Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Random mutagenesis of BoNT/E Hc nanobody to construct a secondary phage-display library

B. Shahi[1], S.L. Mousavi Gargari[1], I. Rasooli[1,2], M. Rajabi Bazl[3] and R. Hoseinpoor[1]

1 Department of Biology, Basic Science Faculty, Shahed University, Tehran, Iran
2 Molecular Microbiology Research Center, Shahed University, Tehran, Iran
3 Department of Clinical Biochemistry, Faculaty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Keywords
affinity maturation, BoNT/E, error-prone PCR, nanobody, phage-display library.

Correspondence
Seyed Latif Mousavi Gargari, Department of Biology, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's shrine, Tehran-3319118651, Iran.
E-mail: slmousavi@shahed.ac.ir

2014/0261: received 7 February 2014, revised 23 March 2014 and accepted 4 April 2014

doi:10.1111/jam.12526

Abstract

Aims: To construct secondary mutant phage-display library of recombinant single variable domain (VHH) against botulinum neurotoxin E by error-prone PCR.
Methods and Results: The gene coding for specific VHH derived from the camel immunized with binding domain of botulinum neurotoxin E (BoNT/E) was amplified by error-prone PCR. Several biopanning rounds were used to screen the phage-displaying BoNT/E Hc nanobodies. The final nanobody, SHMR4, with increased affinity recognized BoNT/E toxin with no cross-reactivity with other antigens especially with related BoNT toxins.
Conclusions: The constructed nanobody could be a suitable candidate for VHH-based biosensor production to detect the *Clostridium botulinum* type E.
Significance and Impact of the Study: Diagnosis and treatment of botulinum neurotoxins are important. Generation of high-affinity antibodies based on the construction of secondary libraries using affinity maturation step leads to the development of reagents for precise diagnosis and therapy.

Introduction

Botulism, a severe disease with a high fatality rate, is caused by highly potent neurotoxins generated by the rod-shaped, Gram-positive, anaerobic sporulating bacterium, *Clostridium botulinum*. The toxins, which block cholinergic synapses, cause foodborne, infant and wound botulisms in humans (Smith 2009; Peck *et al.* 2011). Botulinum toxin induces flaccid paralysis by blocking the release of acetylcholine from presynaptic nerve ends (Sinha *et al.* 2007). Botulinum neurotoxins are the most potent biological toxins known to exist and considered as a potential threat to the public by the Centers for Disease Control and Prevention (CDC; Davletov *et al.* 2005; Dutton and Fowler 2007; Smith 2009). Therefore, early diagnosis and proper management of this potentially lethal disease are necessary. Botulinum neurotoxins (BoNTs) are synthesized as inactive single chains with a molecular mass of about 150 kDa. The toxin becomes active with proteolytic cleavage of holotoxin producing a 100-kDa heavy chain (Hc) and a 50-kDa light chain (LC; Kumaran *et al.* 2009; Smith 2009). The 50-kDa LC contains the catalytic domain, and the C-terminal region of the Hc is the cell-binding domain (Bakherad *et al.* 2013).

Antibodies are powerful and rapidly growing tools in therapy and diagnostics (Weisser and Hall 2009). Despite their advantages, their large size, relative instability and tissue accessibility have limited conventional antibodies application (Kolkman and Law 2010). In 1989, a new type of antibody in sera of the camelidae (i.e. camels, dromedaries and llamas) was detected with the advantages of small molecules besides the benefits of monoclonal antibodies (mAbs; Kolkman and Law 2010; Vanlandschoot *et al.* 2011). Nanobodies are single variable domain of heavy-chain antibody fragments (VHH) with unique properties (Butler *et al.* 2012). Nanobodies are the smallest antigen-binding fragments (~15 kDa) with high homology to the human VH frameworks and can be humanized with some amino acid substitutions in the framework region. Because of their small size, nanobodies can penetrate tissues efficiently and detect hidden epitopes better than conventional antibodies. They are extremely stable against heat, proteases and pH variations and can fold correctly under a wide range of conditions.

Due to substitution of some hydrophobic by hydrophilic residues, nanobodies are naturally soluble in aqueous solution and have no tendency to form aggregates. VHHs are the best candidates to make bispecific antibodies and can be efficiently expressed in *E. coli* with no side effect for host immune system (Lauwereys *et al.* 1998; Muyldermans and Lauwereys 1999; Deffar *et al.* 2009; Butler *et al.* 2012; Ebrahimizadeh *et al.* 2012; Ardekani *et al.* 2013).

Improving the affinity of protein–protein interaction as a therapeutic and diagnostic biomolecules is a challenging problem (Clark *et al.* 2006). Several approaches have been developed for affinity maturation of antibodies derived from the original libraries, but mostly fall into site-directed and random mutagenesis (Antikainen and Martin 2005; Sheedy *et al.* 2007). Therefore, binding proteins such as nanobodies can be easily engineered (Hwang and Park 2008). Error-prone PCR is one of the most popular random methods where point mutations are randomly introduced into a gene (Biles and Connolly 2004; Sheedy *et al.* 2007). The mutation rate in error-prone PCR can be changed by different mutagenic conditions (Wang *et al.* 2006). Phage-display technology with mutagenesis methods can be used for affinity maturation of parent peptides from the random libraries. After generation of the second phage-display library for affinity maturation, the most common selecting method is enrichment of the phage clones by biopanning process (Pande *et al.* 2010). In our previous work (Bakherad *et al.* 2013), the specific nanobody against the binding domain of botulinum neurotoxin E (BoNT/E Hc) was produced from immune phage library.

