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Research paper

Chaperonin GroEL a *Brucella* immunodominant antigen identified using Nanobody and MALDI-TOF-MS technologies

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ABSTRACT

The deployment of today's antibodies that are able to distinguish *Brucella* from the closely similar pathogens, such as *Yersinia*, is still considered a great challenge since both pathogens share identical LPS (lipopolysaccharide) O-ring epitopes. In addition, because of the great impact of *Brucella* on health and economy in many countries including Syria, much effort is going to the development of next generation vaccines, mainly on the identification of new immunogenic proteins of this pathogen. In this context, *Brucella*-specific nanobodies (Nbs), camel genetic engineered heavy-chain antibody fragments, could be of great value.

Previously, a large Nb library was constructed from a camel immunized with heat-killed *Brucella*. Phage display panning of this 'immune' library with *Brucella* total lysate resulted in a remarkable fast enrichment for a Nb referred to as NbBruc02. In the present work, we investigated the main characteristics of this Nb that can efficiently distinguish under well-defined conditions the *Brucella* from other bacteria including *Yersinia*. NbBruc02 showed a strong and specific interaction with its antigen within the crude lysate as tested by a surface plasmon resonance (SPR) biosensor and it was also able to pull down its cognate antigen from such lysate by immuno-capturing. Using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), NbBruc02 specific antigen was identified as chaperonin GroEL, also known as heat shock protein of 60 kDa (HSP-60), which represents a *Brucella* immunodominant antigen responsible of maintaining proteins folding during stress conditions. Interestingly, the antigen recognition by NbBruc02 was found to be affected by the state of GroEL folding. Thus, the Nb technology applied in the field of infectious diseases, e.g. brucellosis, yields two outcomes: (1) it generates specific binders that can be used for diagnosis, and perhaps treatment, and (2) it identifies the immunogenic candidate antigens for developing vaccines.

1. Introduction

Brucellosis is one of the major zoonotic diseases affecting health and economy in large parts of the world and Syria in particular (Boschiroli et al., 2001; Refai, 2002). The disease is caused by members of the genus *Brucella*, a gram negative bacteria that live facultatively within mammalian cells during infection stages. For pregnant wild and domesticated animals the final outcome of this infection is an abortion, resulting in great losses especially in developing

Abbreviations: MALDI-TOF-MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; scFv, single chain variable fragment; Nb, nanobody; HCAbs, heavy-chain antibodies; SPR, surface plasmon resonance; Ni-NTA, nickel-charged nitrilo triacetic acid; RIU, refractive index unit; OMP, outer membrane proteins.

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countries (Pappas et al., 2005). Several methods that bypass the requirement for lengthy and tedious culture conditions to reliably differentiate *Brucella* from other pathogens are available for brucellosis diagnosis (Pappas et al., 2005). One of the most popular method is an immunoassay employing antibodies to LPS, the immunodominant surface antigen (Laurent et al., 2004). However, LPS O-ring epitope, the immunologically distinguishing feature of *Brucella* is also shared by other pathogens such as *Yersinia* (Weynants et al., 1996; Pappas et al., 2005).

Brucella infections in livestock are usually prevented by administration of attenuated live vaccines. The smooth *Brucella abortus* S19 and *B. melitensis* Rev 1 are used for bovine and small ruminant immunization, respectively (Bardenstein et al., 2002; Corbel, 2006). The main drawback with these vaccines is that they induce O-polysaccharide-specific antibodies that interfere with the serologic diagnosis of the disease (Pappas et al., 2005). Also, due to the residual pathogenicity, none of these vaccine strains can be used in humans. Because of its potential use as a biological warfare agent, much effort is being placed on the development of next generation vaccines (Pappas et al., 2006). Other strategies include subunit vaccines, overexpression of a protective antigen in a less virulent *Brucella* vaccine strain, vaccination with *E. coli* expressing *Brucella* antigens, and DNA vaccines (Schurig et al., 2002). The identification of proteins, able to elicit a protective immune response, is essential to develop an efficient vaccination strategy. Therefore, the search for new and specific *Brucella* immunodominant antigens is as important as the development of new antibodies that recognize these targets.

Antibodies are efficient tools used for the prevention, diagnosis and treatment of many diseases and bacterial pathogens including *Brucella*. However, the large size and the complex structure of existing antibodies cause practical and medical limitations, consequently, many targets remain cryptic for intact antibodies (Chester et al., 2004; Holliger and Hudson, 2005). In cases where current antibodies are not practical, smaller recombinant antibodies might provide an elegant solution (Holliger and Hudson, 2005; Van Bockstaele et al., 2009). Hence, the field of recombinant antibody technology has rapidly progressed during the last two decades, mainly with an objective for human therapeutic applications.

Throughout mammalian species, antibodies are invariably composed of two identical H-chains and two identical L-chains, and their recombinant fragment, containing the antigen binding site, is produced by synthetically joining the genes of the variable domains of these chains into a continuous peptide of 25 kDa, known as scFv. The antibodies of all *Camelidae* species (camel, dromedary and four llama species) form a surprising exception to the antibody paradigm structure as their serum contains also a considerable fraction of homodimeric heavy-chain antibodies (HCAbs), which lack the L-chain (Hamers-Casterman et al., 1993). The antigen recognition is accomplished by the variable domain of this camelid heavy-chain antibody. The recombinant form of this autonomous, single variable domain has dimensions in the nm range and is known as Nanobody (Nb). In contrast to scFv – where the in vivo affinity matured VH–VL pair of a classical antibody might get scrambled during the scFv library construction leading to reduced antigen-specificity and affinity – the gene bank of Nbs from an immunized camel is a collection of intact, affinity-matured antigen-binding molecules from which useful antigen-specific binders can be retrieved. In the field of infectious diseases, the Nb technology could provide a valuable approach to identify and study structural and functional proteomics implicated in microbial infections, referred to as infectomics (Huang et al., 2002). Even without access to purified antigen, it is possible to retrieve antigen-specific Nbs since heavy-chain antibodies directed against the most immunodominant antigens are well represented in the blood of a camelid immunized with a crude extract or lysate of the pathogen (Saerens et al., 2008a). Furthermore, by applying reverse proteomic approaches, these cognate antigens can be identified and used subsequently as targets in diagnostic and/or disease management (Saerens et al., 2008a; Groot et al., 2009; Hassanzadeh-Ghassabeh et al., 2011). Today, Nbs represent a powerful tool in many selection systems such as phage, yeast or ribosome display (Harmsen and De Haard, 2007). Especially, the small size and the beneficial biochemical properties (solubility, stability, specificity and affinity) of Nbs are at the origin of their outstanding performance. In addition, Nbs are more resistant to heat and pH, and much simpler in shape and chemical composition, in comparison to complete heterotetrameric antibodies as well as their recombinant antigen-binding fragments. Therefore, these properties make Nbs ideal for numerous diagnostic and therapeutic applications (Dumoulin et al., 2002; Renisio et al., 2002; Dolk et al., 2005).

