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Characterization of folding-sensitive nanobodies as tools to study the expression and quality of protein particle immunogens

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Highlights

- Four families of nanobodies were generated that recognize *Brucella* spp. lumazine synthase (BLS) in its native state only
- These nanobodies also recognize folded BLS *ex vivo*
- Conformation-sensitive Nbs are good molecular probes for quality control of vaccines

Abstract

Vaccination is as one of the most beneficial biopharmaceutical interventions against pathogens due to its ability to induce adaptive immunity through targeted activation of the immune system. Each vaccine needs a tailor-made set of tests in order to monitor its quality throughout the development and manufacturing. The analysis of the conformational state of protein nanoparticles is one of the key steps in vaccine quality control. The enzyme lumazine synthase

from *Brucella* spp. (BLS) acts as a potent oral and systemic immunogen. BLS has been used as a carrier of foreign peptides, protein domains and whole proteins, serving as a versatile platform for vaccine engineering purposes. Here, we show the generation and characterization of four families of nanobodies (Nbs) which only recognize BLS in its native conformational state and that bind to its active site. The present results support the use of conformation-sensitive Nbs as molecular probes during the development and production of vaccines based on the BLS platform. Finally, we propose Nbs as useful molecular tools targeting other protein scaffolds with potential applications in nano-and biotechnology.

Keywords: vaccine; protein particle; quality control; *Brucella* lumazine synthase; nanobodies; conformational probes

1- Introduction

Vaccination is considered the most cost-effective way to control pathogens and prevent diseases both in human and veterinary fields. Vaccines are classified as (i) live-attenuated, (ii)inactivated-killed, (iii) toxoid (inactivated toxin) and (iv) subunit-conjugate. Subunit antigens comprise natural or recombinant antigenic proteins, peptides, capsular polysaccharides or any specific part of the pathogen that stimulate a protective immune response. Examples of subunit vaccines include hepatitis B, pneumococcus and human papillomavirus (HPV) vaccines (Schiller et al., 2012; Schiller and Muller, 2015; Shouval et al., 2015; Skeate et al., 2016; Yum et al., 2012; Zanetti et al., 2008).

Protein nanoparticles (Diaz et al., 2018) can act as powerful immunogens (Luxembourg et al., 2015; Shouval et al., 2015), including virus-sized protein particles vaccines, which combine many of the advantages of whole-virus and recombinant subunit vaccines, integrating key features on immunogenicity, safety and protective potential. Because immunogenicity is related to molecular order and symmetry, virus-like particles (VLPs) are also being used in vaccine engineering. In this case, the highly immunogenic scaffold particles are decorated with otherwise weak antigens in order to increase their immunogenicity (Luxembourg et al., 2015; Smith et al., 2013; Wu et al., 2012).

The enzyme lumazine synthase from *Brucella* spp. (BLS) catalyzes the penultimate step in the riboflavin biosynthetic pathway (Bonomi, 2010; Klinke, 2005). BLS is a highly immunogenic protein (Berguer et al., 2012; Berguer et al., 2006; Velikovsky et al., 2003) that acts as a potent oral and systemic immunogen when used as a protein or as a DNA vaccine

(Velikovsky et al., 2002; Velikovsky et al., 2000). BLS has been used as a protein carrier of foreign peptides and proteins, serving as a platform for vaccine engineering purposes (Alvarez et al., 2013; Bellido et al., 2009; Clausse et al., 2013; Clausse et al., 2017; Craig et al., 2005; Laplagne et al., 2004; Mejias et al., 2014; Mejias et al., 2013; Rossi et al., 2015). The three-dimensional structure of BLS was determined by X-ray crystallography revealing a dimer of pentamers as its quaternary organization. The structure is an intertwined assembly where approximately 45% of the surface of each monomer is engaged in monomer-monomer and pentamer-pentamer contacts (Klinke et al., 2005; Zylberman et al., 2004). Interestingly, BLS folds into a highly stable and compact decamer, smaller than the classical VLPs. For these reasons, we postulate BLS as a better scaffold than VLPs to accommodate entire protein domains or whole proteins. In this sense, BLS is becoming an efficient alternative to VLPs for vaccine design.