In this study, using error-prone PCR, we made an attempt to generate the secondary mutant phage-display library of recombinant VHH derived from the camel immunized against Hc of BoNT/E (Bakherad *et al.* 2013).

Materials & methods

Antigen purification

A synthetic gene coding for Hc (Bakherad *et al.* 2013) was expressed in *E. coli* BL21 (DE3) overnight at 28°C. Purification of the recombinant BoNT/E Hc was accomplished with nickel nitrilotriacetic acid (Ni-NTA) agarose under native conditions. The purified recombinant protein was analysed by SDS-PAGE. Binding activity of the purified recombinant Hc to BoNT/E was verified by ELISA as described previously (Gargari *et al.* 2011; Bakherad *et al.* 2013). The recombinant BoNT/E Hc was confirmed by Western blotting using mouse anti-$(His)_6$ (1 : 10 000, Abcam, Cambridge, UK).

Error-prone PCR

To construct a mutant library, the gene coding for specific VHH antibody against binding domain of BoNT/E is used as template. VHH fragments were amplified by a pair of primers for framework-1 (5′- ACTGGCCCAGGCGGCCGAGGTGCAGCTGSWGSAKTCKG -3′) and framework-4 (5′ -ACTGGCCGGCCTGGCCTGAGGAGACGGTGACCWGGGTC-3′) regions with *Sfi*I restriction sites. Both error-prone PCRs contained 10 ng of plasmid DNA and 10 pmol l^{-1} concentrations of each primer. Combined with descending concentrations of template and ascending concentrations of Taq DNA polymerase, mutation frequencies were further enhanced in both PCRs. First reaction was performed in 100 μl volume containing 10 μl of 10× buffer, 10 pmol l^{-1} of each primers, 10 U Taq DNA polymerase, 10 ng plasmid DNA with 7 mmol l^{-1} $MgCl_2$ and 0·2 mmol l^{-1} deoxyinosine triphosphate (dITP) as mutagenic agents. The second round of PCR was carried out in four reactions with descending concentrations of the dNTPs (0·2 mmol l^{-1} →20 μmol l^{-1}) and adding 0·2 mmol l^{-1} dITP and 0·2 mmol l^{-1} $MnCl_2$. Mutagenic PCR amplifications were performed as follows: 94°C for 10 min, 30 cycles at 94°C for 30 s, 66°C for 35 s and 72°C for 1 min, followed by a 72°C incubation period for 10 min.

Construction of mutagenic library

The products of both error-prone PCR (400 bp) were purified with the gel extraction kit to remove unexpected DNA fragments. Purified mutant PCR products and pComb3x phagemid vector were digested with *Sfi*I restriction enzyme overnight at 50°C. Digested VHH fragments were ligated into the pComb3x vector at the *Sfi*I restriction site overnight at 12°C. Ligation reaction was electroporated into competent cells of *E. coli* TG1. Electroporation was performed several times to increase the diversity of the library. After incubation at 37°C for 1 h, the transformed bacteria were plated onto LB agar plates supplemented with 80 μg ml^{-1} ampicillin (Bakherad *et al.* 2013). Confirmation of positive clones containing the VHH fragments was performed by colony PCR and restriction digestion of the vectors. Calculation of library size was performed by plating serial dilution aliquots on LB agar ampicillin plates and incubated overnight at 37°C. The transformed bacterial stocks were prepared in 20% (v/v) sterile glycerol and stored at −80°C.

Panning and selection of a BoNT/E-specific nanobody

Phage library was generated by infecting the transformed library with M13K07 helper phage at a concentration of

1×10^{12} colony-forming units (CFU) ml^{-1}. Recombinant phages were prepared by the addition of PEG 6000 (20%)/2·5 mol l^{-1} NaCl solution (Alirezapour *et al.* 2013; Bakherad *et al.* 2013). Five consecutive rounds of panning on microtitre plates were performed for enrichment of BoNT/E-specific nanobody library. 10 μg ml^{-1} of purified recombinant BoNT/E Hc was coated in 96-well microtitre plate and incubated overnight at 4°C. The wells were blocked with 3% BSA for 2 h and were then washed three times with 200 μl of TBS (150 mmol l^{-1} NaCl and 50 mmol l^{-1} Tris base, pH7·2) containing Tween 20 (TBST). 100 μl of the concentrated phage library was added to the wells and incubated for 2 h. Washing was intensified by ascending concentrations of Tween 20 in TBST from 0·1% to 0·5% in subsequent panning cycles to arrive at higher-affinity phage. The bound phage particles were eluted with 100 μl of 200 mmol l^{-1} glycine buffer (pH 2·2) for 10 min and were immediately neutralized with 20 μl of 1 mol l^{-1} Tris buffer pH 9. The phage particles were then amplified and purified for the next round of panning as described above. Phage titres for all steps of panning were calculated. After the fifth round of panning, enrichment of phage particles against BoNT/E Hc was determined by polyclonal phage ELISA. 100 μl of purified recombinant BoNT/E Hc at a concentration of 10 μg ml^{-1} in 0·15 mol l^{-1} carbonate bicarbonate buffer pH 9·6 was coated to each well of 96-well plate and incubated overnight at 4°C. After washing the wells three times with TBST and blocking with 150 μl of 5% skimmed milk in TBST for 1 h, 10^{11} units of phage purified from library and each selection round was added to the wells and incubated at 37°C for 2 h. Mouse anti-M13/HRP monoclonal antibody diluted to 1/8000 in PBS was added to each well and incubated on a shaker at 37°C for 1 h. After washing the wells three times, TMB substrate was added and incubated at room temperature for 10–15 min. The reaction was stopped with 3 N H_2SO_4, and absorbance at 450 nm was measured. After five rounds of panning, 19 colonies were randomly chosen from the third round of panning, and VHH binding specificity was measured by monoclonal phage ELISA.