In an initial step to combat brucellosis, a highly diverse Nb library was recently constructed from a camel immunized with a heat-killed *B. melitensis* Riv 1 strain (Abbady et al., 2011). Phage display of the Nbs in this library and panning using *Brucella* total lysate resulted in the identification of Nbs for unspecified antigens within this lysate. One of these Nbs, named NbBruc02 (GenBank accession no. JF313902), is highly reactive even at very low concentrations and is able to distinguish *Brucella* from other bacteria including *Yersinia* (Abbady et al., unpublished data). Thus, the objective of the current work was to characterize this Nb and to identify its antigen using reverse proteomic approaches and MALDI-TOF-MS. The NbBruc02 antigen is presumed to be immunodominant, not only because its phages carrying this Nb were rapidly enriched during panning but also since small quantities of *Brucella* total lysate is sufficient to be captured by this Nb. From our experiments, it appears that the Nanobody technology is a promising strategy to identify new immunogenic antigens of infectious bacteria. Such antigens, and of course their specific Nbs, could be used potentially to diagnose, to prevent and to treat pathogens (such as *Brucella*).

2. Materials and methods

2.1. Antigens and antibodies

Brucella and *Yersinia* reference strains were provided by Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium.

Prior to inactivation, cells were grown in *Brucella* broth medium (HIMEDIA Laboratories) supplemented with 5% heat inactivated horse serum (PAN Biotech GmbH) for at least 48 h at 37 °C. Different types of locally isolated and serotyped bacteria were cultured and maintained according to their preferable conditions and were used mainly to test NbBruc02 cross-reactivity. Stress conditions were applied as described elsewhere (Teixeira-Gomes et al., 2000), briefly, in the experiments with acidic pH, 1.0 N HCl was added to lower the pH from 7.6 to 5.5 and to induce a heat shock response, the culture temperature was shifted from 37 to 42 °C, reincubation was pursued with shaking for an additional 3 h. The inactivation step was performed by washing the bacteria twice with cold phosphate buffer saline (PBS) followed by heat incubation at 60 °C for 30 min. Inactivation was confirmed by inoculating 1 ml of a bacterial suspension onto the solid media.

To prepare soluble lysate from different types of bacteria, 1 g of wet weight bacteria was resuspended in 5 ml ice cold PBS and protease inhibitor solution (1 mM PMSF, 1× complete-protease inhibitor from Roche supplemented with 1 mg/ml of lysosyme), and incubated for 30 min on ice followed by 5 times sonication (15 s pulses) on ice. Bacterial debris was eliminated by centrifugation (15,000 × *g*, 15 min, 4 °C) and the supernatant was saved. To prepare the bacterial subcellular fractions, methods described previously were followed: pure LPS (Yi and Hackett, 2000), outer membrane proteins (OMP) (Chin and Turner, 1990) and periplasmic extract (Rathore et al., 2003). The fractions were dialyzed in PBS and their protein concentration was estimated by Bradford colorimetric assay following the manufacturer's recommendations.

Rabbit anti-camel (Bethyl Laboratories Inc.), Goat anti-rabbit-HRP (BioRad) and Mouse anti-His-HRP (Roche) antibodies were all used according to the manufacturer's instructions.

2.2. Nb-immunoprecipitation (Nb-IP)

500 μl of the bacterial supernatant (1000 μg) from soluble extracts was transferred to a 1.5 ml tube and mixed with 25 μl of pure Nb (1 μg/μl). Mixtures were rotated at 4 °C for 2 h before 50 μl of 50% slurry of nickel-charged nitrilo triacetic acid beads (Ni-NTA; Qiagen, Valencia, CA) was added. Further incubation at 4 °C for 2 h was performed with gentle rotation. Beads with attached protein complexes were recovered by centrifugation at 1000 × *g* for 2 min, then washed four times each with 1 ml wash buffer (50 mM NaH_2PO_4, 150 mM NaCl, 20 mM imidazole, 0.05% Tween-20, pH 8.0) by centrifugation at 1000 × *g* for 2 min. Bound protein complexes were eluted out by boiling washed beads in 50 μl of elution buffer (2× SDS-PAGE sample buffer containing 500 mM imidazole). After brief centrifugation at 12,000 × *g* for 2 min, protein complexes in the supernatant were separated on 12% SDS-PAGE gels and coomassie brilliant blue stained.

2.3. Western blot

Western blot (WB) of SDS-PAGE gels was carried out using a Bio-Rad mini-Protean II system following the manufacturer's instructions. Proteins with known concentrations determined by Bradford assay were blotted onto 0.45 μm nitro-cellulose membranes (BDH, Electran®) using 1× blotting buffer (800 ml SDW containing 14.4 g glycine and 3 g Tris, pH 8.3 and 200 ml methanol). The efficiency of protein transfer was monitored by immersing the blots into Ponceau-S stain (Sigma). For WB, blots were incubated with NbBruc02 purified Nb (1:500 dilution) and mouse anti-His peroxidase-conjugated secondary antibody and detected by AEC (3-amino-9-ethylcarbazole) chromogen substrate in acetate buffer in presence of hydrogen peroxide.

