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Surface plasmon resonance (SPR) based binding studies of refolded single chain antibody fragments

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ABSTRACT

Recent advances in Recombinant antibody technology / Antibody Engineering has given impetus to the genetic manipulation of antibody fragments that has paved the way for better understanding of the structure and functions of immunoglobulins and also has escalated their use in immunotherapy. Bacterial expression system such as *Escherichia coli* has complemented this technique through the expression of recombinant antibodies. Present communication has attempted to optimize the expression and refolding protocol of single chain fragment variable (ScFv) and single chain antigen binding fragment (ScFab) using *E.coli* expression system. Efficiency of refolding protocol was validated by structural analysis by CD, native folding by fluorescence and functional analysis by its binding with full length HIV-1 gp120 via SPR. Results show the predominant β–sheet (CD) as secondary structural content and native folding via red shift (tryptophan fluorescence). The single chain fragments have shown good binding with HIV-1 gp120 thus validating the expression and refolding strategy and also reinstating *E.coli* as model expression system for recombinant antibody engineering. SPR based binding analysis coupled with *E.coli* based expression and purification will have implication for HIV therapeutics and will set a benchmark for future studies of similar kind.

1. Introduction

Antibodies (Ab) are the glycoproteins from immunoglobulin (Ig) superfamily [32,40]. These are large Y-shaped proteins produced from plasma cells and are responsible for the immune defence against invading pathogens such as bacteria and viruses. There are innumerable strategies used by an antibody to kill the pathogens like neutralization, agglutination, opsonization, complement activation and antibody dependent cell mediated cytotoxicity (ADCC) etc. These constitute majority of the gamma globulin fraction of the blood proteins. Each gamma globulin consists of two large heavy and two light chains. Huge heavy chain diversity shown by antibodies is due to five different types of crystallisable fragments (Fc) attached to the antigen-binding sites. Y shaped arm contain sites for antigen, i.e. Fab (fragment antigen-binding) region or paratope, which is specific to an epitope. The Fab region is composed of a constant (C) and a variable (V) region from heavy and light chain of an antibody [39]. The variable domain is the F_V region crucial for binding to antigens.

Variable regions, each from heavy (V_H) and light (V_L) chains of immunoglobulins constitute a single-chain variable fragment (**ScFv**) connected via a small linker peptide of 10–25 amino acids through N/C termini. These linkers are predominant in glycine that provides flexibility and serine or threonine that improves solubility [18,19]. An immunoglobulin can be engineered by the removal of constant region and introduction of linker without affecting the specificity of parent immunoglobulins [35]. These single chain variants of antibody were designed to study phage display to express the fab as a peptide. ScFv can be synthesized by sub-cloning heavy and light chains derived from a hybridoma. ScFvs have potential applications in flow cytometry, immunohistochemistry, and as fab for artificial T cell receptors. ScFvs can be expressed using bacterial expression system, i.e. *E. coli*. This is in contrast to the monoclonal antibodies (mAb) that require mammalian cell culture for expression [35].

The advancement in recombinant antibody technology has revolutionized the genetic manipulation of antibody fragments thus elucidating the structural and functional mechanism of immunoglobulins [26]. Initial attempts to produce recombinant antibodies were met with mixed results due to the issues of folding and precipitation of polypeptides [10]. This has renewed the focus on utilizing only relevant parts of the antibody molecule like Fab or Fv fragments for expression based studies instead of using the whole antibody. The introduction of phagemid vectors has greatly revolutionized the antibody engineering. These vectors have facilitated the soluble antibody secretion into the periplasm due to its oxidizing environment leading to correct disulphide formation between the antibody domains [4]. This was further improvised by introducing 10–25 amino acid (glycine, serine,

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threonine) long linker resulting in enhanced flexibility and solubility [7,19].