Expression and purification of mature nanobody

One clone (SHMR4), with the highest absorbance in monoclonal phage ELISA, was selected for expression of soluble mutant nanobody. The plasmid was purified using Plasmid Mini Extraction Kit and transformed into *E. coli* Top10F′. Expression was performed at OD_{600} of 0·5 at 28°C with isopropyl-1-thio-β-d-galactopyranoside (IPTG) at a final concentration of 1 mmol l^{-1}. For higher expression, the gene coding for nanobody was subcloned into pET28a using the following pair of primers: F2: 5′-ACTTCAGAATTCGAGGTGCAGCTGSWGSA KTCKG-3′ and R2: 5′-ACTACAAAGCTTTTAGGAGACG GTGACCWGGGTC-3′. The ligation product was transformed into *E. coli* BL21 (DE3) competent cells, and the transformants were screened on LB agar containing 70 μg ml^{-1} kanamycin. The transformed cells were expressed after induction at OD_{600} of 0·5 by 1 mmol l^{-1} IPTG for 6 h at 37°C. The nanobody was purified as described above. Bacteria were harvested and sonicated in 8 mol l^{-1} urea, pH 8 with 1·0 mmol l^{-1} phenyl methyl sulfonyl fluoride (PMSF) in order to extract inclusion bodies. Expression was confirmed by 15% SDS-PAGE and Western blotting using mouse anti-His antibody at 1 : 10 000 dilution (Abcam). The recombinant protein was purified by Ni-NTA agarose column. Proteins bound to Ni-NTA column were eluted with 40, 150 and 200 mmol l^{-1} imidazole.

Affinity measurement

The affinity of the parental and mutant nanobodies against recombinant BoNT/E Hc was determined by ELISA. Briefly, recombinant BoNT/E Hc protein was coated with 0, 5, 10, 15, 20, 25 μg ml^{-1} in microplate wells overnight at 4°C. After washing with PBS along with 0·05% Tween 20 (PBST) and blocking with 5% skimmed milk in PBS, 100 μl of nanobody from each of 0, 5, 10, 15, 20, 25 μg ml^{-1} concentrations was added to the above BoNT/E Hc-coated wells and incubated for 2 h at 37°C. After washing three times with PBST, HRP-conjugated anti-His antibody (1 : 10 000) was added to the wells and incubated for 1 h at 37°C. TMB was added as substrate. The reaction was stopped with H_2SO_4 (3 N), and the absorbance was measured at 450 nm (Huang *et al.* 2012). Beatty's equation (Beatty *et al.* 1987) was used to calculate the association constant (K_{aff}) of nanobody to antigen as:

$$[Ag]/[Ag'] = n$$
$$K_{aff} = n - 1/2(n[Ab']_t - [Ab]_t)$$

where Ag and Ag′ are two different concentrations of the antigen (neurotoxin binding domain).

Binding specificity

ELISA assays were carried out to determine the cross-reactivity of matured VHH and various related and unrelated proteins such as BoNT/A and BoNT/B, BSA, UreC from *Helicobacter pylori*, Bap from *Acinetobacter baumannii*, LPS from *Vibrio cholera*e. ELISA was performed using 10 μg ml^{-1} of each cross-reacting molecule coated in microplate and 10 μg ml^{-1} of the purified matured

nanobody (SHMR4). Coating buffer without antigen was used as negative control.

Sequence analysis

Phagemids encoding SHMR4 and parental VHH were sequenced with F1 primer. The results were analysed by NCBI BLAST and CLUSTALW (Ver. 2.1) multiple alignment program (http://www.ebi.ac.uk/clustalw). Mutations were analysed at DNA and protein levels with CLUSTALW program.

Molecular modelling

To identify the substitution locations in mutant gene and mutation effects on nanobody properties, homology models were constructed. PHYRE2 software at http://www.sbg.bio.ic.ac.uk/phyre2 was used to predict the 3-D structure of protein input.

Results

Library construction

A 54 kDa band in Western blotting confirmed the recombinant protein (Fig. 1). The VHH gene against BoNT/E Hc was amplified and mutagenized with error-prone PCR. After digestion and ligation reaction, the phage-display library with $3{\cdot}5 \times 10^6$ members was constructed. One hundred colonies obtained randomly were confirmed by colony PCR in about 80% of library clones.

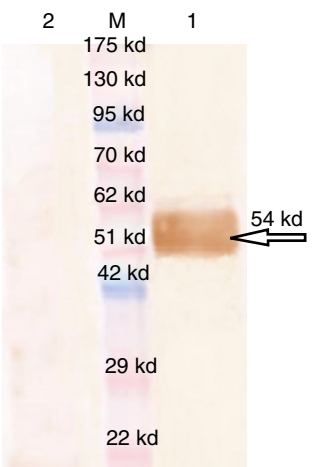

Figure 1 Western blot analysis of recombinant BoNT/E Hc.Lane M: Molecular weight marker; Lane 1: The specific reaction of recombinant BoNT/E Hc after interaction with the HRP-conjugated anti-His-tag antibody; Lane 2: recombinant protein without induction.