2.4. In-gel digestion and MALDI-TOF mass spectrometry

Target protein bands were excised manually from destained SDS-PAGE gels, digested and analyzed by MALDI-TOF-MS as described (Person et al., 2006). Briefly, each excised gel containing the protein of interest was placed in a protein low binding tube (Eppendorf) containing 500 μl of MilliQ-ddH_2O at 4 °C for 24 h. Water was discarded and 300 μl of 50 mM triethylammonium bicarbonate buffer (TEAB, Sigma) was added. The tubes were gently shaken at room temperature (RT) for 15 min before supernatant was replaced with 50 mM TEAB/50% CH_3CN (acetonitrile) solution twice each for 15 min at RT with gentle agitation. Supernatant was removed, 100 μl of CH_3CN was added to rehydrate the protein band for 5 min at RT. Gel pieces were dried in a speed vacuum before they were reduced with 10 mM DTT, 50 mM TEAB for 1 h at 56 °C and alkynated with 55 mM iodoacetamide, 50 mM TEAB for 45 min at RT in the dark. After this treatment, each gel piece was minced and lyophilized, then swollen at 37 °C overnight in 50 mM TEAB containing 50 ng of modified trypsin (Promega, Madison, USA). Protein peptides were collected, and gels were washed with 0.1% TFA in 50% CH_3CN three times to collect the remaining peptides. Peptides were cleaned using C-18 resin ready packed tips and diluted into freshly prepared saturated sinapinic acid dissolved in 50% acetonitrile, 0.3% trifluoroacetic acid (TFA). 2 μl samples were spotted onto a stainless steel plate and spectra were collected by averaging three shots each for 200–300 laser shots. Samples were irradiated using Bruker Microflex MALDI/TOF mass spectrometer (Bruker Daltonics, Germany) with a 377-nm nitrogen laser, attenuated and focused on the sample target using the built-in software (Microflex package). Ions were accelerated with a deflection voltage of 30 kV and differentiated according to their m/z using a time-of-flight mass analyzer.

2.5. Database searches

Peptide masses (mass list) generated from the peptide mass fingerprint (PMF) were used to search the NCBI database with the MASCOT search engine (Matrix Science, UK) for protein identification. The search parameters were set according to (Kerim et al., 2003). MASCOT uses a probability-based molecular weight search (Mowse) score to evaluate data obtained from tandem mass spectra. The Mowse score was reported as $-10 \times \log(p)$ where p is the probability that the observed match between

experimental data and the database sequence was a random event (Perkins et al., 1999). Mowse scores greater than 80 were considered statistically significant ($p < 0.05$).

2.6. Determination of antigen fixation by Nb using biosensor

The capacity of Nb to capture the specific antigen from *Brucella* lysate was evaluated at 25 °C by Surface Plasmon Resonance (SPR) using a SR7000DC instrument (Xantec Inc. Düsseldorf, Germany). The assay was implemented by immobilizing purified Nbs (0.1 μg/μl) dissolved in 10 mM of acetate buffer (pH 5.0) at a flow rate of 50 μl/min, onto a research grade CMD200M sensor chip according to the manufacturer's instructions. All binding experiments were performed at 25 °C in HBS-EP buffer (Xantec) at a flow rate of 50 μl/min. A blank channel was used as an on-line reference during all injections. For binding determination via the direct format, 0.5 mg/ml of *Brucella* extract was injected over the immobilized Nb and control channel. The maximum capacity of binding was determined following three consecutive injections (0.5 ml) of *Brucella* lysate at progressively increasing concentrations of 0.5, 1 and 2 mg/ml, over the chip with immobilized Nb. Pulses of 6 M guanidinium-HCl were used to regenerate the Nb-immobilized chip.

2.7. Solid-phase ELISA

ELISA 96-well plates (MaxiSorp, Nunc) were coated overnight at 4 °C with different antigen preparations at 25 μg/well in PBS. Residual protein binding sites in the wells were blocked for 2 h at RT with 3% milk in PBS. The NbBruc01 or 02 (stock of 1 mg/ml) were diluted 1/500, and camel IgG (1 mg/ml) previously purified from serum of *Brucella* immunized and control camels (Abbady et al., 2011) was diluted 1/1000, and added to separate wells. After 1 h incubation and several washings, the antigen-bound antibodies were targeted with a mouse anti-His-HRP conjugate or with rabbit anti-camel-IgG antiserum, as appropriate. Subsequent detection of the rabbit antiserum was done with a goat anti-rabbit-HRP conjugate. The absorption at 450 nm was measured 15 min after adding the enzyme's substrate TMB (3,3,5,5-tetramethylbenzidine) (Sigma) and stopping solution (1 M H_2SO_4).

3. Results

3.1. Specificity of NbBruc02 toward Brucella

The isolation of NbBruc02 from an "immune" Nb library and its production from bacteria was described previously (Abbady et al., 2011). The NbBruc02 reactivity against antigens from lysates from different *Brucella* species and from different bacteria types causing regularly serum cross-reactivity, e.g. *Yersinia enterocolitica* serotype O9, was tested (Fig. 1A). Reactivity of serum antibodies from the *Brucella*-immunized camel as well as from the control animal was also assessed against the same bacterial lysates using an indirect solid phase ELISA (Fig. 1A). The data clearly show the ability of NbBruc02, but not NbBruc01 (used as negative control), to interact specifically with antigens from the different *Brucella* strains belonging to *B. abortus* (reference strain Nalr, S99, S19 and local strain G30), *B. melitensis* (reference strain Nalr, Riv 1 and local strain S1), and *B. ovis* (local strain S15). The identity of all these Brucella strains was assured by *Brucella* multiplex PCR (data not shown). As expected, and in contrast to NbBruc02, the camel *Brucella* positive serum was clearly able to detect antigens from bacterial lysates as compared to the non-immune serum, but failed in distinguishing *Brucella* from *Yersinia* antigens (Fig. 1A). NbBruc02 strict specificity to *Brucella* was confirmed by ELISA tests against lysates from different types of Gram negative (*Erwinia*, *Pseudomonas*, *Proteus*, *Enterobacter* and *Salmonella*) and Gram positive (*Bacillus*, *Streptococcus* and *Staphylococcus*) bacteria (Fig. 1B).

To identify the NbBruc02–antigen, intact *Brucella* cells as well as various cellular subfractions, e.g. cytoplasmic extract, OMPs, LPS and a periplasmic extract, were tested in ELISA for reactivity with the NbBruc02 (Fig. 1C). According to this experiment, NbBruc02 is able to detect its cognate antigen exclusively in the cytoplasmic fraction of the cell indicating that the antigen is most-likely a protein.

