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A rapid and simple pipeline for synthesis of mRNA–ribosome–V_HH complexes used in single-domain antibody ribosome display†

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The single-domain antibody (V_HH) is a promising building block for a number of antibody-based applications. Ribosome display can successfully be used in the production of V_HH. However, the construction of the expression cassette, confirmation of the translation and proper folding of the nascent chain, and the purification of the ribosome complexes, remain cumbersome tasks. Additionally, selection of the most suitable expression system can be challenging. We have designed primers that will amplify virtually all *Camelidae* V_HH. With the help of a double-overlap extension (OE) polymerase chain reaction (PCR) we have fused V_HH with the F1 fragment (T7 promoter and species-independent translation sequence) and the F2 fragment (mCherry, Myc-tag, tether, SecM arrest sequence and 3′ stem loop) to generate a full-length DNA cassette. OE-PCR generated fragments were incubated directly with cell-free lysates (*Leishmania torentolae*, rabbit reticulocyte or *E. coli*) for the synthesis of mRNA–V_HH–mCherry–ribosome complexes *in vitro*. Alternatively, the cassette was ligated in pQE-30 vector and transformed into *E. coli* to produce ribosome complexes *in vivo*. The results showed that the same expression cassette could be used to synthesize ribosome complexes with different expression systems. mCherry reporter served to confirm the synthesis and proper folding of the nascent chain, Myc-tag was useful in the rapid purification of ribosome complexes, and combination of the SecM sequence and 3′ stem loop made the cassette universal, both for cells-free and *E. coli in vivo*. This rapid and universal pipeline can effectively be used in antibody ribosome display and VHH production.

1. Introduction

Single-domain antibodies (V_HH) are derived from naturally occurring heavy-chain antibodies (HCAbs) in *Camelidae* and cartilaginous fish. Currently, they represent an alternative tool for the diagnostics, prophylaxis, and therapy of various diseases. Their small size (~15 kDa) facilitates easy penetration through tissues and natural barriers composed of endothelial and epithelial cells. HCAbs comprise two constant domains and an antigen-binding site composed of a single variable domain (referred to as V_HH in *Camelidae* and V-NAR in cartilaginous fish). V_HH forms three complementary determining regions (CDRs) and four framework regions.[1,2] HCAbs, V_HH, and V-NAR can be produced *in vitro*, and display techniques such as ribosome display,[3] phage display[4] or surface display[5] have been successfully used in their engineering.

The production of biomolecules by cell-free expression systems (CFes) has become popular in recent years since it is more rapid than conventional expression systems. A variety of cell lysates are used for cell-free expression, originating from *E. coli*, wheat germ, rabbit reticulocyte, *Leishmania*, insect cells, *etc.* These lysates contain whole translation machinery that allows the production of proteins in just one or two steps. They are also suitable for the generation of proteins with unnatural and modified amino acids, toxic molecules or insoluble products, which are difficult to produce in conventional expression systems.[6] With its many advantages, CFes can effectively be used in display technologies, such as ribosome display, for the screening of a large repertoire of proteins or the affinity selection of antibodies.

Ribosome display has also in the past been used in the engineering of human antibodies.[7–9] The antibody–ribosome–mRNA complexes are usually synthesized with CFes, and the antibodies are affinity-selected on an immobilized ligand (antigen) at the selection step (biopanning). Unbound complexes are removed by stringent washing and mRNA molecules from the bound complexes are released for reverse transcription into cDNA, followed by amplification with PCR and sequencing.[10] The chance of selection of antibodies with higher affinity increases

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with increasing size of the library (reviewed in ref. 11). In ribosome display, the diversity of libraries can extend to 10^{14} clones.

Despite the numerous advantages of the *in vitro* ribosome display, cellular factors under *in vitro* conditions may vary between CFes. Moreover, the large-scale production of a mRNA–ribosome–antibody complex is extremely expensive. To overcome these problems, Contreras–Martínez and DeLisa developed a technique to produce ribosome complexes *in vivo*. They used a SecM stall sequence to arrest the translation and thus produce protein–ribosome–mRNA complexes in *E. coli*, which were later isolated and used in biopanning.[12] This novel approach may streamline the production of ribosome complexes for the ribosome display, and is overall less costly than *in vitro*-produced ribosome complexes.

Taking into account the advantages and drawbacks of each expression system, one would anticipate the development of a robust expression system with all the benefits of bacterial and eukaryotic expression or construction of the universal cassette, which would allow the production of the ribosome complex in a variety of expression systems without restriction.

The aim of the present study was thus to construct a universal cassette, which could be used in various expression systems and would streamline downstream applications such as rapid confirmation of the synthesized library or easy purification of ribosome complexes. In this paper we describe a simple and efficient pipeline for the synthesis of mRNA–V_HH–ribosome complexes, both *in vitro* and *in vivo*, which includes:

(1) rapid construction of expression cassettes;

(2) *in vitro* translation of the same cassette with various CFes; and

(3) construction of a mRNA–V_HH–ribosome library *in vivo* in *E. coli* using the same cassette.

2. Material and methods

2.1 Primer design

V_HH contains three hypervariable CDRs, whereas framework regions F1 and F4 are relatively homogeneous. We attempted to construct primers that would amplify V_HH from various *Camelidae*, *e.g. Vicugna pacos*/*Lama pacos*, *Lama glama*, *Camelus dromedarius* and *Camelus bactrianus*. Multiple sequences (ESI,† Table S1) were retrieved from GenBank and aligned using Geneious Pro software (www.biomatters.com). Degeneracy was incorporated into the primers to ensure the maximum probability of amplification of the V_HH region from all the above species. In addition, the forward primer contained 5′ overhang complementary to the overlapping region in the F1 fragment, while the reverse primer contained 3′ overhang complementary to the overlapping region in the F2 fragment (ESI,† Table S2). Details of the F1 and F2 fragments are described below.