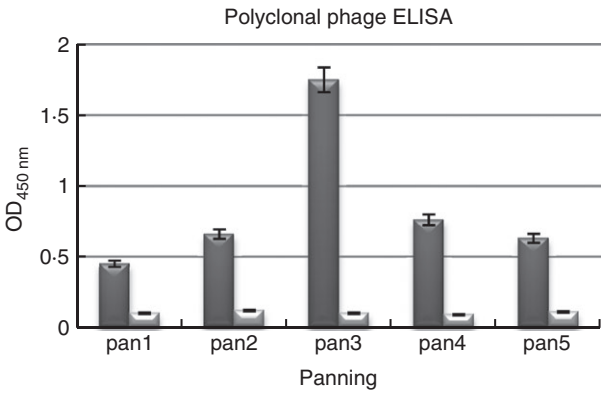

Figure 2 Polyclonal phage ELISA. Maximum enrichment was obtained in the third panning. BSA was applied as a nonspecific binding molecule (NSB). ■ Antigen; ■ BSA.

Panning

The recombinant phages were screened using consecutive rounds of panning against BoNT/E Hc. The strongest enrichment was obtained in third round of panning in the polyclonal phage ELISA (Fig. 2). A total of 19 individual colonies from the third round of panning were randomly selected, and their binding capacity to BoNT/E Hc was tested by monoclonal phage ELISA (Fig. 3). One colony (SHMR4) showing stronger binding to the antigen was selected for the next step.

Expression of soluble VHH

Figure 4a shows expression of a 17 kDa band of soluble SHMR4 on 15% SDS-PAGE. The purified nanobody was confirmed by Western blotting. Presence of a 17 kDa band in Western blotting confirmed the nanobody (Fig. 4b).

Affinity and specificity determinations

SHMR4 nanobody affinity towards recombinant BoNT/E Hc was determined by ELISA (Fig. 5a). The affinity was measured to be $1{\cdot}43 \times 10^{-7}$ $(\text{mol l}^{-1})^{-1}$, and the matured nanobody showed 10% affinity improvement over the parental nanobody. The nanobody was found to be specific for whole toxin compared with other botulinum neurotoxins (BoNT/A, BoNT/B) and other unrelated antigens (Fig. 5b).

Sequence comparison

DNA sequence of parental and SHMR4 nanobodies was aligned to define the mutation status (Fig. 6a). These sequences were translated into protein sequences, and the correct translation frame was chosen and aligned

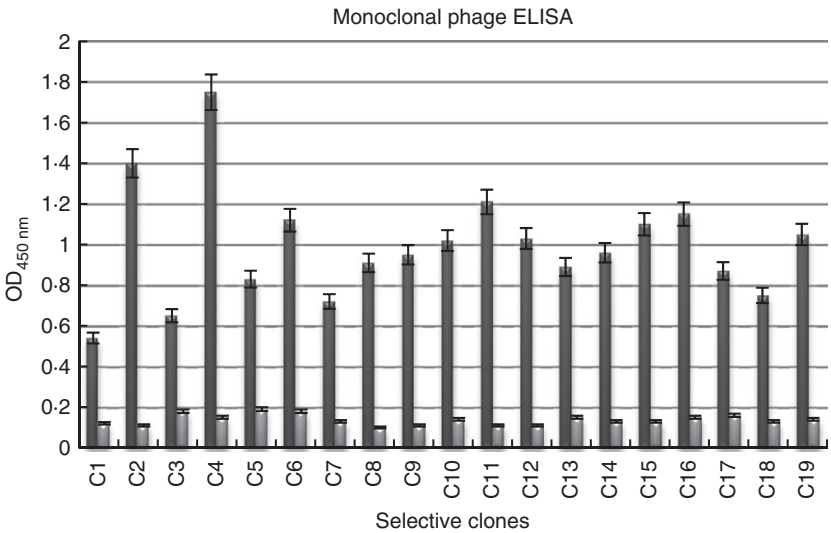

Figure 3 Monoclonal phage ELISA. C4 colony (SHMR4) showed the strongest binding to the antigen. The negative control, that is, coating buffer without antigen was taken as blank. ■ Antigen; ■ BSA.

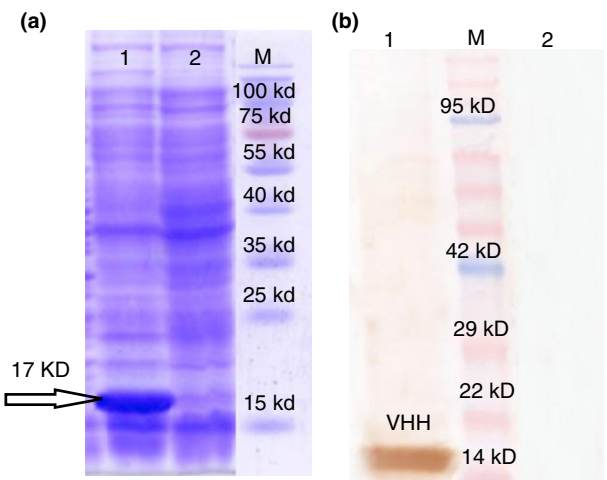

Figure 4 Confirmation of the final VHH (SHMR4) expression by SDS-PAGE and Western blotting. (a) SDS-PAGE of SHMR4 expression. Lane M, molecular weight markers; Lane 1, nanobody expression after 1 mmol l^{-1} IPTG induction; Lane 2: cell lysate without induction. (b) Western blotting of SHMR4 with HRP-conjugated anti-His tag antibody. Lane M: molecular weight marker; Lane 1: specific reaction of nanobody after induction with the anti-His-tag antibody; Lane 2: nanobody without induction.