Skerra and Pl¨uckthun [42] have pioneered the use of *E. coli* bacterial expression system for producing efficient antibody fragments. However, tendency to form aggregates, stability over longer periods, relatively lower affinity of ScFvs over ScFab were still the issues required to be addressed [6,30]. Reasons could be the molecular size, native pairing between two different polypeptides via a disulphide bond, proper folding and assembly of Fab fragments in the periplasm of *E. coli* with reduced efficiency compared to ScFvs [43]. Additionally, Fab fragments have the tendency to form homo-dimers via its light chains, also known as 'Bence Jones' proteins. This leads to decreased solubility and reduced level of expression of Fab fragments in *E.coli* [24,33]. However, Fab fragments are more stable over longer periods [27]. A single-chain Fab (scFab) was constructed recently, and appeared to be compatible with phage display system and soluble expression in *E. coli. Cleland et al., ($year$)* [11,28]. Useful features of ScFv and Fab, i.e. high level of expression and prolonged stability can be combined together generating ScFab that might be useful for antibody display for yeast display technology.

In the present communication, single chain variants of ScFv and ScFab were derived from b12, a broadly neutralizing (bNAb) anti-HIV-1 antibody [46]. Therefore, these fragments will be designated as b12ScFv and b12ScFab throughout the text. These engineered antibody fragments are very significant for HIV based studies and for developing anti-HIV vaccines or drugs particularly epitope based peptide subunit vaccine. Expression of these antibody fragments in *E. coli* leads to the formation of inclusion bodies resulting in inactive protein.

E. coli has emerged as a precious tool for expressing cellular proteins due to well characterized physiology, genetics, rapid growth, high yield of recombinant proteins, easy and optimized molecular biology protocol, low cost, and ease of multiplexing expression screening and protein production. These features have made it invaluable tool for proteomics based cellular, molecular biological and immunological research [3,17,25,29,31,45]. Skerra and Pluckthun (1988) and Pluckthun [38] have reviewed the protocols to express the recombinant antibody fragments. Foremost being the direct expression of ScFv in *E.coli* cytoplasm lacking a signal peptide [7,9]. However, expression of protein in the reducing environment of cytoplasm results in the formation of inclusion bodies that required renaturation to impart native folding / structure to ensure proper function [20]. Periplasmic space however, contains chaperones and disulphide isomerases that aid in the native folding of engineered proteins [3]. Mutation of hydrophobic residues to the hydrophilic ones, length of the linker connecting the variable domains, and composition of amino acids in linker peptide may improve the folding of the recombinant proteins [1,34].

Surface plasmon resonance (SPR), which is employed in the experiment of current manuscript is a powerful technique to analyze the kinetics of label-free biomolecular interactions in real time. This technique provides quantitative measurement of reaction kinetics and affinity constant of biomolecular interactions [14,37]. The biosensing protocols involve receptor immobilization on the surface of a sensor chip followed by injecting analyte (ligand / protein of interest), which is allowed to flow over sensor surface leading to binding. This binding alters the mass of the surface layer which translates into refractive index variation and resonance angle shift. Plotting the variation in resonance angle as a function of time gives the association and dissociation constant values, which is used to calculate the affinity constant [12,47]. In the realms of clinical immunology, this technique monitors real-time analysis of binding of unlabeled antigen with antibody without disrupting the structures of binding partners. Additionally, the amine-coupling permits immobilization with a controlled orientation for the antigens leading to enhanced detection sensitivity [23,41]. SPR technique could also be utilized for biomarker development, quantization of blood group antibodies and as point-of-care (POC) diagnostic devices [23,48].

The current article envisages to test the structural and functional efficacy of refolded proteins, i.e. how efficient are refolded proteins in retaining or retrieving the original native structure and hence the function after being denatured. This could also provide an insight into the effectiveness of protein refolding strategies since, soluble ScFabs are predisposed to dimerize and multimerize [11,28]. It was also the purpose of the study to resolve if the soluble ScFabs would display increased levels of binding due to the effects of avidity.