2.2 Amplification of V_HH

Blood from the *Camelidae* species listed above was collected from healthy individuals raised in the zoological garden or on private farms in Slovakia. Heparinized blood was mixed in a ratio of 1 : 1 with sterile phosphate-buffered saline solution. The suspension was overlaid on lymphocyte separation medium (PAA Laboratories, Germany) and centrifuged at 2060 rpm for 20 min. A buffy coat was carefully transferred and washed with eRDF medium (RPMI-1640 medium and Dulbecco's modified Eagle's medium containing nutrient mixture F-12 Ham, mixed in the ratio 1 : 1; Sigma-Aldrich, Germany).

Total RNA was isolated by PureZol (Bio-Rad, USA) according to the manufacturer's instructions and treated with DNase I (Thermo-Scientific, Slovakia). The first strand of cDNA was synthesized using Maxima H Minus Reverse Transcriptase (Thermo-Scientific) and gene-specific primer (VHHR; ESI,† Table S2) according to the manufacturers' instructions.

Two microliters of cDNA were used for PCR with High-fidelity Phusion Polymerase (Finnzyme, Finland). Cycling conditions were as follows: 2 min at 95 °C, 30 × (20 s at 95 °C, 30 s at 68 °C, and 50 s at 68 °C), and 1 min at 68 °C. Amplicons were separated on 1% agarose gel and purified using a NucleoSpin purification kit (Macherey-Nagel, Germany).

2.3 Construction of F1 fragment

Construction of the F1 fragment was performed as described previously.[13] F1 was amplified from the pLEXSY_*in vitro*_2 vector (Jena Bioscience, Germany), and contained T7 promoter, a species-independent translation sequence (SITS),[14] start codon and overlapping region (complementary to 5′ overhang sequence in VHHF; ESI,† Table S2).

2.4 Amplification of mCherry, tether, SecM arrest and 3′ loop sequences (F2 fragment)

The F2 fragment consisted of several segments, which were amplified from different sources. The sequence of the red fluorescent protein mCherry was amplified from the pLEXSY_I-blecherry3 vector (nucleotides 4033–4735, Jena Bioscience) using mCherryF and mCherryR primers. The mCherryR also contained a sequence encoding Myc-tag. The tether sequence was amplified from pSEX81 plasmid (nucleotides 4130–4337, Progen, Germany) using primers TetF and TetR (ESI,† Table S2). The SecM arrest and 3′ stem loop sequences were relatively small for amplification (75 bp only), and the arrest-loop sequence was therefore designed as an oligonucleotide (ArrestLoop; ESI,† Table S2). The arrest sequence was derived from the bacterial secretion monitor protein SecM,[15] while the loop sequence was acquired from previous work.[16] SecM sequences are necessary to produce V_HH–ribosome–mRNA complex *in vivo* (in *E. coli*).

To construct the F2 fragment, amplicons of mCherry, tether and arrest-loop were fused with double-overlap extension PCR (double OE-PCR), as described previously.[13] Briefly, in the first round of the amplification cycle 5 nM of each segment was mixed with 1 × PCR reaction buffer, 0.2 mM of each dNTP and 20 U of proof-reading Taq polymerase (Jena Bioscience). The cycling conditions were 2 min at 95 °C, followed by 11× (20 s at 95 °C, 30 s at 57 °C, 1 min at 68 °C), and 1 min at 68 °C. The amplified products were column purified (Qiagen) and used as a template for the second round of amplification. The reaction mixture for the second round of double OE-PCR contained

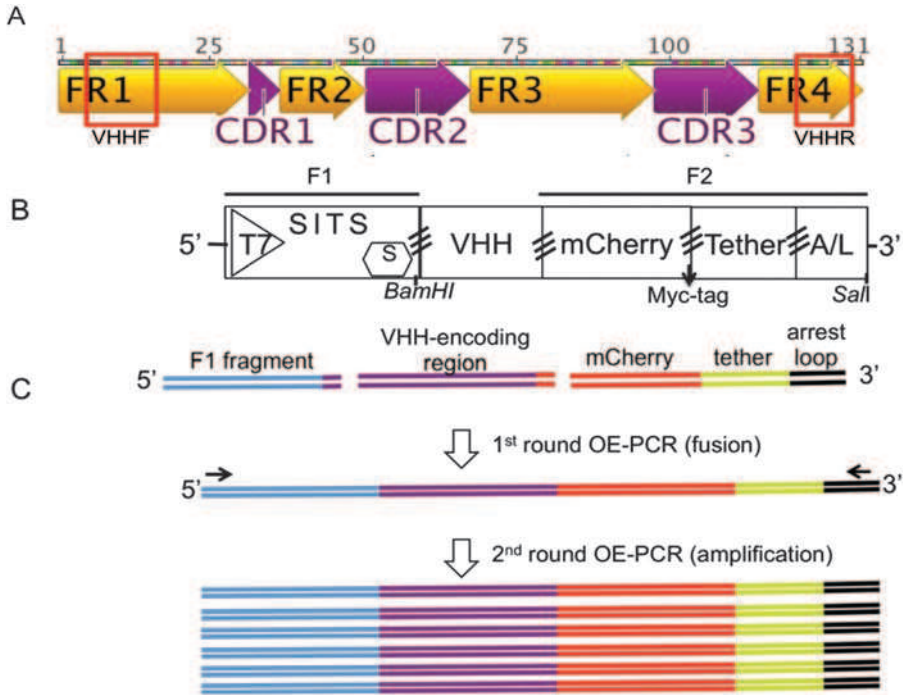

Fig. 1 Schematic overview showing the position of the VHHF and VHHR primers, the expression cassette and OE-PCR. Panel A: position of VHHF and VHHR (rectangles in FR1 and FR4) in the heavy chain; FR: framework; CDR: complementary determining region. Panel B: schematic overview of the expression cassette. F1 contained T7 promoter (T7), species-independent translation sites (SITS) and ATG initiation codon (S). Crosshatching represents the overlap regions necessary for the fusion of each amplified fragment in OE-PCR. F2 was constructed by fusing sequences of mCherry with the Myc-tag, tether and SecM arrest, and 3′ stem loop (A/L). Panel C: principle of the fusion of F1–V_HH–F2 with double OE-PCR. The amplified regions were fused in the first round OE-PCR by overlap regions. The PCR product from the first round was purified and amplified using outer primers in the second round of OE-PCR.