(Fig. 6b). Analysis of these results showed low mutation frequency. No deletion or insertion mutation was observed. Among different positions of nanobody, substitutions occurred in framework regions (FRs), and complementarity determining regions (CDRs) were not changed.

Three-dimensional model comparison

The analysis of parental and mutant nanobodies using three-dimensional models revealed changes in amino acids in homology models. The results illustrated that mutations occurred in framework regions (FRs; Fig. 7).

Discussion

Centers for Disease Control and Prevention (CDC) classified BoNTs as one of the six highest-risk threat agents because of their lethality, the ease of production and transport, and the necessity for prolonged hospitalization for those exposed. The BoNTs are exoneurotoxins that can cause severe paralysis and death in humans and animals (Hill *et al.* 2007). No specific small molecule exists for the prevention or treatment of botulism, except for a pentavalent toxoid vaccine from CDC (Smith *et al.* 2005). This indicates the necessity of rapid and specific pharmaceutical agents for detection of BoNT. Antibodies with high affinity and specificity can be applied in diagnostic and therapeutic measures (Hong *et al.* 2007). Unlike the conventional antibodies, VHH or nanobodies with capability to bind cavities and clefts and unique epitopes inaccessible to other antibody fragments benefit from more therapeutic value (Ebrahimizadeh *et al.* 2012; Bakherad *et al.* 2013). A high-affinity antibody binds its ligand stronger and faster and remains bound longer. Selecting the appropriate technology for antibody affinity maturation depends upon the origin of the antibody and the desired format. The combination of several random and targeted methods including error-prone PCR, DNA shuffling and site-directed mutagenesis can further increase the size and genetic diversity of the mutant library (Wark and Hudson 2006). In this study, error-prone PCR method was employed to create random mutations in our VHH previously obtained against recombinant BoNT/E Hc.

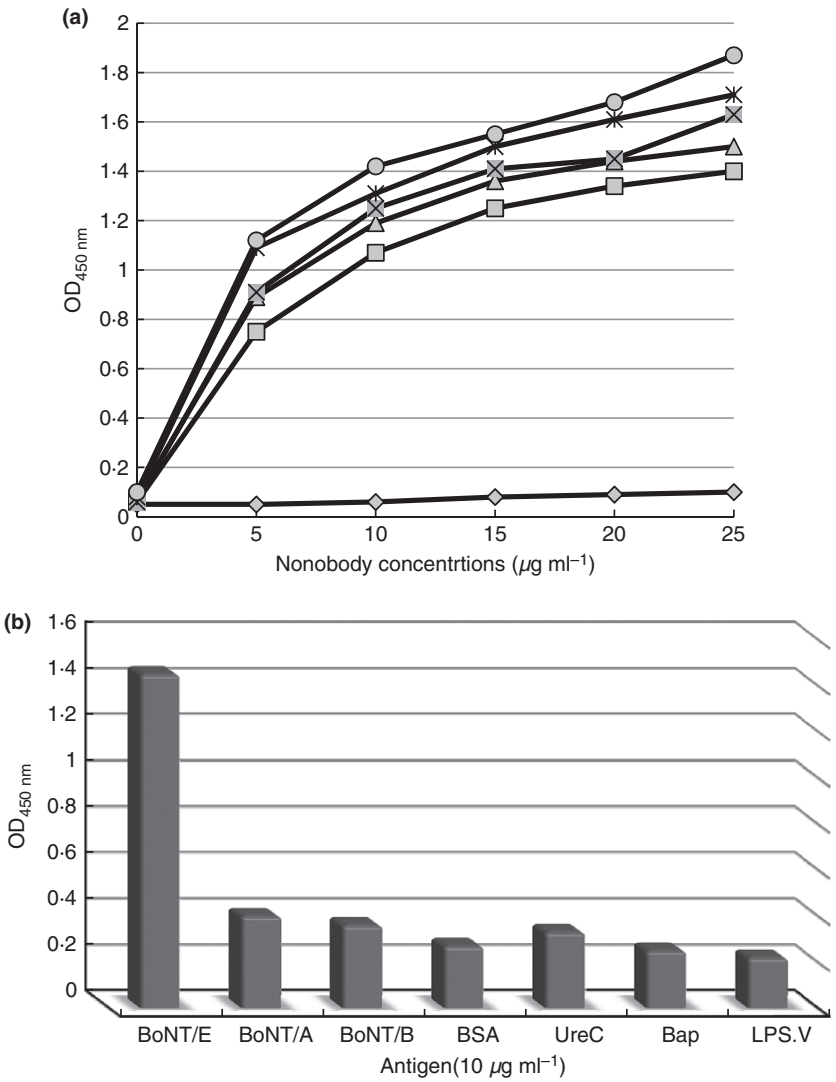

Figure 5 (a) ELISA assay of SHMR4 with recombinant BoNT/E Hc. 2–◇–0 μg ml^{-1}; –□–5 μg ml^{-1}; –△–10 μg ml^{-1}; –✳–15 μg ml^{-1}; –✳–20 μg ml^{-1}; –○–25 μg ml^{-1}. (b) ELISA binding assay to determine the specificity of SHMR4 to recombinant BoNT/E Hc and other proteins. The negative control, that is, coating buffer without antigen was taken as blank.