A crucial step in the vaccine manufacturing process is the quality control of the folded and native structure of its immunogens. The immune system triggers a more effective response if it is challenged with the protein components of a vaccine in their native state, as opposed to denatured antigens, eliciting the production of more competent neutralizing antibodies. Biophysical and biochemical methods, such as circular dichroism (CD), static light scattering (SLS), dynamic light scattering (DLS), enzyme-linked immunosorbent assay (ELISA) and fluorometric studies are commonly used in vaccine quality control (Hamidi and Kreeftenberg, 2014; Metz et al., 2009; Yang et al., 2015; Zhang et al., 2010). The use of monoclonal antibodies (mAbs) can provide additional information about the quality of the vaccine products besides biophysical data, and such information directly relates to the three-dimensional conformational structure of the protein. Furthermore, the use of mAbs allows the design of highly sensitive methods, useful to analyze samples with minimal preparation. Many times, CD and DLS analysis require highly purified samples without interfering agents. Particularly in scaling up processes, there is a demand for simple methods to test the quality of protein particles, and mAbs that recognize only the folded states of the scaffold proteins are being used for this purpose (Keck et al., 2012; Lecouturier et al., 2018; Palomo et al., 2014). Selecting an optimal mAb for this aim for a given protein vaccine may require a significant effort, since many different antibodies with a wide range of specificities are generated, including those that recognize conformational discontinuous and continuous epitopes (Clementi et al., 2013; Klein et al., 2013; Kong et al., 2016; Li et al., 2016; Liang et al., 2016). Thus, the majority of selected mAbs are insensitive to the native folding of the target protein. In contrast, nanobodies (Nbs) (Hamers-Casterman et al., 1993) selected by phage display tend to recognize only

conformational discontinuous epitopes on protein antigens (McMahon et al., 2018; Pardon et al., 2014; Rasmussen et al., 2011; Rothbauer, 2018; Steyaert and Kobilka, 2011). This property makes Nbs attractive molecular tools for vaccine engineering and quality control processes (Koromyslova and Hansman, 2017; Rothbauer, 2018; Virant et al., 2018; Wesolowski et al., 2009). In this work, we generate conformational-sensitive Nbs for monitoring the folded state of the BLS subunit vaccine scaffold through a phage display screening process. The results presented here show that conformation-sensitive Nbs can be easily generated against vaccine scaffolds and antigens and that their use can be directly applied to the vaccine development and production steps.

2-Materials and methods

2.1 BLS expression and purification

The pET11a vector (Stratagene) harboring the *Brucella abortus* BLS ORF (BAB2_0545) (Klinke et al., 2005; Zylberman et al., 2004) was used to transform *Escherichia coli* BL21 (DE3) competent cells (Stratagene). Starter cultures were grown to an OD_{600} of 1 in LB medium containing 100 µg/ml Ampicillin (Amp) at 37 °C with agitation (300 r.p.m.). Five ml of these cultures were diluted to 500 ml of LB medium and grown to an OD_{600} of 1 in the same conditions. The cultures were induced with 1 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) for 4 h at 37 °C with agitation (300 r.p.m). Then, cells were pelleted at 9000*g* for 15 min at 4 °C, resuspended and sonicated in 50 mM Tris pH 8.0, 5 mM EDTA and 1% (v/v) Triton X-100. The homogenates were then centrifuged at 20000*g* for 15 min at 4 °C, pellets were resuspended in 20 ml of buffer A (50 mM Tris pH 8.0 and 1mM dithiothreitol (DTT)) and dialyzed extensively against buffer A. Protein fractions were purified by anion-exchange chromatography in an FPLC apparatus using a Mono-Q column (GE Healthcare Life Sciences) equilibrated in buffer A using a linear gradient of 0–1 M NaCl. The BLS containing fraction was further purified on a size-exclusion chromatography column (GE Healthcare Life Sciences) using phosphate buffered saline buffer (PBS) and 1 mM DTT. The purity of the BLS preparation was determined by 15% SDS-PAGE. The protein was then amidinated with 25 mM iodoacetamide at room temperature (RT) for 1 h to block its free cysteine residues. The purified protein was concentrated to10 mg per mL flash frozen in liquid N_2 and stored for further use at -80 °C.

2.2 Llama immunization and construction of the library

Two llamas (*Lama glama*) were immunized three times subcutaneously every 15 d with 1 mg of purified BLS emulsified with the adjuvant Specol. The humoral immune response was monitored by ELISA using animals sera on plates coated with 1 µg of BLS. Thirty days after the third immunization, the animals were bled. Mononuclear cells were isolated from 120 ml of blood by Ficoll-Paque (GE Healthcare) gradient centrifugation. Total RNA was purified from these cells using Trizol (Invitrogen) and subjected to cDNA synthesis. The nanobodies (Nbs) coding regions were amplified by PCR using specific primers (forward: VH1b-SfiI gctggattgttattactcgcggcccagccggccatggcccaggtsmarctgcagsagtcwgg, VH6b-SfiI cgtggattgttattatctgcggcccagccggccatggccgatgtgcagctgcaggcgtctggrggagg and reverse: Lam07-NotI gatggtgatgatgatgtgcggccgcgctggggtcttcgctgtggtgcg, Lam08-NotI gatggtgat gatgatgtgcggccgctggttgtggttttggtgtcttgg). The amplicons were purified from agarose gels, digested with SfiI and NotI (New England Biolabs) and cloned into the pHEN2 phagemid vector downstream the PelB-leader peptide and upstream the chimeric His6x-Myc epitope tag. Transformation into *E. coli* XL1-Blue yielded a library with a size of 6.6 x 10^6 clones. Ten clones were used for plasmid DNA preparation (QIAprep Spin Miniprep Kit, QIAGEN) and sequenced for library diversity analysis.