2. Material and methods

2.1. Vectors, transformation, expression and affinity purification

b12 is one of the bNAb against HIV-1. It usually binds to a binding site on CD4 (CD4bs) on gp120 glycoprotein of HIV-1 coat protein. Single chain fragments of b12 were cloned into the vector pET22b(+) and pComb3X for its expression in *E. coli* in order to obtain soluble b12ScFv and b12ScFab. These cloned antibody fragments were kindly provided by International AIDS vaccine initiative consortium (IAVI), USA.

Constructs, b12ScFv and b12ScFab were transformed into *E. coli* BL21 (DE3) competent cells and expressed under the control of the T7 promoter at 37 °C. Cells were grown in 1 L of Luria-Broth with ampicilin as marker at 37 °C to the OD_{600} of 0.6–0.8 followed by induction with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration) and pelleted by centrifugation at 5000 × *g* for 15 min after 6–8 h of induction at 37 °C.

2.2. Isolation and solubilization of inclusion bodies

Pellets were solubilized in resuspension buffer (50 mM Tris/pH 7.4 /150 mM NaCl/1 mM EDTA/100 mM PMSF) and sonicated 3 times with 30 s pulse on ice, and centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant was discarded and pellet was washed (thrice) with washing buffer (50 mM PBS/pH 7.4/0.5% Triton X-100) and centrifuged at 9000 × *g* for 30 min at room temperature. Isolated inclusion bodies were solubilized with 8 M GdnHCl in 50 mM Tris–HCl buffer at pH 7.4 containing 100 mM DTT, 1 mM oxidised Glutathione and 10 mM reduced Glutathione and incubated under stirring condition overnight at room temperature. The resulting solution was centrifuged at 15,000 × *g* for 30 min.

The supernatant was bound to 3 ml Ni-NTA affinity matrix resin (GE Healthcare), washed with 30 ml 50 mM imidazole containing 8 M Guanidine hydrochloride in Tris-HCl, pH 7.4 and finally denatured protein was eluted with 8 M GdnCl in Tris-HCl, pH 7.4 containing 500 mM imidazole at room temperature.

2.3. Refolding of antibody fragments (b12ScFv and b12ScFab) from inclusion bodies

The first four eluted fractions (each 3 ml) were pooled together and then rapidly diluted by rapid dilution (10 fold) in 0.1 M cold Tris-HCl, pH 7.4 having 1 mM oxidised Glutathione, 10 mM reduced Glutathione along with 0.5 M Arginine-HCl to reduce the denaturant concentration from 8 M to 0.8 M. The resulting solution was again concentrated back to the original volume in an Amicon concentrator. Purified protein fractions were dialyzed in cold 0.1 M Tris-HCl, 1 mM EDTA, pH 7.4 at 4 °C inside cold room 3-times each for 3 h to remove the arginine. Dialyzed proteins were collected and flash frozen in liquid nitrogen (−80 °C) in aliquots of 200 µl and stored at −80 °C for future studies.

2.4. PAGE & estimation of protein concentration

Purity of proteins were confirmed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using an electrophoresis gel unit from Bangalore Genei, Bangalore, India. The protein