To increase the variety of random mutations, various factors with variable mutation rates such as $MgCl_2$, $MnCl_2$, various dNTP ratios and dITP as a nucleotide analogue were employed in the present study. Taq enzyme without proofreading was used as a DNA polymerase. Different polymerases or combination of polymerases such as Deep vent and Taq and Mutazyme II have been used to increase the mutation rates in other researches (Tuckey and Noren 2002; Kim *et al.* 2011; Huang *et al.* 2012). The mutation rate of Taq polymerase with nonstandard PCR conditions including Mn^{2+}, unbalanced dNTP levels or unnatural mutagenic bases can be further enhanced up to $9{\cdot}8 \times 10^{-3}$ mutation/bp/PCR cycle (Wark and Hudson 2006). In the present study, this number was $6{\cdot}6 \times 10^{-4}$ mutation/bp/PCR cycle.

The size of our mutagenic library was $3{\cdot}5 \times 10^{6}$ clones, a reliable number as against a reported range of 10^{4}–10^{7} clones (Chao *et al.* 2004; Razai *et al.* 2005; Muller *et al.* 2011). For screening the mutagenic library, five rounds of panning against BoNT/E Hc were carried out. The best candidate was selected from the third round and subcloned into expression vector.

SHMR4 VHH with 10% increased affinity against recombinant BoNT/E Hc compared with parental VHH was isolated from mutagenic library. This affinity increase of 10% could be attributed to several factors. First, most of the affinity maturation processes were performed in antibodies obtained from nonimmune libraries (Yau *et al.* 2005; Groves *et al.* 2006; Grimm *et al.* 2012), while in this research, the parental VHH was screened from immune library which was quite specific. Second, all of the random mutations occurring in FR and CDR regions remained unchanged, whereas CDR regions, in particular CDR3, have striking effect in antibody specificity and

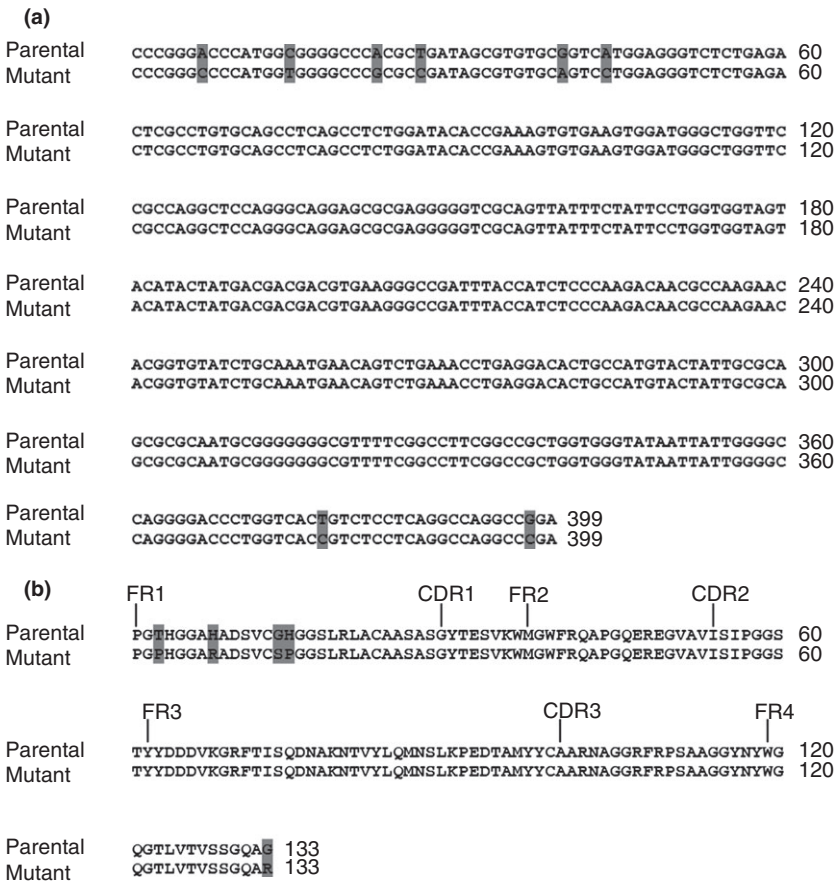

Figure 6 (a) Comparison of the parental and mutant nanobody genes with ClustalW multiple sequence alignment. (b) Comparison of the parental and mutant nanobody proteins with ClustalW multiple sequence alignment. FR and CDR regions are shown. Changed nucleotides and amino acids are highlighted.