1× reaction buffer, 10 ng of purified product from the first round, 0.2 mM each of dNTPs, 20 U ml^{-1} Taq polymerase (Jena Biosciences, Germany) and 200 nM of each forward primer (mCHF; ESI,† Table S2) and ArrestLoop (in this case serving as a reverse primer). The reaction was performed as follows: 2 min at 94 °C, followed by 30 cycles of amplification (30 s at 94 °C, 30 s at 53 °C and 1.20 min at 72 °C), and final extension for 2 min at 72 °C. The amplified and fused F2 fragment was checked on 0.7% agarose gel and purified.

The yield of translated proteins (nascent chain) attached to a ribosome complex is often small, making the standardization of the overall protocol cumbersome. To avoid this, a stop codon was inserted downstream to Myc-tag (using primer mCherryStop (ESI,† Table S2); the position of Myc-tag is illustrated in Fig. 1). This allowed us to confirm (a) frame fusion of the V_HH and mCherry, and (b) the ability of each expression system to translate the V_HH–mCherry fusion under the SITS.

2.5 Construction of an expression cassette for *in vitro* synthesis of the ribosome complex

The V_HH, F1 and F2 fragments were fused with double OE-PCR as described above. An equimolar concentration of three fragments was used in the first step of OE-PCR. In the second round of amplification, F1F and ArrestLoop (ESI,† Table S2) served as outer primers (Fig. 1). The cycling conditions were as follows: 2 min at 94 °C, 31 × (30 s at 94 °C, 30 s at 60 °C, 120 s at 72 °C) and 3 min at 72 °C. The other conditions were as described above. The amplified cassette was gel purified (Qiagen).

2.6 *In vitro* synthesis of the ribosome complex (mRNA–ribosome–V_HH library)

Three different cell-free translation systems, *E. coli*, rabbit reticulocyte and *Leishmania*, were used to synthesize ribosome complexes using the same cassette. For translation with *E. coli*, CFes, 200 ng of expression cassette, 5 μl of amino acid mixture minus methionine, 20 μl of S30 Premix without amino acids, 1 μl of 1 mM methionine, and 15 μl of S30 linear *E. coli* lysate (Promega, USA) were mixed gently together and incubated 1 h at 37 °C. The translation was terminated by placing the tube on ice. For translation with *Leishmania* CFes (Jena Bioscience, Germany), 1 μg of the cassette was mixed with 40 μl of cell lysate and incubated 2 h at 20 °C. A detailed description of the *in vitro* translation with *Leishmania* CFes is presented elsewhere.[13]

For translation with rabbit reticulocyte CFes, the TNT® T7 Quick for PCR DNA kit was used (Promega, USA). Briefly, the concentration of the expression cassettes was set at 50 ng μl^{-1}, and 5 μl (250 ng) were mixed with 40 μl of TNT T7 Quick master mix, 1 μl of 1 mM methionine, 1 μl of Transcend® Biotin–Lysyl–tRNA (Promega, USA). The translation mix was incubated for 1 h at 30 °C. The reaction was terminated by placing the tube on ice for 5 min.

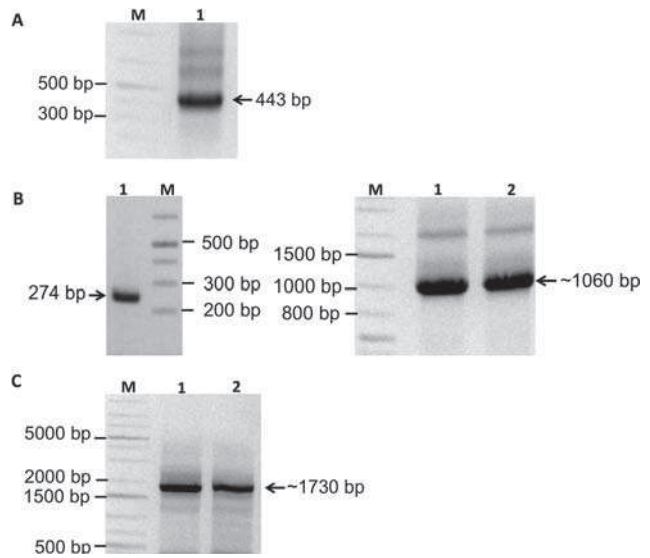

Fig. 2 Amplification of V_HH and construction of the cassette. Panel A: representation of the amplified V_HH region of the *Camelidae* heavy chain. Amplified V_HH (443 bp) of *Vicugna pacos* is shown in this panel. Panel B: lane 1: F1 fragment. Lane 2: OE-PCR- constructed F2 fragment, containing sequences for mCherry, tether, arrest and 3′ stem loop. Lane 3: F2 fragment with stop codon. Panel C: final expression cassettes after second round of OE-PCR. Lane 1: expression cassette F1–V_HH–F2. Lane 2: the same cassette with stop codon. M indicates DNA marker.