2.3 Phage display selection of BLS-specific Nbs

The panning was performed by ELISA. Briefly, four wells were sensitized with 1 µg of BLS and two wells were left uncoated as negative controls. After blocking with 1% skim milk in PBS, 2.3 x 10^9 plaque formation units (p.f.u) VCS-M13 (Stratagene) diluted in 1% skim milk in PBS were added to each well and incubated for 2 h at RT. Wells were extensively washed with PBS and bound phages were eluted with 100 mM glycine-HCl pH 2 and quickly neutralized with 2 M Tris-HCl, pH 8. Eluted phages were titrated and subjected to a second round of panning following the same procedure. Output phage titers were estimated by infection of XL1-Blue cells and plating them on LB + Amp + 2% glucose plates. Ninety-six individual colonies were grown in culture plates in 2XTY + Amp + 2% glucose for 3 h at 37 °C with agitation, and superinfected with 2.3 x 10^9 p.f.u for 30 min. Cells were pelleted, resuspended in 2XYT + 2% glucose + Amp + Kanamycin A (Kan) and incubated for 16 h at 30 °C. Culture phage supernatants from individual colonies were tested for BLS specificity by ELISA on plates sensitized with 1 µg of BLS. After washing with PBS + 0.05% Tween 20 (PBST), bound phages were revealed with peroxidase-conjugated anti-M13 mAb (Sigma-Aldrich). Plasmidic DNA was isolated from single colonies and sequenced using pHEN2-specific primers. Nbs with a

highly similar CDR3 sequence were grouped in families (Protein multiple sequence alignments performed using *Clustal Omega,* https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.4 Production and purification of Nbs

Representative clones for each family of Nbs and a Non-Related Nb (NRNb) (Alzogaray et al., 2011) were expressed as soluble periplasmic proteins in *E. coli* HB2151 non-suppressor strain and purified by HiTrap chelating HP column (Amersham Biosciences) followed by size-exclusion chromatography using a Superdex 75 column (GE Healthcare Life Sciences). Purity was assessed by 15% SDS-PAGE and their concentration was determined by absorbance at 280 nm using their theoretical molar absorption factor obtained from the amino acid sequence using ProtParam software from ExPASy server (https://web.expasy.org/protparam/).

2.5 ELISA and Western blotting

ELISA plates were sensitized with 0.5 µg of native or heat denatured BLS samples for 1 h, blocked with 3% skim milk overnight (ON) and incubated with 0.5 µg of Nbs or anti-BLS monoclonal antibody BI24 (mAb BI24) (Goldbaum et al., 1993) for 2 h at RT. The BLS heat denatured sample was obtained by heating at 95 °C for 10 min as previously reported (Zylberman et al., 2004). The mAb BI24 and NRNb were used as positive and negative controls, respectively. Nbs were revealed with a monoclonal anti-polyHistidine antibody (Sigma-Aldrich). The plates were subsequently incubated with anti-mouse IgG conjugated to horseradish peroxidase (HRP) enzyme (Sigma-Aldrich). The color reaction used O-Phenylenediamine (OPD, Sigma-Aldrich) as substrate and was stopped with 2 M H_2SO_4. Absorbance at 492 nm was measured using a plate reader (Thermo Fisher Scientific).

Native and heat denatured BLS samples were resolved in 10% SDS-PAGE and 10% PAGE (native-PAGE), transferred to nitrocellulose membranes (Immobilon, Millipore) and blocked with 3% skim milk in PBST. Afterwards, membranes were incubated with Nbs (1/800 dilutions) and anti-BLS mAb BI24 (1/1000 dilution) for 1 h at RT. Nbs treated membranes were incubated with anti-His (Sigma-Aldrich). Both blots were incubated with anti-mouse IgG-HRP (1/1000 dilution) (Sigma-Aldrich). Detection was achieved using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) on an ImageQuant LAS 400 apparatus (GE Healthcare Life Sciences).

2.6 Affinity measurements

The kinetics of association and dissociation between BLS and different Nbs were studied in an IAsys plus Affinity Sensor Biosensor (Thermo LabSystems Inc.). A solution of BLS in 10 mM malic acid pH 6 was used for immobilization on a carboxymethyl dextran matrix cuvette using the EDC/NHS coupling kit as recommended by the supplier. Dilutions of each Nb in PBST were added to the cuvette. Binding traces were recorded for at least six different concentrations. The dissociation was measured at the higher concentrations of Nbs. In all cases, rate constants for association and dissociation were obtained by globally fitting the data using the Fast plot software (Thermo LabSystems Inc.). All experiments were performed at 25 °C.