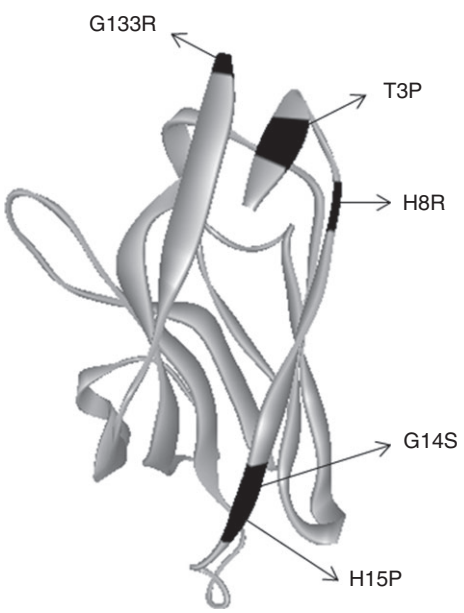

Figure 7 Homology models of mutant nanobody against recombinant BoNT/E Hc. Changed amino acids were illustrated in black colour on three-dimensional models. Amino acid substitutions are threonine to proline, histidine to arginine, glycine to serine, histidine to proline and glycine to arginine.

affinity. Analysis of parental and mutant VHH gene showed that random mutations occurred in FR regions. Five amino acid changes were observed in FR1 and FR4 regions, and other segments remained unchanged. A 10% increase in nanobody affinity could be due to these substitutions. Some of these amino acids substituted with similar amino acids with no significant impact on physicochemical features. Third, great affinity enhancements by random mutagenesis have not yet been reported for VHHs, and most of the affinity improvements by random or site-directed mutagenesis have been performed for scFvs that are not comparable with nanobodies in overall structure and other features. Fourth, affinities of selected binders for specific targets can differ widely, depending on several parameters including binder class, selection methods, library size and target characteristics (Grimm *et al.* 2012). SHMR4 nanobody recognizes BoNT/E toxin with no cross-reactivity with other antigens, especially with related BoNT toxins.

Because of the appropriate affinity and specificity of the recombinant VHH, the nanobody produced here, that is, SHMR4, could be a suitable candidate for the production of VHH-based biosensor to detect the *Clostridium botulinum* type E. Further improvements such as switching

onto diabodies formation and other modifications can accommodate the product in therapeutic applications.

Acknowledgements

The authors would like to thank Shahed University, Tehran, Iran, for the sanction of grants to support this study.

Conflict of Interest

The authors declare no conflict of interest.

References

Alirezapour, B., Rajabibazl, M., Rasaee, M. and Omidfar, K. (2013) Production and characterization of recombinant scFv against digoxin by phage display technology. *Monoclon Antib Immunodiagn Immunother* **32**, 172–179.

Antikainen, N.M. and Martin, S.F. (2005) Altering protein specificity: techniques and applications. *Bioorg Med Chem* **13**, 2701–2716.

Ardekani, L.S., Gargari, S.L.M., Rasooli, I., Bazl, M.R., Mohammadi, M., Ebrahimizadeh, W., Bakherad, H. and Zare, H. (2013) A novel nanobody against urease activity of *Helicobacter pylori*. *Int J Infect Dis* **17**, 723–728.

Bakherad, H., Gargari, S.L.M., Rasooli, I., RajabiBazl, M., Mohammadi, M., Ebrahimizadeh, W., Ardakani, L.S. and Zare, H. (2013) In vivo neutralization of botulinum neurotoxins serotype E with heavy-chain Camelid antibodies (VHH). *Mol Biotechnol* **55**, 159–167.

Beatty, J.D., Beatty, B.G. and Vlahos, W.G. (1987) Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J Immunol Methods* **100**, 173–179.

Biles, B.D. and Connolly, B.A. (2004) Low-fidelity *Pyrococcus furiosus* DNA polymerase mutants useful in error-prone PCR. *Nucleic Acids Res* **32**, 176.

Butler, D.C., McLear, J.A. and Messer, A. (2012) Engineered antibody therapies to counteract mutant huntingtin and related toxic intracellular proteins. *Prog Neurobiol* **97**, 190–204.

Chao, G., Cochran, J.R. and Dane Wittrup, K. (2004) Fine epitope mapping of anti-epidermal growth factor receptor antibodies through random mutagenesis and yeast surface display. *J Mol Biol* **342**, 539–550.

Clark, L.A., Boriack Sjodin, P., Eldredge, J., Fitch, C., Friedman, B., Hanf, K.J., Jarpe, M., Liparoto, S.F. *et al.* (2006) Affinity enhancement of an in vivo matured therapeutic antibody using structure based computational design. *Protein Sci* **15**, 949–960.

Davletov, B., Bajohrs, M. and Binz, T. (2005) Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. *Trends Neurosci* **28**, 446–452.

Deffar, K., Shi, H., Li, L., Wang, X. and Zhu, X. (2009) Nanobodies – the new concept in antibody engineering. *Afr J Biotechnol* **8**, 2645–2652.

Dutton, J.J. and Fowler, A.M. (2007) Botulinum toxin in ophthalmology. *Surv Ophthalmol* **52**, 13–31.

Ebrahimizadeh, W., Gargari, S.L.M., Rajabibazl, M., Ardekani, L.S., Zare, H. and Bakherad, H. (2012) Isolation and characterization of protective anti-LPS nanobody against V. cholerae O1 recognizing Inaba and Ogawa serotypes. *Appl Microbiol Biotechnol* **97**, 4457–4466.

Gargari, S.L.M., Rasooli, I., Valipour, E., Basiri, M., Nazarian, S., Amani, J. and Farhadi, N. (2011) Immunogenic and protective potentials of recombinant receptor binding domain and a C-terminal fragment of Clostridium botulinum neurotoxin type E. *Iran J Biotechnol* **9**, 181–187.