2.7 *In vivo* synthesis of the ribosome complex (mRNA–ribosome–V_HH library)

The expression cassette was digested with *BamH*I and *Sal*I enzymes (Thermo Scientific, Slovakia) and ligated into pQE-30 plasmid (Qiagen, USA; ESI,† Fig. S1), using T4 ligase (Jena Bioscience, Germany) according to the manufacturer's instructions. The *BamH*I site is incorporated in VHHF primer and the *Sal*I site is present in the 5′ overhang of ArrestLoop (ESI,† Table S2). The ligation mix was purified by the standard phenol–chloroform method and transformed into electro-competent *E. coli* SG13009 cells (Qiagen). Transformed *E. coli* cells were selected on LB agar containing 100 μg ml^{-1} ampicillin. All transformants were harvested by scraping, and inoculated into 500 ml of Terrific Broth (TB) medium supplemented with 0.1% glucose, 0.1 mg ml^{-1} carbenicillin and 0.1 mg ml^{-1} kanamycin. A culture was grown at 30 °C to obtain OD_{600} 6. The culture was then centrifuged 10 min at 6000 rpm and the pellet resuspended in TB medium, supplemented with 1 mM IPTG, 0.1 mg ml^{-1} carbenicillin and 0.1 mg ml^{-1} kanamycin, for 2 h at 20 °C. The culture was placed on ice for 10 min and centrifuged for 10 min at 6000 rpm at 4 °C. The pellet was resuspended in cold R buffer (50 mM TRIS, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl) and held at −80 °C for 1 h. The suspension was allowed to thaw at room temperature and 1 mg ml^{-1} of lysozyme added. The mixture was incubated 30 min on ice, then 1 h at −80 °C. Finally, the cell suspension was again allowed to thaw at room temperature, 50 mM of $MgSO_4$ and 100 U of DNaseI were added and the mixture incubated 30 min at 4 °C. The lysate was then centrifuged at 14 000 rpm for 50 min at 4 °C and the supernatant retrieved.

2.8 Purification of ribosome complexes and analysis of the nascent chain

The ribosome complexes synthesized *in vivo* and *in vitro* were captured on anti-c-Myc-affinity beads (Sigma, USA) according to the manufacturer's instructions. The complexes captured on the beads were eluted directly in SDS–sample buffer (Invitrogen) and heated at 90 °C 10 min to dissociate the V_HH–mCherry-tether from the ribosome. The proteins were separated on 10% polyacrylamide gel, as described previously.[17] The proteins were either stained (Coomassie or silver staining) or electro-transferred onto the nitrocellulose membrane (30 V for 1 h in X-cell miniblotter; Invitrogen). The membrane was blocked for 1 h in a blocking buffer (TBS containing 0.05% Tween 20 and 2% skimmed milk), washed twice with TBST (TBS containing 0.05% Tween 20 alone), and incubated with anti-Myc tag antibody (HRP conjugated, dilution 1 : 2500; Abcam, UK) for 1 h. Subsequently, the membrane was washed six times with TBST and then incubated with chemiluminescence substrate (Pierce, UK) for 5 min. Signals were detected using a LICOR C-Digit scanner (Licor, USA). As a negative control, anti-c-Myc-affinity beads were incubated with RIPA buffer only. All the experiments were repeated three times.

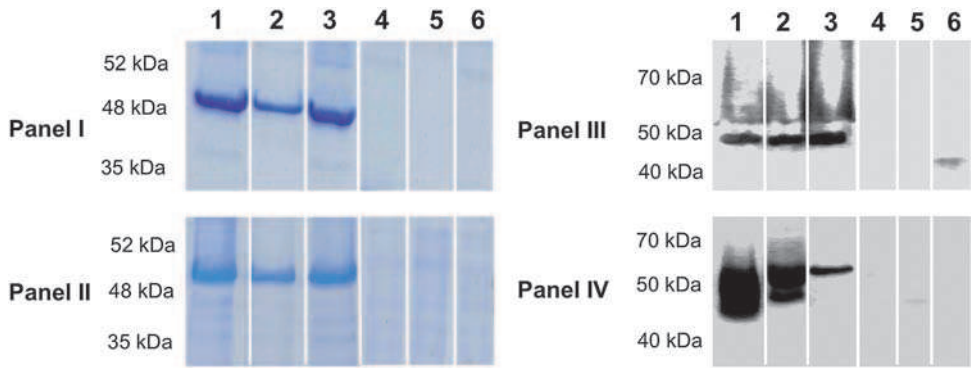

Fig. 3 The expression cassettes were translated with CFes and analyzed by SDS-PAGE or Western blot. Translated V_HH–mCherry (with stop codon) resolved on SDS-PAGE (Panel I) and detected by WB (Panel III). V_HH–mCherry-tether nascent chain dissociated from ribosome and resolved on SDS-PAGE (Panel II) and analysed by WB (Panel IV). *E. coli* CFes with (lanes 1) or without (lanes 4) exogenous DNA; *Leishmania* CFes with (lanes 2) or without (lanes 5) exogenous DNA; and rabbit reticulocyte CFes with (lanes 3) or without (lanes 6) exogenous DNA.

Fluorescence microscopy was also used to assess the translation of VHH–mCherry. Anti-c-Myc affinity beads with captured ribosome complexes were placed on a glass slide and observed at 650 nm. Similarly, 5 μl of translated lysate were added to non-fluorescent silica beads (Sigma-Aldrich, Germany) and placed on the glass slide. The slides were air-dried and observed under a 10× objective at 650 nm (Zeiss, Germany).

2.9 Screening for genetic variability of the library

90 randomly selected *E. coli* clones (colonies) from LB agar were resuspended in 50 μl Milli-Q water. DNA was isolated by incubating the suspension for 10 min at 97 °C. The V_HH–F2 inserts in pQE-30-UA were amplified by the vector-specific primers, UA-INF and UA-INR. Purified PCR products were sequenced (Avant ABI 3100, Applied Biosystems), and the sequence of V_HH from each clone was aligned by CLUSTAL W method to assess the genetic variability of the library.