2.7 Riboflavin displacement assay

Riboflavin (RF) fluorescence was achieved by exciting at 445 nm and collecting emission at 520 nm using 3-nm band passes for both excitation and emission. The BLS-RF complex was generated by mixing 5 µM BLS (monomer) with 5 µM RF solutions and incubated for 30 min at RT. Experiments were performed in PBS, pH 7, in the presence of increasing concentrations of each Nb (0-23 µM). All fluorescence measurements were determined at 25 °C on a FP-770 spectrofluorometer (Jasco).

2.8 Transfection of HEK-293 cells

The coding region of BLS (GenScript) was cloned into the pCI-neo expression vector (Promega). Plasmid DNA were isolated from single colonies and isolated using Maxiprep plasmid isolation columns (QIAGEN), following the manufacturer's instructions. Adherent epithelial HEK-293 cells (ATCC® CRL-1573™) were cultured on 12-mm coverslips inside the wells of 24-well tissue culture plates in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (PAA Laboratories). Transfection of HEK-293 cells with BLS expression vector pCI-neo was performed using jetPEI transfection reagent (Polyplus Transfection Inc.) according to the manufacturer's instructions. At 24 h after transfection, cells were washed with Hank's balanced salt solution (HBSS; Gibco), fixed in 4% formaldehyde for 15 min, washed twice with HBSS, and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Samples were then incubated with Nbs (1/800 dilutions), followed by monoclonal anti-His antibody incubation (1/1000 dilution) for 1 h at RT. The mAb BI24 (1/2000 dilution) was used as positive control. Immunofluorescence was revealed using an Alexa350 conjugated anti-mouse IgG (Molecular Probes). Finally, the coverslips were mounted on microscope slides and visualized

in an LSM 510 confocal microscope (Zeiss). Images were processed using ImageJ 1.51u software (https://imagej.nih.gov/ij/).

2.9 Quantitative ELISA to measure the expression levels of a BLS subunit vaccine

The chimera BLS-Omp31 was expressed in *E. coli* as previously described (Laplagne et al., 2004). First, to perform the calibration curve of BLS-Omp31 for the quantitative ELISA, plates were sensitized with 0.5 µg of Nb7 in PBS as capture antibody ON at 4 °C and then blocked with 3% skim milk 1 h at RT. Purified recombinant BLS-Omp31 was added in a range from 0.05 ng to 1.0 ng per well. The plates were subsequently incubated with anti-BLS mAb BI24 as the detection antibody and then with anti-mouse IgG conjugated to horseradish peroxidase (HRP) enzyme (Sigma-Aldrich) as a secondary antibody. The reaction was developed by adding 50 µl of a solution containing 2 µg/µl ortho-phenylenediamine (OPD, Sigma-Aldrich) and 0.03% H_2O_2 in 0.1 M citrate-phosphate buffer and was stopped with 50 µl of 2 M H_2SO_4. Absorbance at 492 nm was measured using a plate reader (Thermo Fisher Scientific).

E. coli BL21 (DE3) competent cells (Stratagene) expressing BLS-Omp31 were grown in 100 ml of LB medium containing 100 µg/ml Ampicillin (Amp) at 37 °C with agitation (300 r.p.m.) to an OD_{600} of 0.6-0.7 and induced with 1 mM IPTG for 4 h at 37 °C with agitation (300 r.p.m). Aliquots were taken every 30 min (until 210 min), pelleted and stored at -20 °C. Then, pellets were resuspended in 50 mM Tris pH 8 and 5 mM EDTA to an OD_{600} of 0.5 and sonicated. The homogenates were then centrifuged at 20000 g for 15 min at 4 °C and different dilutions of the supernatants were analyzed by ELISA as described for the calibration curve. The same samples were analyzed by 15% SDS-PAGE and quantified using Image Lab 5.2.1 (Bio-Rad).

3-Results

3.1 Generation of BLS-specific Nbs

A crucial step to obtain antibodies that recognize the conformational folded proteins is to immunize the experimental animals with a folded immunogen. Hence, it is key to perform a quality control of the protein prior to the injections. BLS-based vaccines can be easily characterized by performing thermal denaturation monitoring the CD signal and SLS analysis (Zylberman et al., 2004). To generate Nbs specific for folded BLS, two llamas were immunized three times with native BLS protein. The induction of a humoral immune response was evaluated by ELISA determining the titers of llama antibody directed towards BLS using

immune sera obtained from the animals 30 d after immunization (a.i.). In both animals, a humoral immune response was induced with very high titers of IgG antibodies (data not shown). A Nbs phage display library of 6.6 x 10^6 clones was generated from one of the llamas and BLS specific Nbs were selected after two rounds of panning. Ninety six randomly chosen clones were grown and tested by phage ELISA, where 95% of the clones were specific against BLS. Twenty positive clones were selected for sequencing analysis. The results revealed four distinct families of Nbs based on the length and variability of the CDR3 region (**Figure 1**). One representative clone for each family was selected for Nb expression, purification and further characterization.