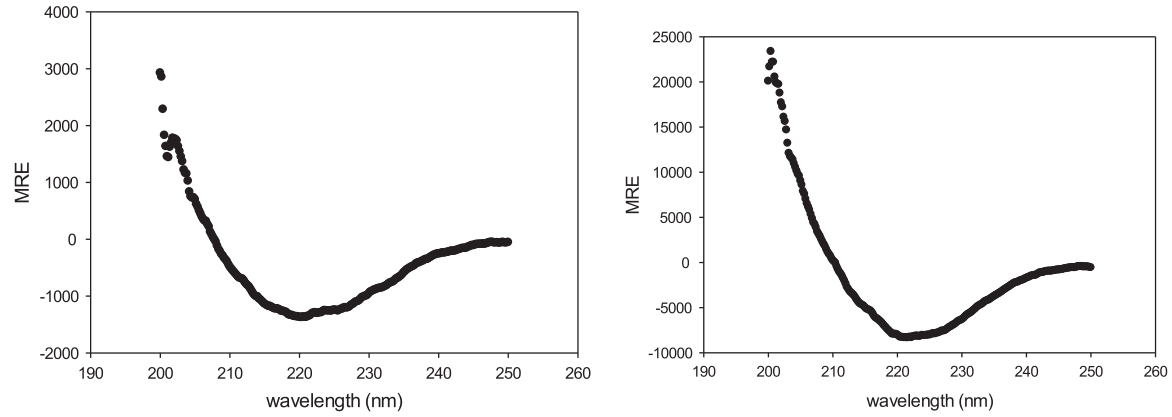

Fig. 1. Far-UV CD spectrum of b12ScFv and b12Scfab. The spectrum was buffer corrected and was obtained at 25 °C with 10 μM of protein in 10 mM Tris HCl buffer, pH 7.4 with a 0.1 cm path length cuvette, a scan-rate of 50 nm/min, a response time of 4 s and a bandwidth of 2 nm. Data reported are averaged over 3 scans.

bands on the gel were visualized using Coomassie blue stain. Protein concentration was estimated by CD (MRE), explained in following section.

2.5. Circular dichroism (CD) measurements

Far-UV (200–250 nm) circular dichroism spectroscopy acts as a good marker for determination of secondary structural contents of proteins [2]. Circular dichroism (CD) spectra were recorded on a Jasco J-715C spectropolarimeter flushed with nitrogen gas. Measurements were recorded in a 1 mm path length quartz cuvette with a scan rate of 50 nm/min, response time of 4 s and a bandwidth of 2 nm. Each spectrum was an average of three scans. The concentration of protein sample used was 10 μM and buffer used was 10 mM Tris–HCl, pH 7.4. Buffer spectra were also acquired under similar conditions and subtracted from protein spectra, before analysis. Mean residue ellipticities (MRE) in deg cm^2 $dmol^{-1}$ as a function of wavelength were calculated as described in Kelly et al. Kelly et al., ($year$) [22].

$$[\theta]_{\mathbf{MRE}} = \frac{\mathbf{MRW} \times \theta_{obs}}{\mathbf{10} \times \boldsymbol{d} \times \boldsymbol{c}}$$

where MRW is the mean residue weight obtained from the formula M/N-1, where M is the molecular weight in dalton of the polypeptide chain and N is the number of amino acids residues in polypeptide chain; obs is the observed ellipticity expressed in degrees; d refers to the path length measured in centimeter, and c is the protein concentration (g ml^{-1}).

2.6. Fluorescence measurements

Tryptophan fluorescence spectra were recorded at 25 °C on a JASCO FP-6300 spectrofluorimeter. For intrinsic fluorescence measurements, protein concentration used was 1 μM. The excitation was at 280 nm and emission was recorded from 300 to 400 nm. The excitation and emission slit widths were 3 and 5 nm, respectively. Each spectrum was an average of three scans. Buffer spectra were also acquired under similar conditions and subtracted from protein spectra before analysis.

2.7. SPR experiments

All surface plasmon resonance (SPR) experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden) optical biosensor at 25 °C. 800–900 resonance units (RU) of *Bal* strain (subtype B) derived full-length HIV-1 gp120 was immobilized on sensor surface of CM5 chip by amine coupling. A sensor surface (without gp120 or any antibody) that has been activated and deactivated served as a negative control for each binding interaction. wt gp120, b12ScFv and b12ScFab in varied concentrations were allowed to flow over sensor surface in a running buffer of phosphate-buffered saline (PBS, pH 7.4) containing 0.01% P20 surfactant. The protein concentrations ranged from 245 nM to 1.86 μM for b12ScFv and 925 nM to 1.23 μM for b12ScFab. The association and dissociation were measured for 300 s. In each case, sensor surface was regenerated by repeated washing with 10 mM HCl and 10 mM NaOH for 30 s at the rate of 30 μl/min each. In each case binding curve was corrected by subtracting the signal collected from negative control flow cell, i.e. nonspecific binding. Data were fitted to simple 1:1 Langmuir interaction model using BIA Evaluation 3.1 software to obtain the kinetic parameters.