2.10 Assessment of antigen binding

The antigen-binding ability of V_HH translated *in vivo* and *in vitro* was assessed. *Borrelia* (*B. afzelii* – strain SKT4, *B. burgdorferi* sensu stricto – SKT2, *B. garinii* – Rio2, *B. garinii* – PBi) and *Francisella* (*F. tularensis* subsp. *holarctica* – LVS and *F. tularensis* subsp. *holarctica* – TUL4) were cultivated as described previously.[18,19] The bacteria were sonicated and the proteins separated on polyacrylamide gel by SDS-PAGE, electro-transferred on to nitrocellulose

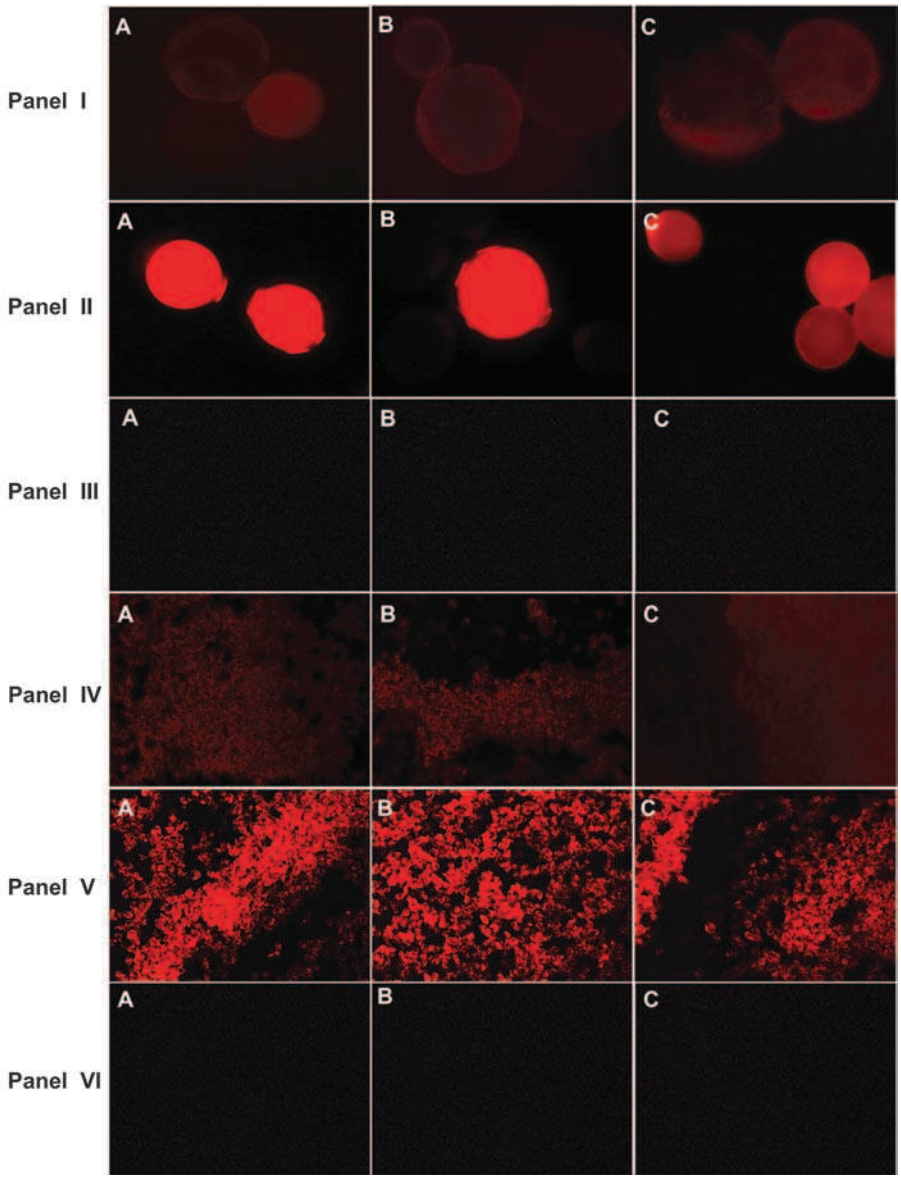

Fig. 4 Detection of translated nascent chain by fluorescence microscopy. Ribosome complex captured on c-Myc affinity beads (40×, Panel I) or mixed with silica gels (10×, Panel IV) and observed under microscope at 650 nm. V_HH–mCherry (with stop codon) captured on c-Myc affinity beads (40×, Panel II) or mixed with silica gels (10×, Panel V) and observed under microscope at 650 nm. Negative controls – translation lysate without exogenous DNA captured on c-Myc affinity beads (Panel III) or silica gel (Panel VI). A – *E. coli* CFes, B – *Leishmania* CFes, C – rabbit reticulocyte CFes.

membranes and the membranes cut into 3 mm strips. The strips were blocked for 1 h in blocking buffer (TBS containing 0.05% Tween 20 and 2% skimmed milk) and incubated overnight at 4 °C with translated lysates containing V_HH (200 μl of each CFes lysate or 500 μl of *E. coli* lysate from *in vivo* expression, diluted with 2 ml blocking buffer). The strips were washed three times and incubated with anti-Myc antibody (HRP conjugated, dilution 1 : 2500; Abcam, UK) for 1 h and examined as described above. As a negative control in the procedure, translation lysates without exogenous DNA were used. As an input control, 2 μl of each translated lysate were immobilized on a membrane, incubated with anti-Myc antibody and the signals examined.

3. Results

3.1 Amplification of V_HH of various *Camelidae* with a single set of primers

A total of 22 sequences were aligned to find the most homologous sequences in the FR1 and FR4 regions of the heavy chain of *Camelidae*. The FR1 and FR4 regions encompassed all the CDRs (CDR 1 to 3) required to form an antigen-binding pocket (Fig. 1, panel A). Attempts were made to maintain minimum degeneracy in these primers to avoid amplification of non-specific amplicons. VHHF and VHHR were able to amplify V_HH of all *Camelidae* included in the study from a minimum 5 ng of cDNA, without the significant issue of non-specific amplification. Representative amplified V_HH is illustrated in Fig. 2, panel A.

3.2 F1 and F2 fragments

The size of the amplified F1 fragment (Fig. 2, panel B, lane 1) was 274 bp, and it contained T7 promoter and a species-independent translation sequence. The double OE-PCR was used to construct F2 fragments, with or without stop codon. Various features were combined in the F2 fragments to speed up and simplify the overall workflow of ribosome complex production in different CFes, as well as the *in vivo* expression system. The length of the F2 fragment was 1060 bp (Fig. 2, panel B, lanes 2 and 3). The sequence of the reporter molecule, mCherry, which fused to the C-terminus of V_HH, was incorporated to simplify detection of the translated nascent chain. Myc-tag was included to capture the ribosome complexes on anti-c-Myc-affinity beads and to purify the library efficiently. The tether sequences served as spacers that tethered the protein to the ribosome and maintained proper folding. The SecM sequence caused the translation to end, with formation of a stable ribosome complex *in vivo* in *E. coli*, while the 3′-loop sequence protected mRNA from exonuclease (Fig. 1, panel B).