3.2 Four families of Nbs only recognize BLS in its native state

To determine whether these Nbs recognize BLS in its native and/or unfolded state, ELISA and Western blot analyses were performed using folded and heat denatured BLS. **Figure 2A** shows that the four selected Nbs recognize only the BLS folded conformation in an ELSA. However, the mAb BI24 is able to recognize both states of BLS, as previously reported (Velikovsky et al., 2000). The reactivity of mAb BI24 with both native and unfolded BLS clearly demonstrates that the lack of reactivity of Nbs against the latter is not due to poor binding of the denatured protein to the solid phase of the ELISA plate. The reactivity of the Nbs and mAb BI24 against folded and heat denatured BLS was further analyzed by Western Blot after native-PAGE or SDS-PAGE using Nb7 as a representative Nb. It can be seen in **Figure 2B** that both Nb7 and mAb BI24, strongly react with decameric BLS, separated in a native-PAGE (running band of ~180 kDa). In contrast, after SDS-PAGE the mAb BI24 is able to react against denatured monomeric BLS (running band of ~18 KDa) whereas Nb7 shows complete absence of reactivity (**Figure 2B, Figure S1**). Taken together, these results indicate that the four families of Nbs recognize conformational epitopes only present in the oligomeric native state of this protein particle.

3.3 Nbs bind to BLS active site-related epitopes

A biosensor analysis was performed to determine Nbs-BLS binding affinities. The results show that dissociation constants (K_D) are mostly in the sub-micromolar range (**Table 1 and Figure S2**). Because Nbs commonly recognize the active sites of enzymes (Desmyter et al., 2015; Desmyter et al., 1996; Duhoo et al., 2017; Rasmussen et al., 2011) we wondered whether the four Nbs selected were able to recognize the active site of BLS or alternatively to recognize

allosteric sites that modify the active site. There are ten active sites per BLS particle that bind to ten RF molecules. In **Figure 3A** one active site is indicated in the decameric structure (colored in magenta), constituted by two neighboring monomers (cyan and green colored) from the same pentamer. RF binding to the BLS protein produces a quenching of its fluorescence (Zylberman et al., 2004). Therefore, the release of RF bound to the BLS active site can be monitored by an increase in its fluorescence. Nbs were assayed for their capacity to displace BLS-bound RF. The BLS-RF complex was incubated with increasing amounts of the four selected Nbs or a NRNb. Only the specific Nbs are able to displace bound RF (**Figure 3B**), indicating that they bind (or affect) directly the BLS active site. As the active site of BLS is built by residues belonging to neighboring monomers, this result highlights again the conformational nature of the recognized epitopes. Nb8 produces a displacement of RF of 100% at equimolar concentrations, whereas the other specific Nbs shows either lower affinity or partial RF displacement (**Figure 3B**), indicating differences in the recognized epitopes.

3.4 Nbs recognize folded BLS *ex vivo*

BLS is an effective vaccine carrier also when it is used as a DNA vaccine. Several reports show that BLS can be successfully expressed either in eukaryotic cell lines for quality control as well as in immunized host muscle cells (Estein et al., 2009; Mejias et al., 2013; Velikovsky et al., 2000). We pose the question whether BLS is in its native state fold when it is expressed *ex vivo* in control cells as previously used in DNA vaccination assays (Mejias et al., 2013; Velikovsky et al., 2000). To answer that question, we performed immunofluorescence microscopy on transfected HEK-293 cells expressing BLS and revealed them using Nb7 as the primary antibody. **Figure 4** shows that Nb7 is able to detect BLS, similarly as the mAb BI24 positive control, indicating that BLS is indeed natively folded within the eukaryotic cell. This result indicates that Nbs can be used as molecular probes to analyze protein particle immunogens during vaccine development.

3.5 Nbs are useful to design a quantitative ELISA to measure correctly folded BLS nanoparticles in a fermentation culture

BLS has been successfully decorated with a 27-mer peptide pertaining to the outer membrane protein 31 (Omp31) of *Brucella* spp. The resulting chimera, BLS-Omp31, has shown promising results as an experimental vaccine against ovine and canine brucellosis (Clausse et al., 2013; Estein et al., 2009). Here we show the development and application of a quantitative sandwich ELISA to measure the expression levels of BLS-Omp31 in a fermentation process.