3. Results

SDS-PAGE (15%) was utilized to ascertain the purity of the proteins. Molecular mass of the proteins were determined correctly by ESI-MS. The size of b12ScFv and b12ScFab came around 27 kDa and 50 kDa respectively.

3.1. Circular dichroism (CD) measurements

CD was done to check if the refolded proteins have any secondary structural contents in terms of α-helix or β-sheet. CD spectra showed secondary structure with high β-sheet content having a characteristics single dip at 220 nm and typical V shape for both the fragments. The secondary structure in form of β-sheet is characteristic of these antibody fragments (Fig. 1) [13].

3.2. Fluorescence emission spectra

Once it was confirmed that the antibody fragments have typical secondary structure, Tryptophan fluorescence was done to see if these secondary structures can impart these fragments their native folded structure. The fluorescence emission spectra of b12ScFv and b12ScFab are given in Fig. 2. Fluorescence emission spectra of native protein in buffer showed a λ_{max} at 340 nm for both b12ScFv and b12ScFab, which is similar, a characteristic of native structure. Similar λ_{max} (emission) exhibited by the proteins show that the proteins have adopted correctly folded native-like state. Incubating refolded proteins in 8 M GdnHCl leads to a characteristic red shift that corresponds to 350 nm for b12ScFv and 355 nm for b12ScFab. For, b12ScFv the red shift was accompanied by decrease in fluorescence intensity while for b12ScFab, a slight increase in the fluorescence was observed. This observation is due to the exposure of tryptophan residues from the quenched environment in an unfolded state [2].

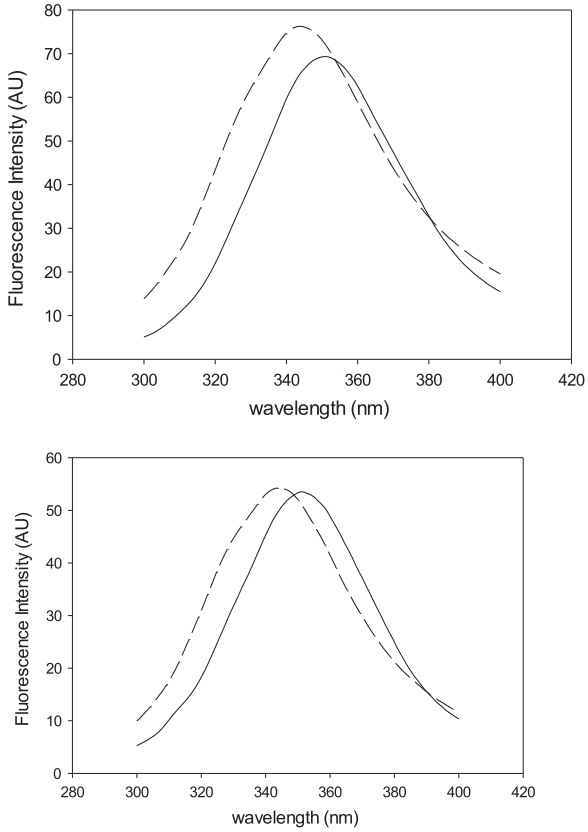

Fig. 2. Fluorescence emission spectrum of native and unfolded b12ScFv (left) and b12ScFab (right). The spectrum was obtained at 25 °C with a final protein concentration of 5 μM in 10 mM Tris HCl buffer, pH 7.4 (dashed line) or in presence of 8 M Guanidine Hydrochloride in 10 mM Tris HCl buffer, pH 7.4 (solid line). Each spectrum was buffer corrected. The excitation was at 280 nm and emission was recorded from 300 to 400 nm.

