antigen-specific VHH isolated

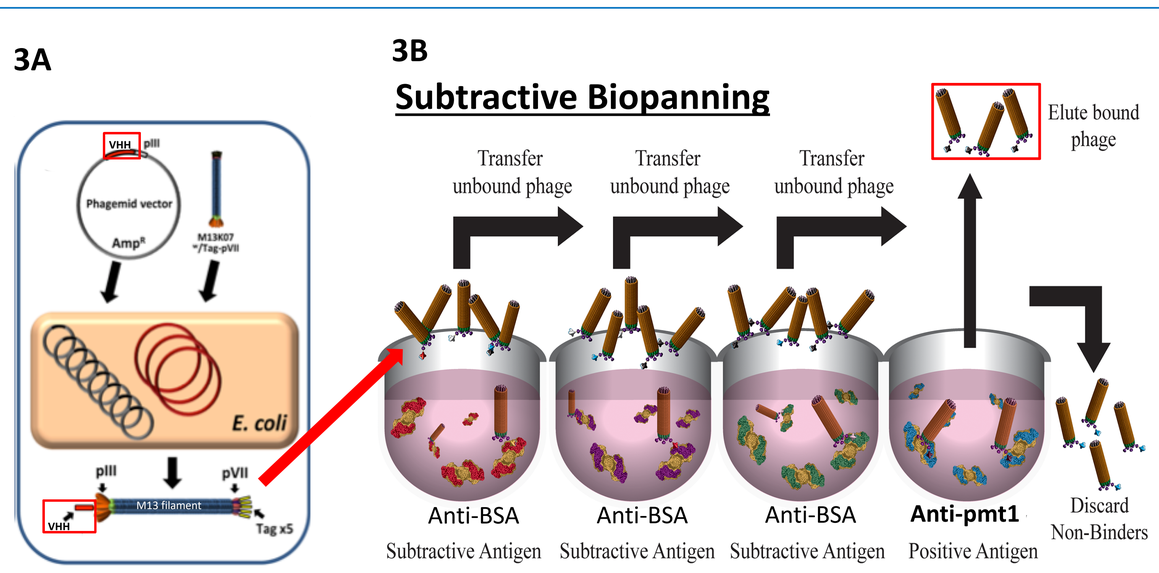


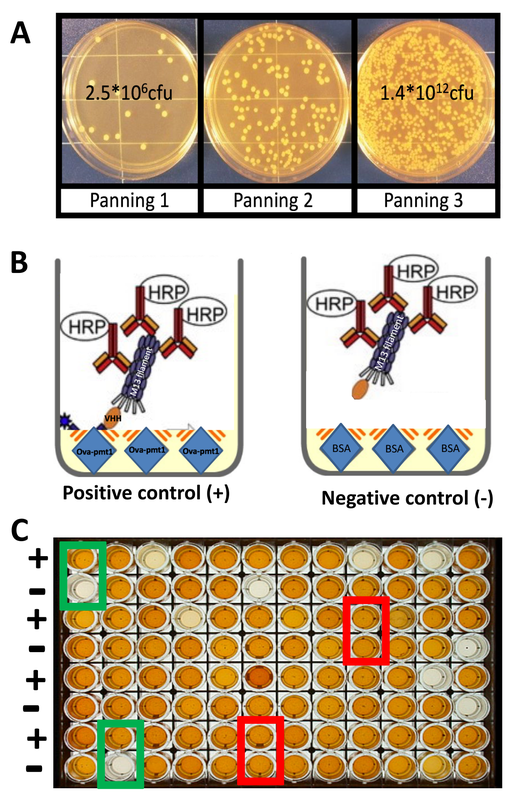
Figure 3A. Phage Display: VHH gene is cloned into the pHEN-6 vector in fusion with the gene encoding the bacteriophage surface protein p3. This vector Is then transfected into bacteria. To allow VHH expression on the phage surface, bacteria are co-transfected with helper phage (MK1307)
Figure 3B. Biopanning: VHH-expressing bacteriophage are incubated with an antigen coated on a solid phase and the unbound phages are removed by extended washing. Remaining VHH bound to pmt1 are eluted using Triethylamine.
- Eluted phages can be used to infect bacteria for the next round of panning.
- Increasingly stringent washing at every round of panning removes less specific VHH, allowing selection of more specific phage-VHH.
- Then individual clones are tested by ELISA (see below)

4. Pmt1-Specific nanobodies were isolated and verified by ELISA

Figure 4A. Plating of bacteria show enrichment of pmt1-specific VHH
- Each stage of panning was plated at concentrations 10^{-1} to 10^{-7}.
- Isolated colonies were picked and grown to form individual VHH-expressing bacteria on every well of each 96 well plate.
- Each ELISA well corresponds to a unique VHH.

Figure 4B. ELISA against pmt1-ovalbumin, and BSA
- Two indirect ELISA's were performed per panning cycle.
- One plate lined with BSA-pmt1 as the positive control and the other lined with BSA alone as the negative control.
- Phage-VHH was introduced to bind to the antigen. A secondary anti-phage Ab-HRP was then added.

Figure 4C.
- A colour change was seen when 100 uL OPD was added.
- The colour change (clear – orange) shows positive affinity of each unique VHH for pmt1– BSA and are cross-referenced with the negative control (BSA).
- Results which show maximum colour change (≥3.0) against pmt1- BSA and minimal colour change to BSA (≤0.2) are good candidates for sequencing.
- Results showed ten positive colonies which were sequenced, of which three were unique.