3.3 Speeding the workflow in construction of the cassette for synthesis of mRNA–ribosome–V_HH

Laborious molecular cloning steps are needed to generate the expression cassette for translation. The work involved limits the throughput of cell-free protein production, especially when different expression systems are involved in testing. To overcome this obstacle double OE-PCR was again used, in which V_HH was fused with F1 and F2 to obtain the expression cassette (Fig. 1, panel C). In the first step, all the fragments were hybridized due to the complementary overlapping between F1, V_HH, and F2. These overlaps ensured directional fusion of all three fragments in addition to serving as a priming site for elongation in PCR. In the second step, the fused fragments were amplified with the end primers, generating a full-length expression cassette (Fig. 2, panel C). We found it possible to construct the cassette within a single day.

Shifting from the *in vitro* to *in vivo* expression system required *de novo* construction of the expression cassette, which again could be cumbersome. The expression cassette synthesized with OE-PCR could be simply treated with *BamH*I and *Sal*I restriction enzymes and ligated into a suitable vector, *e.g.* in this case, pQE-30-UA plasmid (ESI,† Fig. S1).

3.4 Expression of ribosome complexes

The synthesized expression cassette contained the SITS sequence, which should allow initiation of translation in different CFes, irrespective of their origin. To evaluate its universal applicability, expression cassettes were tested using *E. coli*, *Leishmania tarentolae* and rabbit reticulocyte CFes. Purified expression cassettes F1–V_HH–F2 and F1–V_HH–F2 with a stop codon were incubated with translation mixes, and the synthesized products were evaluated using a variety of tests.

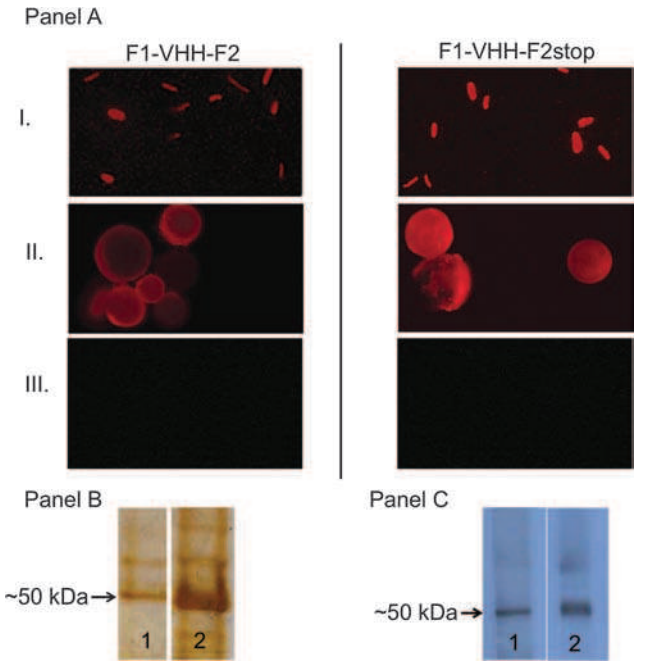

Fig. 5 Detection and analysis of *in vivo* expressed ribosome complexes. Panel A: rluorescent microscopy (650 nm). Lane I: live *E. coli* expressing ribosome complex (F1–V_HH–F2) or V_HH–mCherry with stop codon (F1–V_HH–F2 stop). Lane II: ribosome complex (F1–V_HH–F2) or V_HH–mCherry with stop codon (F1–V_HH–F2 stop) captured on c-Myc affinity beads. Lane III: negative control, only affinity beads. Panel B: V_HH–mCherry-tether nascent chain dissociated from ribosome. Lane 1: resolved on SDS-PAGE. Panel C: lane 1: analysed by WB. Translated with V_HH–mCherry (with stop codon), resolved on SDS-PAGE (panel B, lane 2) and detected by WB (panel C, lane 2).

Firstly, the nascent V_HH–mCherry was captured on anti-c-Myc affinity beads and checked directly under the fluorescence microscope (Fig. 3, panels I and II). The Myc affinity beads were incubated with translation reaction only, without the expression cassette (negative control), and showed no fluorescence (Fig. 3, panel III). Fluorescence of the nascent chain of the ribosome complex was however detectable when a small volume (5 μl) of the translation reaction was mixed with non-fluorescent silica beads (with no auto-fluorescence) and examined under the microscope at 650 nm (Fig. 3, panels IV and V). To confirm the translation of the V_HH–mCherry-tether, ribosome complexes captured on Myc affinity beads were dissociated in SDS sample buffer, separated on polyacrylamide gel and examined using Coomassie staining or anti-Myc antibody in western blotting (WB). In the case of V_HH–mCherry with stop codon, we found a band at approximately 44 kDa, both with Coomassie staining and WB (Fig. 4, panels I and III), while in case of the V_HH–mCherry-tether dissociated from the ribosome, the band occurred at approximately 50 kDa (Fig. 4, panels II and IV).

Synthesis of mRNA–mCherry–V_HH fused to ribosome was also successful in the *in vivo* expression system (Fig. 5, panel A). The complex was isolated by anti-c-Myc affinity chromatography. V_HH–mCherry-tether fusion was dissociated from the ribosomes and detected using SDS-PAGE and WB (Fig. 5, panels B and C).

3.5 Genetic variation in V_HH

Diversity of the library is very important in display technology. To assess its diversity, the V_HH region from 90 randomly selected *E. coli* clones were sequenced and aligned. We found a 95.5% sequence diversity in V_HH among the clones analyzed (out of 90 clones, 86 had unique CDR sequences) (Fig. 6). The size of the library (*i.e.*, the number of clones) was approximately 10^9.