3.2. SPR analysis of NbBruc02/antigen interaction

The capacity of NbBruc02 to interact specifically and strongly with its antigen within a *Brucella* lysate was confirmed using a double channel SPR biosensor (Xantec, Germany) (Fig. 2). The SPR enables the measurement of the direct biomolecular interaction between an antibody fragment such as NbBruc02 and its antigen with several advantages (fast-reaction time, no-labeling, real-time measurement with minimal sample volume) over traditional methods, e.g. ELISA (Chuang et al., 2009). Nbs were covalently immobilized on activated matrix of one sensor channel by a method of amine coupling and the second channel was left empty to obtain a constant reference value. SPR signals from both channels were recorded in a sensorgram of refractive index units (RIU) as a function of time (Fig. 2). In contrast to the results obtained with NbBruc01 (Fig. 2A, left panel), the immobilized NbBruc02 was able to bind and capture its target antigen from an injected flow of *Brucella* total lysate (Fig. 2A, middle panel). The interaction between NbBruc02 and its specific antigen seems to be very stable since the elevated SPR signal is maintained even after flowing the washing buffer over the chip (Fig. 2A, right panel). Moreover, since the SPR signals are affected by the amount and type of molecules attached to the sensor chip, the sensorgram reveals that immobilized NbBruc02 is able to capture increasing amounts (corresponding to ~2200, ~4700 and ~7000 μRIU) of its antigen from a sample containing increasing concentrations of *Brucella* lysate (Fig. 2B).

3.3. Isolation of NbBruc02 specific antigen by Nb-IP method

Using a new approach of immunoprecipitation with Ni-NTA agarose beads (Thys et al., 2011), the capacity of the 6×His-tagged NbBruc02 to pull down its antigen from a *Brucella* total lysate was tested (Fig. 3A and

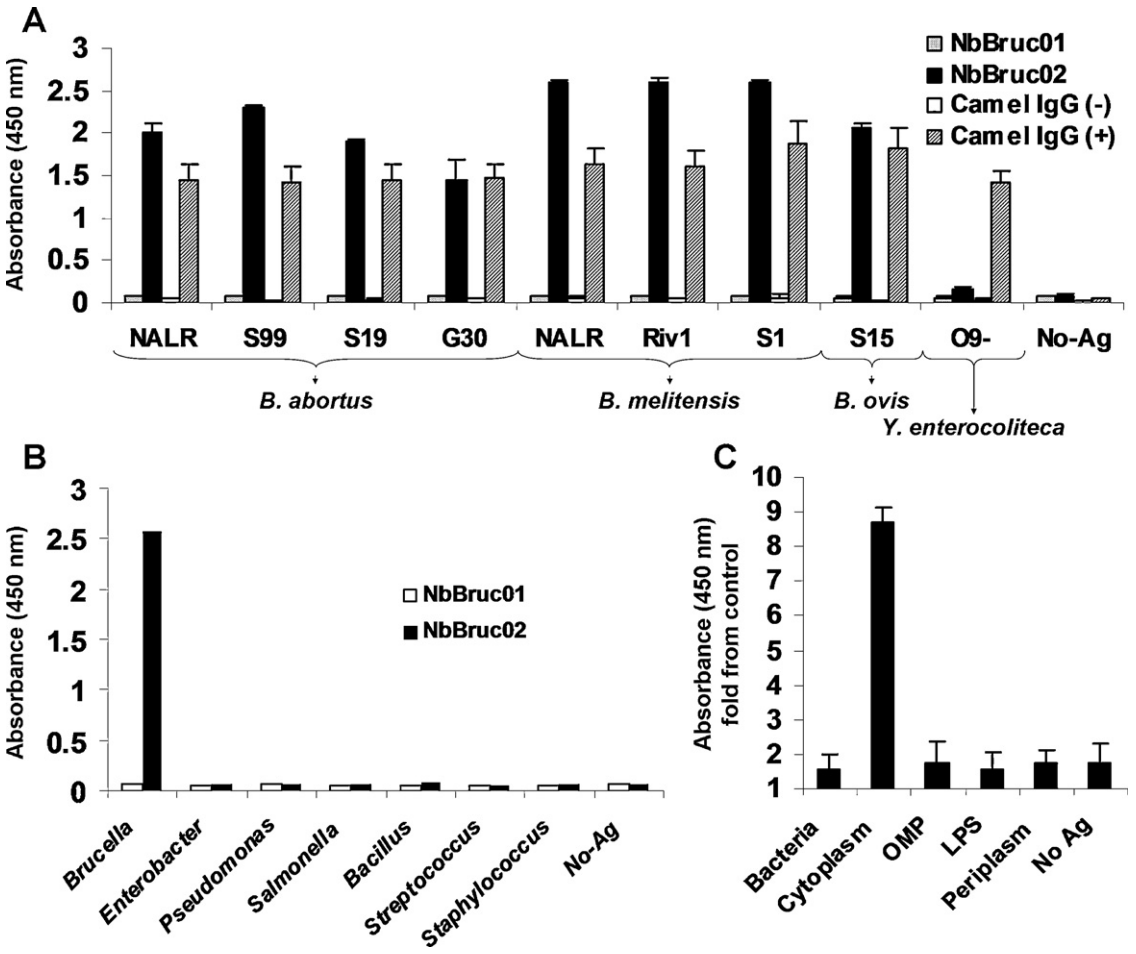

Fig. 1. Specificity of NbBruc02 toward antigens prepared from several bacterial types, as analyzed by ELISA. (A) Reactivities of NbBruc01 or NbBruc02 (2 μg/ml) or purified IgGs (1 μg/ml) from *Brucella* immunized (+) and control non-immunized (−) camels, were tested against total lysates (25 μg/well) prepared from several strains of bacteria: *B. abortus* (NALR, S99, S19 and G30), *B. melitensis* (NALR, RivI and S1), *B. ovis* (S15), *Y. enterocolitica* (O9-) and in the absence of antigens (No Ag). (B) Specificity of the same Nbs was tested against total lysate (25 μg/well) prepared from different types of bacteria belonging to the indicated bacterial genera. (C) Reactivity of NbBruc02 was tested against *B. melitensis* in the form of intact cells (bacteria, 10^7 cells/well) as well as 25 μg/well of cytoplasmic, OMP, pure LPS or periplasmic extracts or without antigen (No Ag). The data are expressed as the *x*-fold higher signal over a well without added NbBruc02.