The calibration curve (**Figure S3**) shows that the assay is very sensitive and lineal between 0.1 and 0.5 nanograms of the chimera. Although the affinity of Nb7 is low (226 nM) compared with others Nbs and with mAbs mentioned in bibliography, it behaves as an excellent capture antibody presumably because of the polymeric nature of BLS. This would be a common feature for other protein nanoparticles, indicating that the specificity of the Nbs for the folded particle is more important than its intrinsic affinity for.

The concentration of BLS-Omp31 after inducing an *E.coli* recombinant culture increases from zero to 10 mg/ml in about three hours (**Figure 5A**), allowing for a fast quantification of correctly folded nanoparticles. **Figure 5B** shows the parallel analysis of the same samples by SDS-PAGE. A clear correlation between both assays can be observed.

4-Discussion

The successful development of vaccines is a lengthy and costly process, requiring input from different areas of expertise, such as research and development, quality control, quality assurance, production, regulatory affairs, marketing and sales. A cornerstone in vaccine development is the availability of a panel of efficient assays useful for quality control to reduce the risk of failure in the process (Metz et al., 2009). Nbs are molecules relatively easy to obtain compared to mAbs, and exhibit an exquisite capacity to recognize conformational epitopes. Vaccine development can take advantage of these positive characteristics in order to perform quality control of protein particle immunogens throughout the production process. Here we show the generation and characterization of four families of Nbs with useful properties for applications in the BLS platform. This protein scaffold has an extraordinary stability against thermal and chemical denaturation, and due to its compact and small size, it is suitable to be decorated with large antigens, increasing the stability of the inserted foreign structures (Mejias et al., 2014; Mejias et al., 2013). The scaling up of the production process of these chimeric antigens often take advantage of the stability of BLS to 8 M urea to achieve discontinuous folding procedures. The use of conformation-sensitive Nb molecular probes may become a useful control tool in the folding steps as well as in the bulk fermentation processes. For example, by using these tools, the quality of new BLS chimeras may be followed in every step of the process. Analyzing the reactivity of the samples in a sandwich ELISA, the native, decameric scaffold fold can be determined and easily quantified (**Figure 5**). A similar analysis may be conducted to analyze the native state of the protein *ex vivo* as shown in **Figure 4**. In this

sense, Nbs could be used in a similar fashion against other protein scaffolds, with potential applications in imaging and nanotechnology.

Conclusion

In the present work we generated different Nbs that can bind only to the native state of the decameric BLS structure and we show several assays using these Nbs as molecular probes. The results provided here suggest that similar approaches could be useful in the characterization of other protein scaffolds for vaccine development.

Author Contributions: VA assisted on the construction of the library, purification and expression of BLS and Nbs, performed ELISA, Western blotting, transfection of HEK cells, performed SDS-quantitative, analyzed and discussed the results and wrote the paper. MU performed the llama immunization, construction of the library, phage display selection of BLS-specific Nbs, affinity measurements and riboflavin displacement assay and analyzed and discussed the results. PB analyzed and discussed the results. AR helped with *ex vivo* binding analysis and preparation of the figures. VZ performed riboflavin displacement assays and discussed the results. RP and VZ designed the ELISA and SDS-PAGE quantitative analysis. HRB analyzed and discussed the results. FAG designed the study, analyzed and discussed the results and wrote the paper. All authors commented on the manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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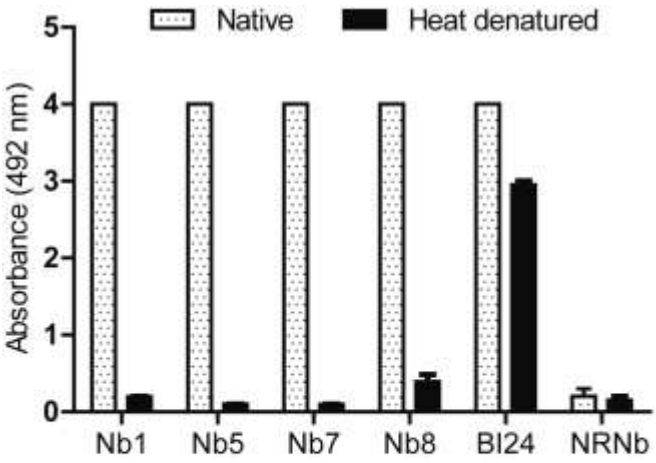
Figures and table

Figure 1. BLS-specific nanobody families. Amino acid sequence alignment of BLS-specific Nbs after two panning rounds. Twenty positive phage ELISA clones were classified into four families according to their CDR3 length and variability. The sequences of representative clones for each family are represented. CDR3 lengths: clone 8 (family 1) = 18 aa; clone 7 (family 2) = 16 aa; clone 1 (family 3) = 13 aa and clone 5 (family 4) = 10 aa. Framework regions (FR) and complementarity determining regions (CDR) are indicated. An * (asterisk) indicates positions that have a single, fully conserved residue; **:** (colon) indicates conservation between groups of strongly similar properties; and **.** (period) indicates conservation between groups of weakly similar properties.