3.3. Binding kinetics studies via SPR

The functionality of the refolded antibody fragments were analyzed through binding to full length HIV-1 gp120. Binding study was done by measuring the K_D via SPR (Biacore) using full length immobilized HIV-1 gp120 in vitro (Figs. 3 and 4). A comparison of all the kinetic parameters for binding is listed in Table 1. The K_D values calculated for b12ScFv was (116 nM) and for b12ScFab (68 nM). This confirms the findings from earlier studies that ScFab show better binding (1.5 fold higher) with full length immobilized HIV-1 gp120 as compared to b12ScFv (Table 1).

4. Discussion

The envelope protein gp120 is the only part of the HIV that can be recognized by our immune system. gp120 makes the first interface with human CD4 receptor cells through its spikes. Since gp120 is a large protein, it displays potentially large number of epitopes most of which are non-neutralizing. b12 is a broadly neutralizing antibody (bNAb) and sera from rabbit primed with variants of b12, i.e. b122a followed by boosting with full length gp120 neutralized 16 virus panels [5]. Therefore, binding activity of single chain fragments of b12, i.e. ScFv and ScFab are crucial in anti HIV-1 therapy.

4.1. Protein purification and refolding

The protein was expressed in *E. coli* BL21(DE3) cells and purified on a Ni-NTA affinity matrix after resolubilization from inclusion bodies. SDS-PAGE studies confirmed that the protein was at least 90% pure.

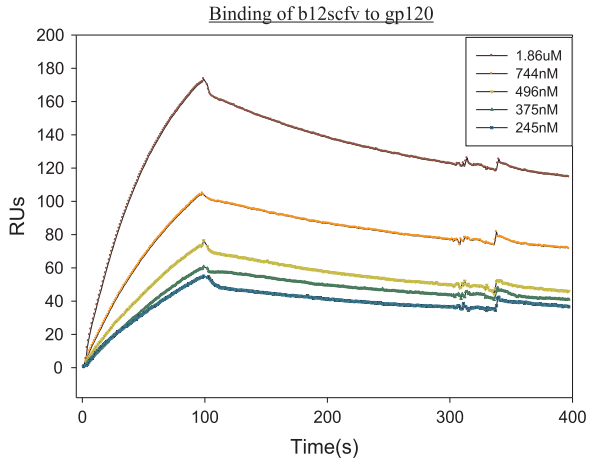

Fig. 3. Biacore 2000 Sensorgram overlays for the binding kinetics of different concentrations of refolded b12 ScFv to surface-immobilized HIV-1 gp120. Curves 1, 2, 3, 4, and 5 indicate 245, 375, 496, 744, and 1860 nM concentrations of b12 ScFv, respectively. Surface density: 1000 resonance units; buffer: 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% P20; flow rate: 30 μl/min; temperature: 298 K.

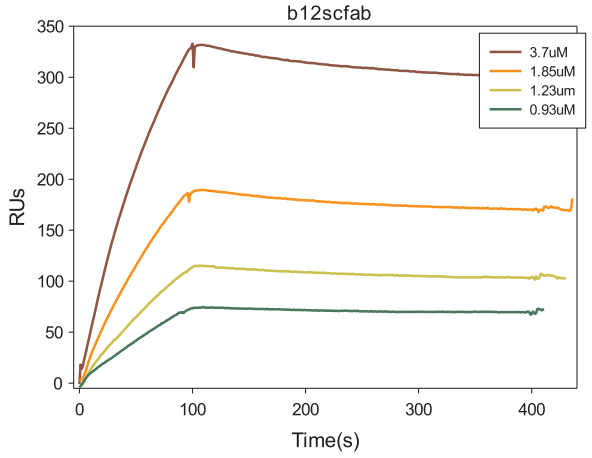

Fig. 4. Biacore 2000 Sensorgram overlays for the binding kinetics of different concentrations of refolded b12 ScFab to surface-immobilized HIV-1 gp120. Curves 1, 2, 3, 4, and 5 indicate 245, 375, 496, 744, and 1860 nM concentrations of b12 ScFv, respectively. Surface density: 1000 resonance units; buffer: 10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005% P20; flow rate: 30 μl/min; temperature: 298 K.