B). The NbBruc02 was incubated with bacteria lysates, and Nb/antigen protein complexes were captured with Ni-NTA beads, eluted and then separated by SDS-PAGE (Fig. 3A). Interestingly, the NbBruc02 specifically pulls down a protein of ~60 kDa. Incubation of a fixed amount of this Nb (25 μg) with increasing amounts of *Brucella* lysates (from 50 to 5000 μg) results in the immunoprecipitation of increasing quantities of this target protein (Fig. 3B, lanes 2–7). In absence of NbBruc02, this 60 kDa protein apparently fails to bind to the beads as it is absent from the gel (Fig. 3B, lanes 8 and 9). Applying the Nb-IP method using NbBruc01, failed as well in capturing the NbBruc02–antigen or any other protein (data not shown).

3.4. Sensitivity of NbBruc02 to the folding state of its cognate antigen

To confirm the specific interaction between NbBruc02 and its antigen within the *Brucella* lysate, we performed a WB assay. To this end, proteins from cell lysates from three types of bacteria, *B. melitensis*, *B. abortus* and *Y. enterocolitica*, were separated onto SDS-PAGE and then either stained with coomassie brilliant blue (Fig. 4A, left) or blotted and used in WB with NbBruc02 as a probe detected with an anti-His-HRP conjugated antibody (Fig. 4A, right). This experiment reveals that, using similar amounts of bacterial lysates, the NbBruc02 is able to detect the same ~60 kDa protein from both *Brucella* species lysates (Fig. 4A, lanes 1 and 2). Unexpectedly, a protein around ~60 kDa is also detected in the *Yersinia* extract which was supposed to be a negative control in the experiment (Fig. 4A, lane 3) (since this NbBruc02 does not recognize a *Yersinia* compound in an ELSIA).

This seemingly contradictory behavior of antigen recognition by NbBruc02 in ELISA and WB experiments might be explained by a deviating sensitivity of the NbBruc02 to the folding state of a conserved target antigen in *Brucella* and *Yersinia*, and perhaps other types of bacteria.

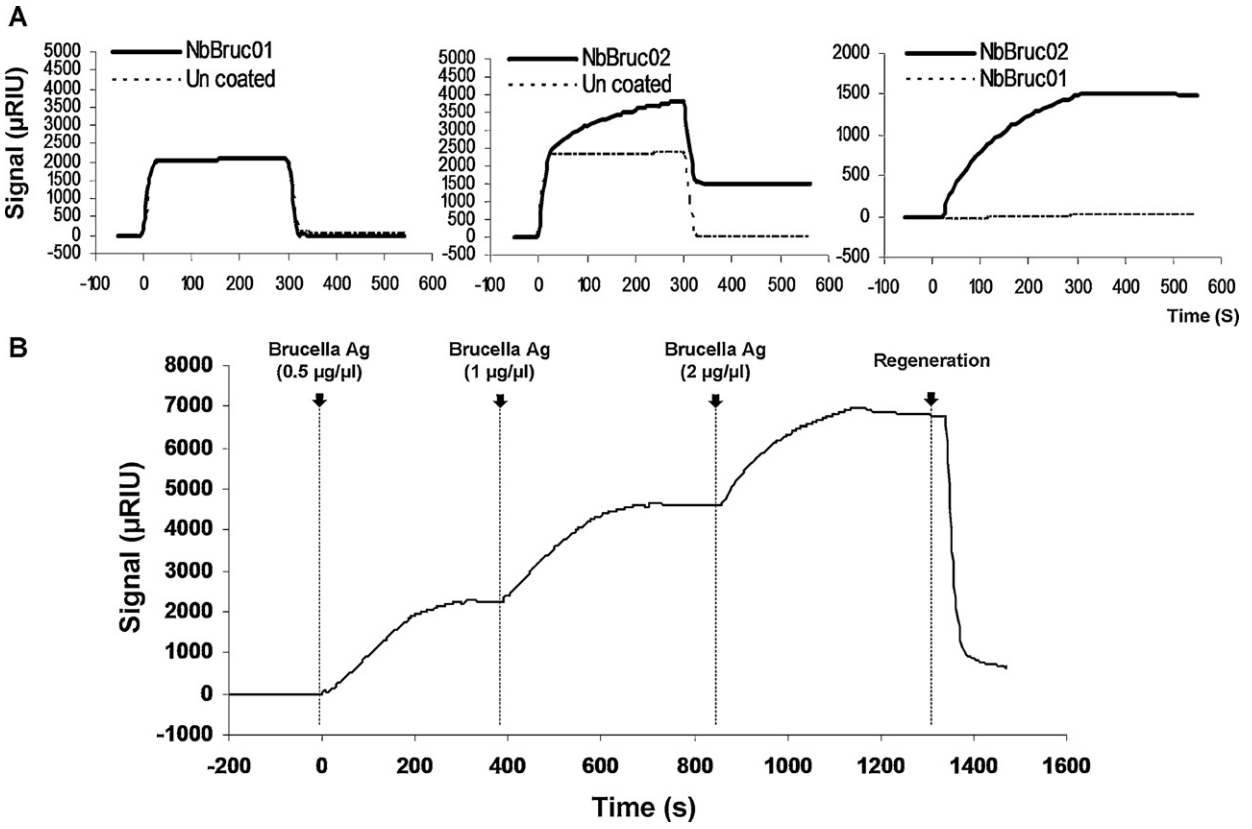

Fig. 2. SPR assay of the interaction between Nb and *Brucella* total extract. (A) Double channels SPR were used to detect the interaction of antigen in *Brucella* total lysate with immobilized Nbs (either NbBruc01 (left panel) or NbBruc02 (middle panel)) (continuous line) compared to empty channel (dashed line). Subtractive signals (i.e. the signal from channel with Nb–signal from channel without Nb) were calculated for both Nbs (NbBruc01 and 02) and are shown together (right panel). (B) Subtractive signal of the interaction of the immobilized NbBruc02 with serially increasing concentrations (as indicated) of *Brucella* lysate flowed over the channels. Surface regeneration was performed by flowing 6 M guanidin-HCl solution over the sensor chips.