Figure 2. Specific Nbs recognize BLS in its native state. A) Indirect ELISA analysis sensitizing plate wells with native BLS (white dotted bars) or heat denatured BLS (black bars) in same amounts. Negative control: Non-related Nb (NRNb). B) Western blot revealing BLS after native-PAGE and denaturing SDS-PAGE. Nb7 and mAb BI24 were used as primary antibodies.



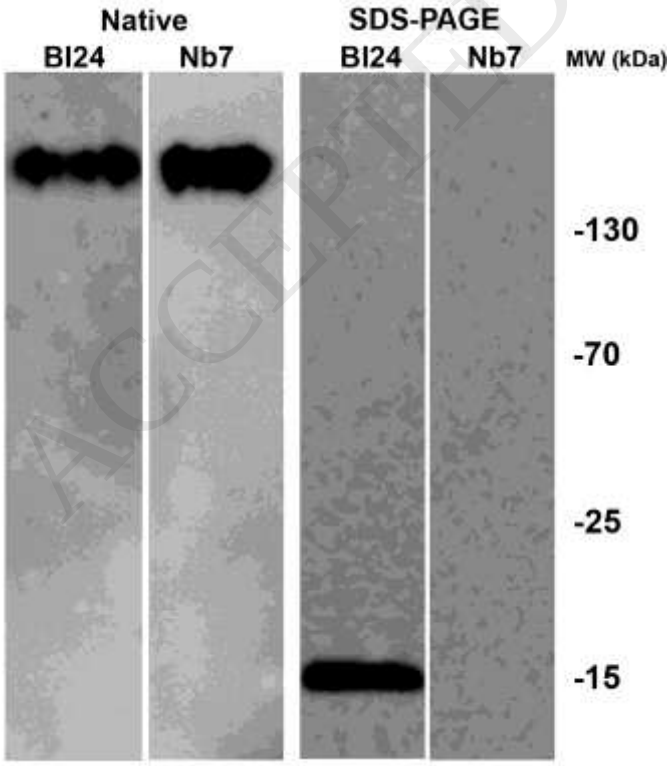
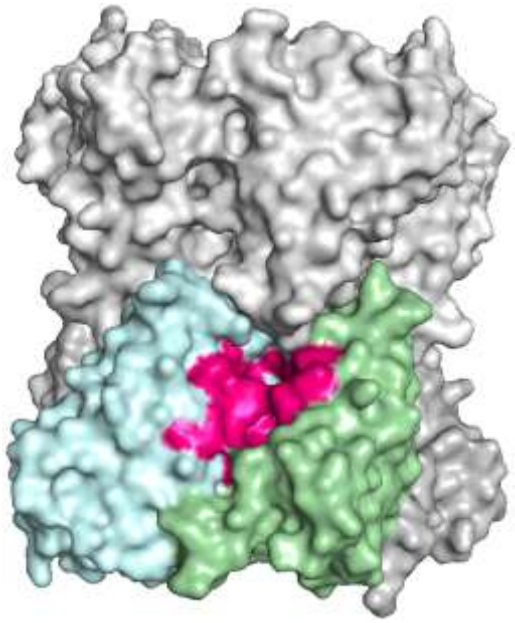


Figure 3. Nbs recognize the active site of BLS. A) The active site of BLS is represented in pink color constituted by residues from neighboring monomers within the same pentamer. One riboflavin (RF) molecule binds to this pocket. There are ten active sites per BLS particle. B) Bound RF displacement curves. The BLS-RF complex was titrated by increasing Nbs concentrations. RF fluorescence was monitored exciting at 445 nm and measuring at 520 nm. Data represent the fluorescence increments in percentage (%) relative to the highest value (Nb8). Specific Nbs: 1, 5, 7 and 8; Control non-specific Nb: NRNb.