3.6 Antigen binding ability of V_HH complexes

In the present study the V_HH library was synthesized from the naïve B cells of llama, and no specific antigen was therefore used to assess the ligand-binding ability of V_HH. However, epidemiological studies performed previously showed the prevalence of *Borrelia burgdorferi* and tick vectors in the region in which the llamas had been reared.[20–22] Whole cell antigen derived from three different *Borrelia* species were thus used in this assay. On the other hand, antigen from *Francisella* was included in the assay, since the prevalence of this pathogen was very low in the given area (and it might serve as a negative control). The binding ability of the V_HH complex to antigen derived from *Borrelia burgdorferi sensu stricto* strain SKT-2 was clearly observed in this assay (Fig. 7). All V_HH complexes were synthesized with CFes, and the *in vivo* expression system maintained

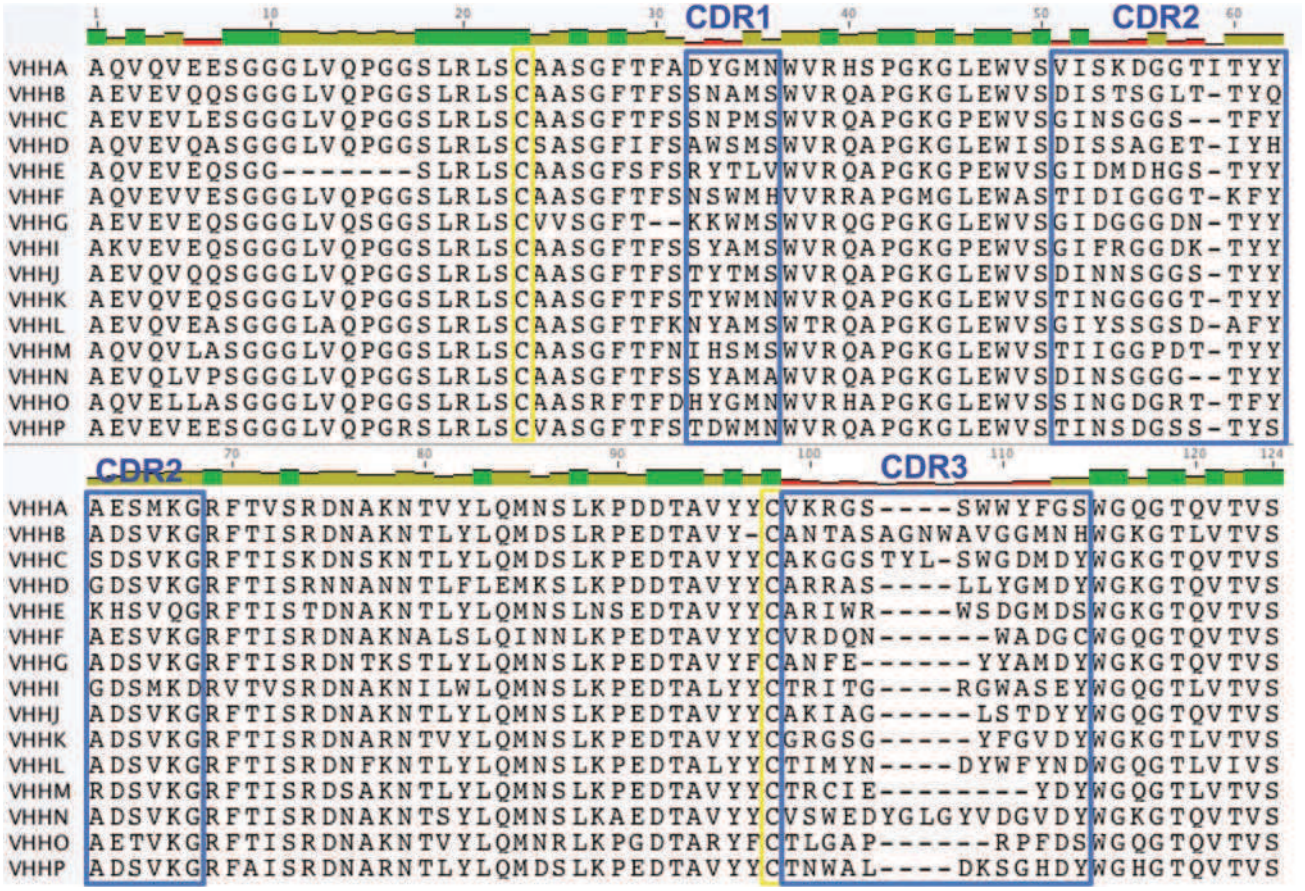

Fig. 6 Fifteen representative amino acid sequences of V_HH from randomly picked colonies of *E. coli* are shown. Residues are numbered according to the IMGT numerical system. The yellow rectangle indicates the position of disulfide bonds in cysteine. The blue rectangles represent the position of CDRs.