To investigate this possibility, the reactivity of NbBruc02 was tested in an ELISA against *Brucella* and *Yersinia* lysates prepared in PBS in absence or presence of 6 M guanidinium hydrochloride (GuHCl) as protein solubilizing and unfolding agent. Remarkably, NbBruc02 was only reactive with the 'native' *Brucella* lysate and conversely, it recognized only the GuHCl-unfolded lysates of *Yersinia* in ELISA (Fig. 4B).

3.5. Identification of NbBruc02 specific target

The protein, putatively interacting with NbBruc02, was identified by MALDI-TOF mass spectrometry analysis and database search. Peptide mass values (mass list, Fig. 5A) of the antigen were used for its identification using MASCOT peptide mass fingerprint (PMF) search that identified the antigen as chaperonin GroEL with a Mowse score of 92 (Fig. 5B) considering that Mowse scores greater than 80 were statistically significant ($p < 0.05$).

GroEL (or HSP-60) is one of *Brucella* proteins whose level of synthesis is up-regulated under stress conditions (Teixeira-Gomes et al., 2000). To confirm the ability of NbBruc02 to monitor this expression upregulation of its target antigen, equal amounts of protein extracts from *Brucella* cultured in different stress conditions (normal, acid and heat) were separated onto SDS-PAGE and then either stained with coomassie brilliant blue or immuno blotted with NbBruc02 as a probe and then detected using anti-His-HRP conjugated antibody (Fig. 5C). Interestingly, NbBruc02 was able to detect the increase in GroEL expression in the extracts of stressed *Brucella* comparing to the untreated control, which showed a very low level of GroEl expression similar to our previous results (Fig. 4A).

4. Discussion

The current data represent a characterization of a new *Brucella*-specific Nb, referred to as NbBruc02, which was isolated previously from a large 'immune' Nb library prepared from a dromedary immunized with *Brucella*. In fact, phages carrying this Nb were rapidly enriched during phage display pannings on *Brucella* total antigens (Abbady et al., 2011). Possibly, this NbBruc02 reacts with a very immunogenic *Brucella* antigen so that B cells expressing heavy-chain antibodies reacting with this antigen were highly represented in the blood sample used to make the Nb library, which facilitated its retrieval during pannings. Moreover, the camel immunization step provides access to in vivo affinity-matured and intact antigen-binding units (Muyldermans et al., 2009). Our findings here confirm also

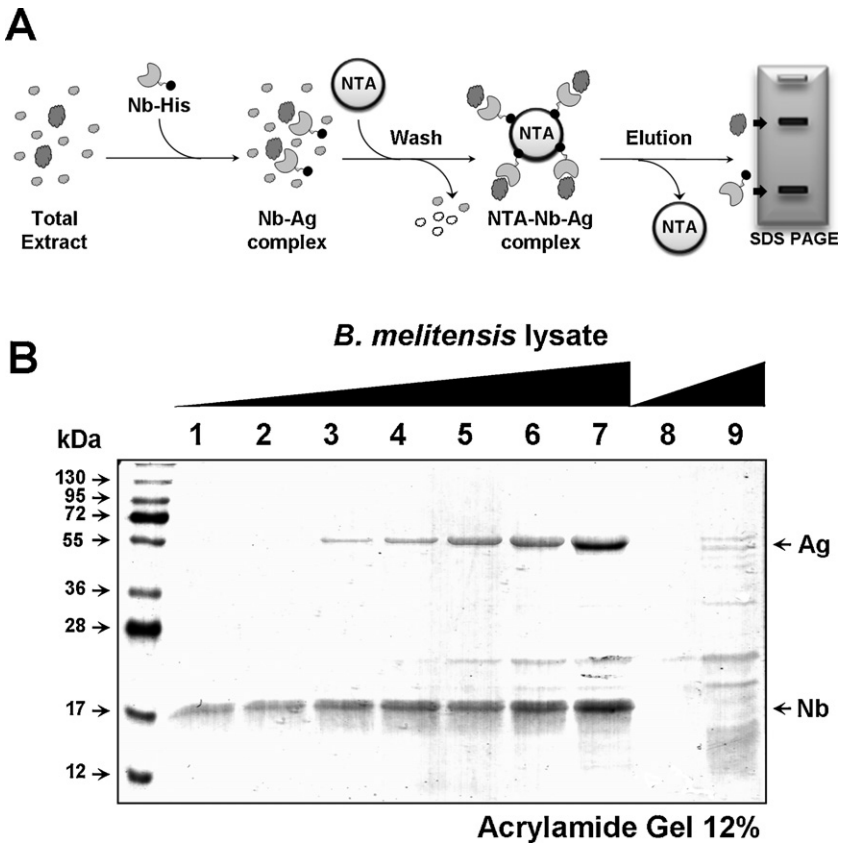

Fig. 3. NbBruc02 immunoprecipitation (IP) of antigen from a *Brucella* total lysate. (A) Schematic representation of the different steps of the IP experiment. The NbBruc02 is incubated first with the total bacterial lysate containing its cognate antigen. The Nb-antigen complexes are pulled down from the solution with Ni-NTA beads, interacting with the oligo His-tag of the Nb. After several wash steps, the Nb/antigen complexes are liberated from beads and separated by an SDS-PAGE (12%) and then revealed with coomassie blue staining. (B) Nb-IP was performed by incubating NbBruc02 (25 μg) with increasing amounts of *Brucella* lysate (0, 50, 250, 500, 1000, 2500 and 5000 μg; lanes 1–7, respectively). Two amounts of *Brucella* lysate (1000 and 5000 μg; lanes 8 and 9, respectively) were incubated in the absence of Nb. The migration of captured (precipitated) antigen (Ag) as well as the NbBruc02 (Nb) is indicated by arrows along the gel.

the conclusion from a previous report describing the feasibility to isolate interesting infectome-specific binders from an immunized camel without having a priori knowledge of the antigens involved (Saerens et al., 2008b). The gene of NbBruc02 was successfully re-cloned into expression vector (pHEN6) and its encoded protein was expressed and purified in large amounts from *E. coli* culture.