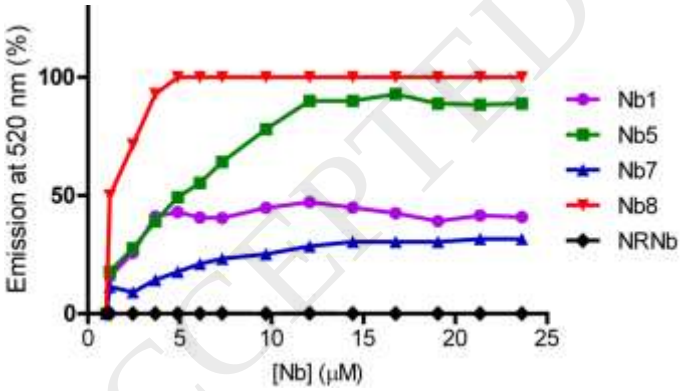


Figure 4. Nbs are able to recognize BLS *ex vivo*. HEK-293 cells were transiently transfected with a vector for BLS expression. The expression of the protein was evaluated by immunofluorescence microscopy using Nb7 and BI24 as primary antibodies. Cell nuclei were stained with DAPI. Control cells were not transfected.

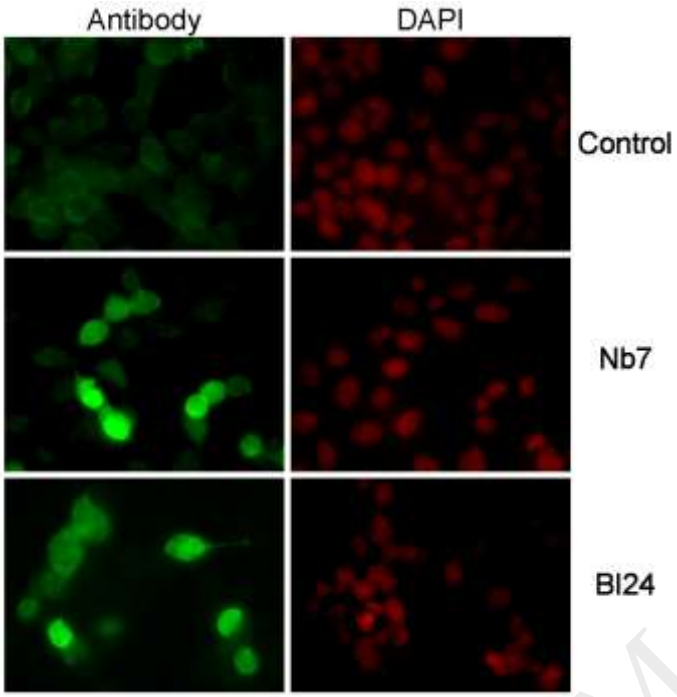
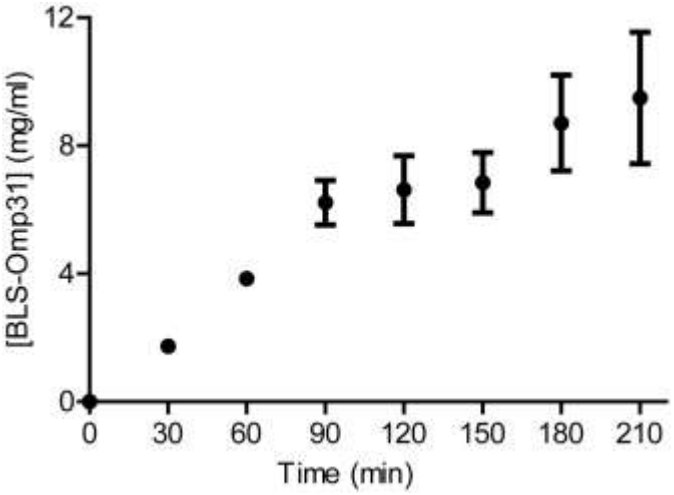


Figure 5. Quantitative sandwich ELISA using Nb7 as capture antibody to measure BLS nanoparticles in a fermentation culture. A) ELISA plates were sensitized with Nb7 as capture antibody and the presence of BLS particles was detected by using anti-BLS mAb BI24. Values are expressed as mean ± SD as a function of the time of induction of the cultures (n = 3 replicates). Data are representative of two independent experiments. B) The same (right lanes) samples were analyzed by SDS-PAGE using purified BLS-Omp31 as standard (left lanes).



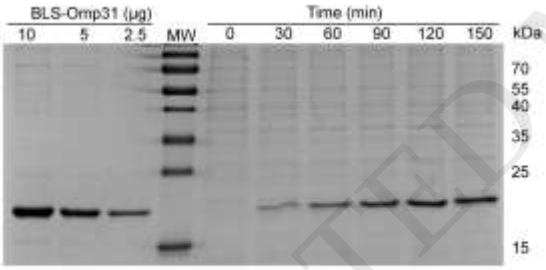


Table 1. Nb affinity constants for BLS determined by biosensor kinetic analysis.

Nb	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM) *
5	3.2±0.2x10^4	6.1±0.1	191±15
7	5.3±0.1x10^4	1.2±0.1	226±23
1	7.2±0.1x10^3	5.6±0.1	778±25
8	3.8±0.6x10^4	3.8±0.1	1000±184

*$K_D = k_{off} / k_{on}$