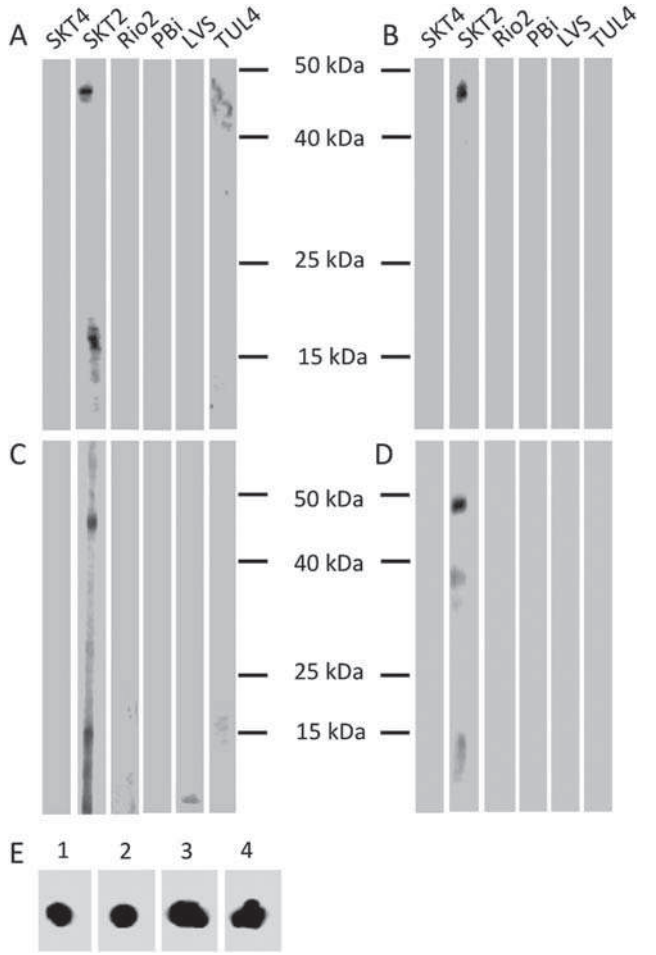

Fig. 7 Assessment of the binding ability of V_HH complex to antigens of *Borrelia* (strains SKT4, SKT2, Rio2 and PBi) or *Francisella* (strains LVS and TUL4). A naïve V_HH library was synthesized with *E. coli* CFes (panel A), *Leishmania* CFes (panel B), rabbit reticulocytes CFes (panel C), and *E. coli in vivo* expression system (panel D). Panel E: input control for V_HH–mCherry expressed with *E. coli* CFes (1), *Leishmania* CFes (2), rabbit reticulocytes CFes (3), and *E. coli in vivo* expression system (4).

their antigen binding ability. No non-specific signals were observed in the negative controls, when translation lysates without exogenous DNA were used in the assay (data not shown).

4. Discussion

A number of *in vivo* and *in vitro* expression systems are currently available, based on a variety of prokaryotic and eukaryotic sources. Despite their availability it is challenging to find an accurate expression system for the expression of the protein and antibodies of interest. *In vivo* expression is time-consuming and the toxicity of the protein can present a challenge, but it remains the preferred method. For ribosome display, on the other hand, an *in vitro* expression system is preferable. The benefits of *in vitro* expression have been described in a recent review,[23] but the protein yield in *in vitro* systems is relatively low.

Our main aim was to construct a universal expression cassette for V_HH–ribosome display that would speed and streamline selection of the most suitable expression systems. Our expression cassette was successfully translated in at least three CFes (*E. coli*, *Leishmania* and rabbit reticulocytes) as well as in *E. coli in vivo*. Due to the presence of SITS, it is a translation initiation sequence. Previously it was shown that a DNA fragment that contained SITS, by promoting the assembly of the active ribosome, replaced the untranslated 5region (5′ UTR) in mRNAs that were recognized by the translation initiation machinery, apparently initiated translation without a requirement for species-dependent translation initiation factors.[14] Thus, the SITS sequence in the expression cassette expanded the organism range used for the production of ribosome libraries.

The main difference between the various systems is the availability of post-translation machinery and protein folding. Although the antibodies or other disulfide-bonded proteins have previously been produced using *E. coli* translation machinery,[24,25] their proper folding remains questionable. Success of the ribosome display is dependent on the proper folding of the nascent chain. During translation the C-terminus of the nascent chain is covalently tethered to the peptidyl transferase center, and as the nascent chain grows in length its N-terminus exits the ribosome tunnel but remains held close to the outer surface of the ribosome. The narrowness of the exit tunnel places a restriction on the extent of nascent chain folding. A tether sequence incorporated in the expression cassette downstream to mCherry, should maintain the distance between the exit tunnel and nascent chain and allow proper folding. To examine folding on the ribosome, mCherry reporter was chosen, owing to its chemical stability, and since it required no exogenous cofactors, it was brighter than GFP, it had improved photostability and it was codon-optimized compared to other red fluorescent proteins, such as DsRed or mRFP1.[26] To form the chromophore, GFP and mCherry must be folded correctly.[27] Libraries translated in our study showed a high intensity of fluorescence (Fig. 3 and 5). Earlier work showed that reporter (GFP and its derivatives) at the C-terminus of the protein of interest gave a signal directly proportional to the amount of correctly folded protein.[27]

V_HH derived from *Camelidae* and IgNAR from shark have initiated a new era of antibody development. Their capability of recognizing unusual epitopes not detected by classical antibodies is promising.[28] A popular method for the generation of recombinant V_HH is phage display,[4,29] but the library size is often limited by the transformational efficiency of the host strains.[30] The library size in phage display and bacterial surface display may range from only 10^5 to 10^7.[5,31] The key benefit of ribosome display is the size of the library – up to 10^{15} members – which is essential mainly for naïve libraries. Although we did not assess the size and diversity of the *in vitro* library created, its diversity achieved *in vivo* was 95.5% (diversity of V_HH in the *E. coli* clones). Such high diversity indicated that OE-PCR could be successfully used in constructing the ribosome library. The 10^9 size of the library obtained *in vivo* confirmed that the V_HH–mCherry was not toxic to *E. coli* and could readily be used to produce mRNA–ribosome–V_HH complexes for the selection of single-domain antibodies.

5. Conclusions

The pipeline described offers a promising choice for the rapid construction of a universal expression cassette for the production of mRNA–ribosome–V_HH–mCherry complexes of high diversity. The overall technique is cost- and time-saving (one working day to fuse F1, V_HH and F2). The primers designed to amplify V_HH may be used to amplify V_HH of various members of *Camelidae*. The cassette can be used in various CFes as well as *in vivo* (*E. coli*) to produce ribosome complexes. A reporter (mCherry) and Myc-tag provides a rapid indication of the efficiency of translation and easy purification of ribosome complexes. We believe that the pipeline described will be of assistance to researchers in the production of single-domain antibodies.

Conflict of interest

The authors wish to declare that no conflict of interest occurred in the work described.

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