In agreement with our previous findings, the ELISA tests shows that NbBruc02 is specific toward *Brucella* that belongs to a mono-specific genus sharing an inter-species homology above 87% (Pappas et al., 2005). It is able to recognize several strains, both reference and local strains, belonging to the most important species of *Brucella* in Syria; *B. abortus* and *B. melitensis*. In addition, this NbBruc02 discriminates *Brucella* from many other bacterial strains tested, including *Y. enterocolitica O9* which shares many antigens with *Brucella*, mainly the LPS O-ring epitope (Pappas et al., 2005) that leads often to cross-reactivity in serological tests. A proteinaceous nature of the cognate antigen for NbBruc02 was assumed since NbBruc02 shows good reactivity to a cytosolic extract and no reactivity to LPS, OMP or even to intact *Brucella* cells, despite being the source of the most immunogenic parts of the bacteria (Tumurkhuu et al., 2006). This strategy of avoiding LPS-specific antibodies by using monoclonal Nbs to detect relatively rare but unique and specific epitopes should allow a rapid identification of bacteria within mixed populations. Such Nbs might also proof adequate in applications of environmental sensing.

Apart from the employment of Nbs for diagnostic purposes, a therapeutic utilization might be envisaged as well. In fact, the identification of new specific *Brucella* immunodominant antigens is of great importance to develop serological tests as well as specific and efficient vaccines. In this objective, the identification of this interesting antigen targeted by NbBruc02 was carried out using proteomic approaches. Firstly, we tested the capacity of NbBruc02 to recognize its antigen target via a biosensor instrument which measures the direct interaction between Nb/antigen without the interference of secondary detecting systems, as it is the case in ELISA and WB. Moreover, Nbs have been proposed previously as a novel class of biosensor probes rivaling conventional antibodies and their derived antibody fragments (Saerens et al., 2005). Covalently immobilized NbBruc02 is clearly able to bind its antigen from a total *Brucella* extract flowed over the functionalized biosensor chip, and this interaction increased in a dose dependant manner. The SPR results encouraged us to move to a modified IP

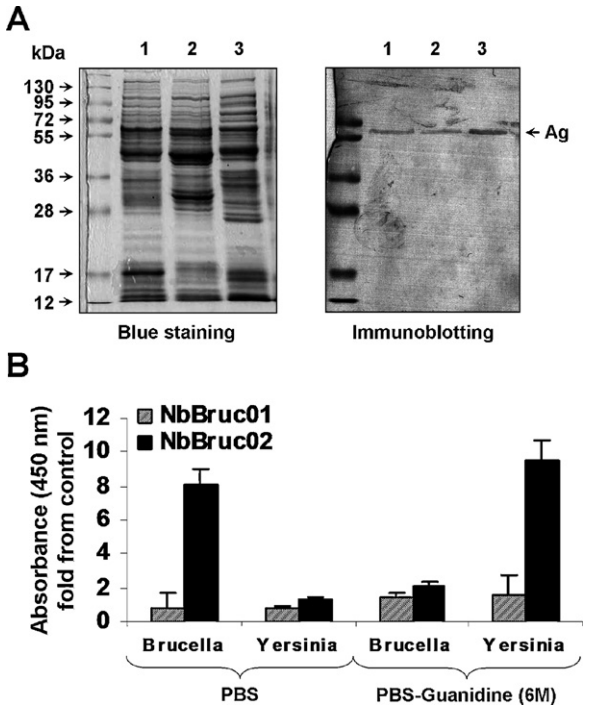

Fig. 4. Susceptibility of NbBruc02 toward the folded state of its antigen. (A) Bacteria total lysate (25 μg/well) from *B. abortus* (lane 1), *B. melitensis* (lane 2) and *Y. enterocolitica* (lane 3) were separated by SDS-PAGE and then stained with coomassie blue (left) or immuno blotted with NbBruc02 (1/500) a probe and detected by anti-His-HRP conjugated antibody (1/500). (B) The ELISA test demonstrating the reactivity of NbBruc02 (1/500) against protein lysates (25 μg/well), prepared from *Brucella* and *Yersinia*, extracted by sonication in PBS, in presence (+) or in absence (−) of 6 M guanidine-HCl as unfolding reagent.

technique to isolate the antigen from *Brucella* crude extract. This Nb-IP experiment, based on the ability of NbBruc02 to bind to NTA beads charged with nickel ions via its recombinantly added oligo-His tail, resulted in the purification of a single protein with a molecular mass of about 60 kDa. Obviously, the extra disulfide bond in the NbBruc02 structure, occurring between the first and the third antigen-binding loop, is expected to confer an increased stability to the Nb structure and thus allowing its functional immobilization and assisting in its high reactivity toward its antigen during antigen binding and bead precipitation. Further investigation is required to determine the exact kinetic binding rate constants, k_{on} and k_{off}, and to calculate the equilibrium binding constant of NbBruc02 with its specific antigen. These parameters are essential to predict the efficacy of the NbBruc02 in future diagnostic or therapeutic applications. These data can be obtained most easily using the SPR biosensor as it avoids artifacts from (antibody or antigen) labeling and/or from the use of secondary antibodies. Moreover, the purified antigen, which is indispensable for such SPR measurements, could be obtained using an immuno-chromatography with the NbBruc02 as affinity adsorbent to isolate the GroEL from a *Brucella* extract and thus avoiding the need for a recombinantly produced GroEL that might be problematic to obtain in a properly folded state. Previous work of Rothbauer et al. (2008) proved indeed that Nbs directly coupled to Sepharose to generate their Nanobody-trap column-based purification system is an extremely powerful approach to purify the green fluorescent proteins (GFPs) and its variants of fusion proteins from total protein extracts.

Using the MALDI-TOF technique, applied directly on antigen extracted from coomassie blue stained SDS-PAGE after Nb-IP experiment, the NbBruc02 target protein was identified as chaperonin GroEL, also known as the heat shock protein (HSP-60). The heat shock response is highly conserved and presumably allows the organisms to adapt to-, and cope with stress (Teixeira-Gomes et al., 2000). GroEL proteins have been found to be immunodominant targets of both humoral and cellular immune responses. GroEL was found as an immune dominant antigen among many other proteins from the outer membrane of *B. abortus* and may play a critical role in virulence to both human and bovines (Al Dahouk et al., 2006; Connolly et al., 2006). Furthermore, GroEL reacts with serum from humans, cattle and sheep that naturally acquired brucellosis (Connolly et al., 2006; Amirmozafari et al., 2008). Previous studies already investigated the role of GroEL as a potential vaccine in mice (Bae et al., 2002).