Table 1
Kinetic parameters for binding of refolded b12ScFv and b12ScFab to full-length HIV-1 gp120 by surface plasmon resonance.

Ligand	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
b12ScFv	4.3×10^4	5×10^{-3}	116
b12ScFab	1.04×10^5	7.1×10^{-3}	68

Mass spectrometric analysis (ESI-MS) confirmed the identity of the protein.

Arginine hydrochloride was added to the refolding buffer as it is a well-known stabilizing osmolyte which is known to help in refolding of proteins by destabilizing expanded states like the unfolded state of the protein [8,44]. We attempted to modify the refolding protocol by using arginine hydrochloride in the dilution step to reduce the denaturant (GdnHCl) concentration. The remaining quantity of GdnHCl was further removed by dialysis in the presence of arginine, and finally arginine was completely removed by dialysis.

4.2. Biophysical characterization

Both the constructs showed an expected red shift and a change in emission intensity upon denaturation, showing that the protein is likely to be folded with burial of some tryptophan residues in the native state. CD spectrum of the arginine refolded protein showed a characteristic spectrum of a beta-sheet. This showed that the Arginine-HCl as an osmolyte helped in taking up a better secondary structure for the antibody fragments.

4.3. Binding experiments with SPR

Both refolded antibody fragments, b12ScFv and b12ScFab can neutralize HIV-1 [46]. A protein with appreciable secondary structure and proper native folded structure should perform the assigned function. SPR experiments were done to analyze the kinetics and strength of binding of antibody fragments with full length immobilized HIV-1 gp120. Both fragments show nanomolar value of K_D. ScFab show better binding (1.5 fold higher) with full length immobilized HIV-1 gp120 as compared to b12ScFv. This is surprising as both fragments bind to identical residues in the gp120 binding site. Only in few cases ScFvs have shown to bind with elevated affinity than the linked Fab [21]. The plausible reasons might be the multimerization that enhances the affinity for solube Scfabs. Multimerization may also lead to higher saturation signal for b12 and 4E10 ScFabs when compared to their Fab counterpart.

Secondary structure mediated folding and stability which determines protein function is largely decided by link between V_H/V_L and C_{H1}/C_L interfaces for Fab fragments and stability of these domain interactions enhance antigen bindings. C_{H1}/C_L interface in ScFv improves the flexibility of binding pocket that leads to increased entropic cost to binding [15,16,36].

5. Conclusion

Present communication establishes that appreciable expression and yield of recombinant antibodies can be achieved using *E.coli* expression system. The refolding strategy also worked well with these fragments as deduced by structure analysis by CD spectroscopy and folding by fluorescence spectroscopy. Finally, binding with good affinity to full length gp120 analyzed via SPR has generated hope for further improvising these fragments for HIV-1 therapy.

An antibody in scFv (single chain fragment variable) having variable regions of heavy (V_H) and light (V_L) chains joined together through a peptide linker can be expressed in functional form in *E.coli*. This will facilitate protein engineering to improvise the properties of scFv (single chain fragment variable) such as increase of affinity and alteration of specificity. Further, an antibody design combining stability and assay compatibility of Fab fragments with high level bacterial expression of single chain Fv fragments would be desirable. The desired antibody fragment should be both suitable for expression as soluble antibody in *E. coli* and antibody phage display.

It remains to be seen whether any further mutations in this construct can help it to fold better and thereby improve binding with other bNAbs including b12, CD4 and VRC01. Improved versions can be generated via yeast display or other selection technologies.

Acknowledgements

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.04.005.

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