A rapid and simple pipeline for synthesis of mRNA–ribosome–V₃H₃ complexes used in single-domain antibody ribosome display†

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The single-domain antibody (V₃H) is a promising building block for a number of antibody-based applications. Ribosome display can successfully be used in the production of V₃H. 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 V₃H. With the help of a double-overlap extension (OE) polymerase chain reaction (PCR) we have fused V₃H 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–V₃H–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 (V₃H) 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 V₃H in Camelidae and V-NAR in cartilaginous fish). V₃H forms three complementary determining regions (CDRs) and four framework regions. HCAbs, V₃H, and V-NAR can be produced in vitro, and display techniques such as ribosome display, phage display or surface display have been successfully used in their engineering.

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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 stalling sequence to arrest the translation and thus produce protein–ribosome–mRNA complexes in E. coli, which were later isolated and used in biopanning. 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–VHH–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–VHH–ribosome library in vivo in E. coli using the same cassette.

2. Material and methods

2.1 Primer design

VHH contains three hypervariable CDRs, whereas framework regions F1 and F4 are relatively homogeneous. We attempted to construct primers that would amplify VHH 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 VHH 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 VHH

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. Auffy 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, Slovak). 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 x (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. F1 was amplified from the pLEXSY_in vitro_2 vector (Jena Bioscience, Germany), and contained T7 promoter, a species-independent translation sequence (SITS), 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-blecherry 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, while the loop sequence was acquired from previous work. SecM sequences are necessary to produce VHH–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. 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 x (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...
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 cloned 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 VHH and mCherry, and (b) the ability of each expression system to translate the VHH–mCherry fusion under the SITS.

2.6 In vitro synthesis of the ribosome complex (mRNA-ribosome-VHH 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 TNT³ 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 Transcend³ 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.
2.7 *In vivo* synthesis of the ribosome complex (mRNA–ribosome–VHH library)

The expression cassette was digested with BamHI and SalI 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 BamHI site is incorporated in VHHF primer and the SalI 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 OD₆₀₀ 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 MgCl₂, 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 MgSO₄ 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 VHH–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.
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 V1H–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 V1H 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 V1H 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. 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. V1H–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 VHH (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 VHH 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 VHH of all Camelidae included in the study from a minimum 5 ng of cDNA, without the significant issue of non-specific amplification. Representative amplified VHH 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 VHH, 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 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–VHH

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 VHH 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, VHH, 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 BamHI and SalI 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–VHH–F2 and F1–VHH–F2 with a stop codon were incubated with translation mixes, and the synthesized products were evaluated using a variety of tests.

Panel A

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<tr>
<th>F1-VHH-F2</th>
<th>F1-VHH-F2stop</th>
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Panel B

Panel C

~50 kDa

1 2

Fig. 5 Detection and analysis of in vivo expressed ribosome complexes. Panel A: fluorescent microscopy (650 nm). Lane I: live E. coli expressing ribosome complex (F1–VHH–F2) or VHH–mCherry with stop codon (F1–VHH–F2 stop). Lane II: ribosome complex (F1–VHH–F2) or VHH–mCherry with stop codon (F1–VHH–F2 stop) captured on c-Myc affinity beads. Lane III: negative control, only affinity beads. Panel B: VHH–mCherry-tether nascent chain dissociated from ribosome. Lane 1: resolved on SDS-PAGE. Panel C: lane 1: analysed by WB. Translated with VHH–mCherry (with stop codon), resolved on SDS-PAGE (panel B, lane 2) and detected by WB (panel C, lane 2).
Firstly, the nascent 
\(V_{\text{HH}}\)-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 \(V_{\text{HH}}\)-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 \(V_{\text{HH}}\)-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 \(V_{\text{HH}}\)-mCherry-tether dissociated from the ribosome, the band occurred at approximately 50 kDa (Fig. 4, panels II and IV).

Synthesis of mRNA–mCherry–\(V_{\text{HH}}\) 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. \(V_{\text{HH}}\)-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 \(V_{\text{HH}}\)

Diversity of the library is very important in display technology. To assess its diversity, the \(V_{\text{HH}}\) region from 90 randomly selected \(E.\ coli\) clones were sequenced and aligned. We found a 95.5% sequence diversity in \(V_{\text{HH}}\) 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 \(V_{\text{HH}}\) complexes

In the present study the \(V_{\text{HH}}\) library was synthesized from the naïve B cells of llama, and no specific antigen was therefore used to assess the ligand-binding ability of \(V_{\text{HH}}\). However, epidemiological studies performed previously showed the prevalence of \(Borrelia burgdorferi\) and tick vectors in the region in which the llamas had been reared. 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 \(V_{\text{HH}}\) complex to antigen derived from \(Borrelia burgdorferi sensu stricto\) strain SKT-2 was clearly observed in this assay (Fig. 7). All \(V_{\text{HH}}\) complexes were synthesized with CFes, and the in vivo expression system maintained

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Fig. 6 Fifteen representative amino acid sequences of \(V_{\text{HH}}\) 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.
expression have been described in a recent review, but the preferred method. For ribosome display, on the other hand, the toxicity of the protein can present a challenge, but it remains antibodies of interest.

accurate expression system for the expression of the protein and sources. Despite their availability it is challenging to find an available, based on a variety of prokaryotic and eukaryotic systems is relatively low.

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, but the protein yield in in vitro systems is relatively low.

Our main aim was to construct a universal expression cassette for V₈H–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 5′ region (5′ UTR) in mRNAs that were recognized by the translation initiation machinery, apparently initiated translation without a requirement for species-dependent translation initiation factors. 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, 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 photo-stability and it was codon-optimized compared to other red fluorescent proteins, such as DsRed or mRFP1. To form the chromophore, GFP and mCherry must be folded correctly.

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.

V₈H 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. A popular method for the generation of recombinant V₈H is phage display, but the library size is often limited by the transformational efficiency of the host strains. The library size in phage display and bacterial surface display may range from only 10^5 to 10^7. 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 V₈H 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 V₈H–mCherry was not toxic to E. coli and could readily be used to produce mRNA–ribosome–V₈H complexes for the selection of single-domain antibodies.

Fig. 7 Assessment of the binding ability of V₇H complex to antigens of Borrelia (strains SKT4, SKT2, Rio2 and PBi) or Francisella (strains LVS and TUL4). A naïve V₇H 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 V₇H–mCherry expressed with E. coli CFes (1), Leishmania CFes (2), rabbit reticulocytes CFes (3), and E. coli in vivo expression system (4).
5. Conclusions

The pipeline described offers a promising choice for the rapid construction of a universal expression cassette for the production of mRNA–ribosome–VHH–mCherry complexes of high diversity. The overall technique is cost- and time-saving (one working day to fuse F1, VHH and F2). The primers designed to amplify VHH may be used to amplify VHH 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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