Molecular chaperones play a major role in protein folding and degradation by means of mechanisms involving a reversible conformational change in their structure (Lund, 2009). Interestingly, our results show that NbBruc02 is able to sense the GroEL folded state in *Brucella* extracts, and in addition to recognize the unfolded *Yersinia* GroEL after being treated with guanidinium hydrochloride or SDS. The susceptibility of Nbs for properly folded antigens is not surprising since Nbs distinguishing subtle changes in the structure or conformation of their target antigens have been reported already (Saerens et al., 2004; Habicht et al., 2007; Hendrickx et al., 2011). However, the opposite dependence of the NbBruc02 recognition of the folded or unfolded GroEL from *Brucella* or *Yersinia*, respectively was totally unexpected. Only after treating the antigen under the harsh conditions of denaturation, e.g. during WB sample preparation, the NbBruc02 is able to recognize its GroEL target from *Yersinia* lysates. Although it remains puzzling, it could be explained since chaperones are highly conserved throughout evolution and if the epitope recognized on the *Brucella* GroEL native structure is possibly hidden in the GroEL of other bacteria types, e.g. *Yersinia*. This leads to the hypothesis that NbBruc02 binds an epitope that adopts easily an alternative conformation upon different experimental conditions. More structural insight is required to fully conceive the picture of this surprising interaction between NbBruc02 and the different folding states of GroEL.

Owing to their high stability and excellent folding capacity under different environmental conditions, Nbs have also been targeted successfully to the cytosol by removing the leader peptide and to the intracellular vesicles, such as the endoplasmic reticulum, by genetic fusion to suitable targeting sequences (Rothbauer et al., 2008; Serruys et al., 2009; Blanco-Toribio et al., 2010). Inside the living cell environment, Nbs have been found to be able to inhibit the physiological function of their target antigens (Kirchhofer et al., 2010). Since *Brucella* establishes a long-term residence within the host macrophages, it is

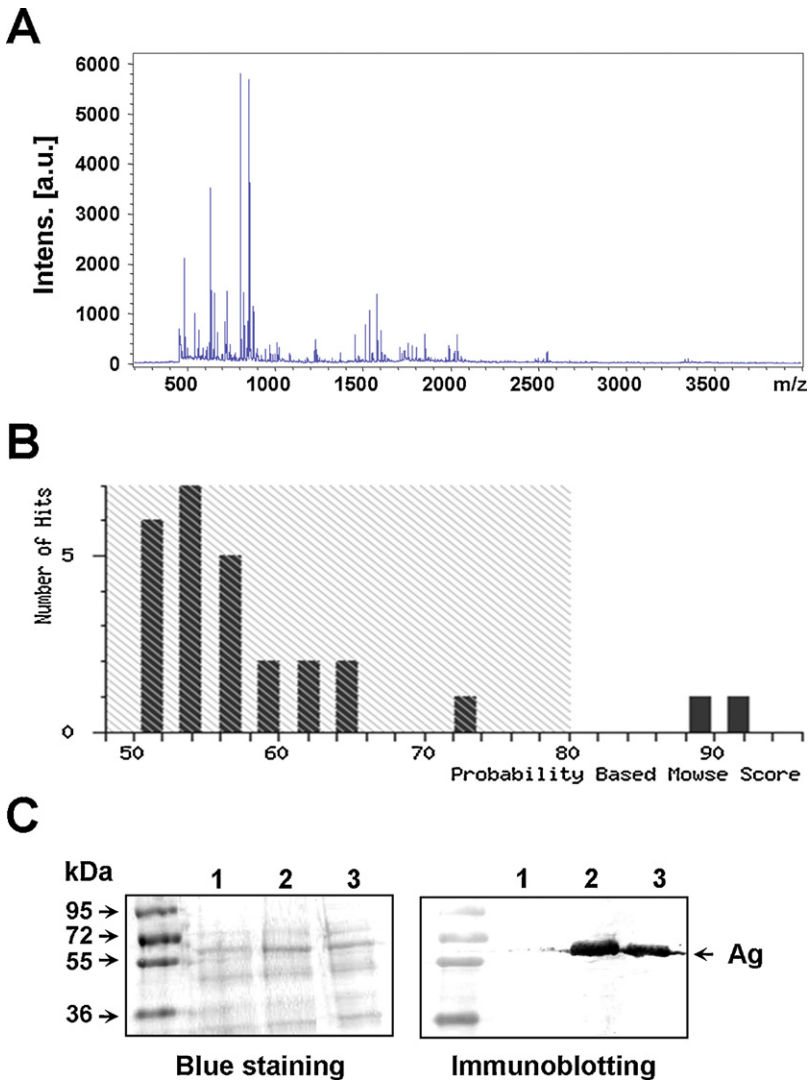

Fig. 5. Identification of NbBruc02–antigen by MALDI-TOF-MS. (A) Spectra of GroEL generated by MALDI-TOS-MS. (B) MASCOT results showing the significance of GroEL identification. (C) SDS-PAGE of different extracts (25 μg) of *Brucella* cultured under normal (lane 1) and stress conditions such as elevated growth temperature at 42 °C (lane 2) and low pH (lane 3). Detection was carried out by blue staining (left) or by WB analysis using NbBruc02 (right). The arrow at the right of the blot indicates the GroEL antigen. (For interpretation of the references to color in text and figure legend, the reader is referred to the web version of the article.)

subjected to harsh conditions including extremes of pH and oxidative and nutritional stress. Under such conditions, the expression of heat shock proteins is vital for the survival of this pathogen in this hostile environment (Teixeira-Gomes et al., 1997). Furthermore, *B. abortus* GroEL was reported to be involved in bacterial internalization and intracellular signal transduction (Watarai et al., 2003). According to this scheme, the *Brucella* entry through lipid rafts is specifically mediated by the binding of surface-exposed Hsp60 to the cellular prion protein PrP of macrophages (Watarai et al., 2003). The possibility to employ NbBruc02 in vivo to target GroEL to interfere with *Brucella* adhesion and invasion, and to disrupt the cellular entry system of *Brucella* inspires an interesting perspective to explore a new treatment approach against brucellosis.

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