5. Subcloning Of Pmt1 – Positive VHH into pET23, an expression Vector Plasmid

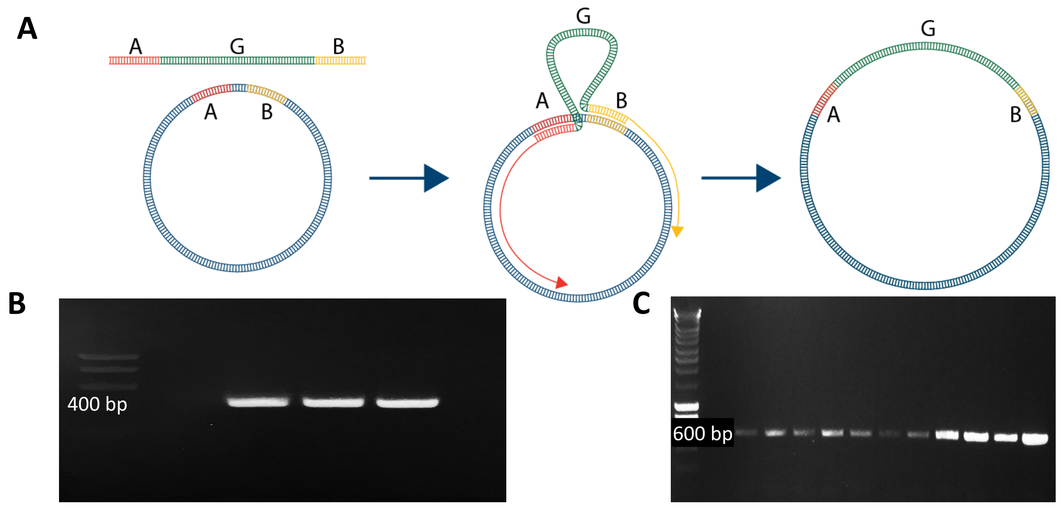


Figure 5A. Restriction-free cloning of B11 VHH into a pET23 vector.

(B) PCR1 of B11 VHH shows 3 strong bands at 450bp. Segments of the vector (A and B) were added to 5' and 3' of the B11 VHH Primers. The 450bp sample is purified from the agarose gel. The new segments ligated to the VHH gene are complementary to a plasmid sequence 100bp apart. This complementary binding to the pET23 vector forms a loop of approximately 400bp. **(C)** The second PCR transcribes the pET23 vector with the VHH inside. Parental vectors are eliminated by Dpn1 cleavage at a methylated site. The correct 600bp PCR2 product is verified by agarose gel electrophoresis. PCR2 product is purified on an agarose gel, and DNA is sent for sequencing. Sequences that match the first sequence is transfected into VHH expressing bacteria. The VHH raised to pmt1 can then be produced.

Disulfide bond

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> pmt1 B11
MADVQLVESGGGLVQAGGSLSLSCAASGRYTMGWFRQAPGREREFVAAITRNFGSTYYEDSIKGRFAISRDNAKNTVYLQMNSLKPEDTAVYYCAARRPTYRSTTYNDPNEYDYWAQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHHGS*
> pmt1 F9
MADVQLVESGGELVQAGGSLRLSCAASGRYTMAWFRQAPGKEREFVAAITRNIGATIYADSVEGRFTISRDNAKNTVYLQIDNLKPEDTAVYYCTARRPTYRSTTYSDPNEYHYWGQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHHGS*
> PEP3 G4P3
MAEVQLVESGGGLVQAGDSLRLSCAASGRYTMAWFRQVPGKEREFVAAITRNIGATLYADSVKGRFTISRDNADNTIYLQMNSLIPEDTAFYYCAARRPTYRSITYDDSNEYHYWGQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHHGS*
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FR1 FR2 CDR2 FR3 CDR3 HIS Tag

Conclusion

Here we describe the production process of VHH over a 6-month timeline from initial immunization to production of a functional nanobody. The VHH is specific to a protein of a cloned fission yeast gene pmt1 m5C-Mtase (pombe methyltransferase). This VHH is produced for the research interest of another department within the Pasteur Institute. Pmt1 has significant homology in prokaryotic and eukaryotic m5c-Mtase but no in–vitro tests can elucidate its methyltransferase activity as of yet. Anti-SMT1 VHH will be used to study the DNA Methylation of this enzyme. This research can be used to elucidate why fission yeast spores contain more methylated cytosines than their mycelial counterparts, and whether the methylation is an essential part of this developmental change. This project concluded after isolated VHH were resent for sequencing (VHH sequence did not return before the completion of this project). If the nanobody sequence results show identical sequence with the first sequence, the next step would be to transform the new sequence in gene expressing shuffle cells, selection of positive transformation with a selective marker, picking isolating colonies, induce VHH expression with IPTG, purification and isolation of VHH, and confirm VHH B11 expression with coomassie staining protein gel.

References

Smolarek, D., Bertrand, O. & Czerwinski, M. 2012, "Variable fragments of heavy chain antibodies (VHHs): a new magic bullet molecule of medicine?", *Postepy higieny i medycyny doswiadczalnej (Online),* vol. 66, no. 855199, pp. 348-358.
Wilkinson, C.R., Bartlett, R., Nurse, P. & Bird, A.P. 1995, "The fission yeast gene pmt1+ encodes a DNA methyltransferase homologue", *Nucleic acids research,* vol. 23, no. 2, pp. 203-210.

Acknowledgement

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