Grimm, S., Salahshour, S. and Nygren, Pk (2012) Monitored whole gene in vitro evolution of an anti-hRaf-1 affibody molecule towards increased binding affinity. *N Biotechnol* **29**, 534–542.

Groves, M., Lane, S., Douthwaite, J., Lowne, D., Rees, D.G., Edwards, B. and Jackson, R.H. (2006) Affinity maturation of phage display antibody populations using ribosome display. *J Immunol Methods* **313**, 129–139.

Hill, K., Smith, T., Helma, C., Ticknor, L., Foley, B., Svensson, R., Brown, J., Jhonson, E. *et al.* (2007) Genetic diversity among botulinum neurotoxin-producing clostridial strains. *J Bacteriol* **189**, 818–832.

Hong, W.W., Yen, Y.-H. and Wu, S.-C. (2007) Enhanced antibody affinity to Japanese encephalitis virus E protein by phage display. *Biochem Biophys Res Commun* **356**, 124–128.

Huang, R., Fang, P. and Kay, B.K. (2012) Improvements to the Kunkel mutagenesis protocol for constructing primary and secondary phage-display libraries. *Methods* **58**, 10–17.

Hwang, I. and Park, S. (2008) Computational design of protein therapeutics. *Drug Discov Today* **5**, 43–48.

Kim, H.S., Lo, S.C., Wear, D.J., Stojadinovic, A., Weina, P.J. and Izadjoo, M.J. (2011) Improvement of anti-Burkholderia mouse monoclonal antibody from various phage-displayed single-chain antibody libraries. *J Immunol Methods* **372**, 146–161.

Kolkman, J.A. and Law, D.A. (2010) Nanobodies – from llamas to therapeutic proteins. *Drug Discov Today Technol* **7**, 139–146.

Kumaran, D., Eswaramoorthy, S., Furey, W., Navaza, J., Sax, M. and Swaminathan, S. (2009) Domain organization in Clostridium botulinum neurotoxin type E is unique: its implication in faster translocation. *J Mol Biol* **386**, 233–245.

Lauwereys, M., Ghahroudi, M.A., Desmyter, A., Kinne, Jr, Halzer, W., De Genst, E., Wyns, L. and Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* **17**, 3512–3520.

Muller, B.H., Savatier, A., L'Hostis, G., Costa, N., Bossus, M., Michel, S., Ott, C., Becquart, L. *et al.* (2011) In vitro

affinity maturation of an anti-PSA antibody for prostate cancer diagnostic assay. *J Mol Biol* **414**, 545–562.

Muyldermans, S. and Lauwereys, M. (1999) Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. *J Mol Recognit* **12**, 131–140.

Pande, J., Szewczyk, M.M. and Grover, A.K. (2010) Phage display: concept, innovations, applications and future. *Biotechnol Adv* **28**, 849–858.

Peck, Mw, Stringer, S.C. and Carter, A.T. (2011) Clostridium botulinum in post-genomic era. *Food Microbiol* **28**, 183–191.

Razai, A., Garcia-Rodriguez, C., Lou, J., Geren, I., Forsyth, C., Robles, Y., Tsai, R., Smith, T.J. *et al.* (2005) Molecular evolution of antibody affinity for sensitive detection of botulinum neurotoxin type A. *J Mol Biol* **351**, 158–169.

Sheedy, C., Roger MacKenzie, C. and Hall, J.C. (2007) Isolation and affinity maturation of hapten-specific antibodies. *Biotechnol Adv* **25**, 333–352.

Sinha, D., Karri, K. and Arunkalaivanan, A. (2007) Applications of Botulinum toxin in urogynecology. *Eur J Obstet Gynecol Reprod Biol* **133**, 4–11.

Smith, L.A. (2009) Botulism and vaccines for its prevention. *Vaccine* **27**, 33–39.

Smith, T., Lou, J., Geren, I., Forsyth, C., Tsai, R., Laporte, S., Tepp, W., Bradshaw, M. *et al.* (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. *Infect Immun* **73**, 5450–5457.

Tuckey, C.D. and Noren, C.J. (2002) Selection for mutants improving expression of an anti-MAP kinase monoclonal antibody by filamentous phage display. *J Immunol Methods* **270**, 247–257.

Vanlandschoot, P., Stortelers, C., Beirnaert, E., Ibaez, L.I., Schepens, B., Depla, E. and Xavier, Saelens (2011) Nanobodies: new ammunition to battle viruses. *Antiviral Res* **92**, 389–407.

Wang, T.-W., Zhu, H., Ma, X.-Y., Zhang, T., Ma, Y.-S. and Wei, D.-Z. (2006) Mutant library construction in directed molecular evolution. *Mol Biotechnol* **34**, 55–68.

Wark, K.L. and Hudson, P.J. (2006) Latest technologies for the enhancement of antibody affinity. *Adv Drug Deliv Rev* **58**, 657–670.

Weisser, N.E. and Hall, J.C. (2009) Applications of single-chain fragment antibodies in therapeutics and diagnostics. *Biotechnol Adv* **27**, 502–520.

Yau, K.Y., Dubuc, G., Li, S., Hirama, T., MacKenzie, C.R., Jermutus, L., Hall, J.C. and Tanha, J. (2005) Affinity maturation of a V_HH by mutational hotspot randomization. *J Immunol Methods* **297**, 213–224.