

Original Article

Tailoring the binding properties of SpA Ig binding domains by *in vitro* molecular evolution

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Abstract: Background: *Staphylococcus aureus* protein A (SpA) is a bacterial immunoglobulin (Ig)-binding protein (IBP) and has fundamental applications in medical and biological sciences associated with IgG. In addition to its high affinity for IgG Fc, SpA also has a low affinity for the VH3 regions of IgG, IgM and IgA Fab, which may complicate its IgG applications. Methods: To diminish its VH3 binding potential and preserve the Fc binding potential, the amino acids which are involved in the interaction with VH3 at positions 29 and 30 in SpA A domain and at positions 36 and 37 in SpA C domain were randomly mutated respectively, meanwhile, a combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA was constructed. Then, a combination of A_{L29I30}-A_{V29K30} was generated by *in vitro* molecular evolution using human IgG as bait. Results: The binding assays demonstrated that comparable to that of its equivalent (A-A), A_{L29I30}-A_{V29K30} exhibited affinity for IgG, but diminished affinity for IgM and IgA. Horse-radish peroxidase (HRP) conjugated A_{L29I30}-A_{V29K30} presented enhanced IgG binding potential and improved effects for antibody detection against HIV core antigen in serum. Further, A_{L29I30}-A_{V29K30} affinity chromatography recovered purified IgG in amount comparable to that for SpA but recovered no detectable IgM and IgA, thus demonstrating the former's application advantages. Conclusions: This study demonstrates a successful example of functional protein engineering via *in vitro* molecular evolution and provides a useful approach to remold the Ig binding property of SpA for application purposes.

Keywords: NEIBM, IgG, phage-based molecular evolution, antibody detection, antibody purification

Introduction

Bacterial immunoglobulin (Ig)-binding proteins (IBPs) specifically bind to immunoglobulins (Igs) and play important roles in the pathogenicity of bacteria [1]. Protein A of *Staphylococcus aureus* (SpA) [2], protein L of *Finnegoldia magna*, formerly *Peptostreptococcus magnus* (PpL) [3], and protein G of groups C, G streptococci (SpG) [4], are three well-defined IBPs. Among them, SpA with a molecular weight of 57 kDa comprises 524 amino acid residues [5]. SpA has a tandem repeat sequence that containing five highly homologous IgG-binding domains, which named (from the N terminus) E, D, A, B and C. Each domain comprises of approximately 58 amino acid residues with a characteristic secondary structure consisting of three up-down

α -helices that mediate the binding with IgG Fc region [6]. In addition, SpA also binds to a fraction of Ig molecules other than IgG Fc, for instance, the VH3 heavy chains in Fab region of Igs [7-9]. Crystal structure studies have shown that SpA B domain binding with Fc mainly involves residues in helix I and less involves in helix II [10, 11], whereas D domain interacting with VH3 of Fab involves residues in helix II and helix III [5].

SpA and other IBPs have fundamental applications in medical and biological area, e.g. diagnostic antibody detection, antibody purification, immunoprecipitation assays, and immunoadsorption therapy [12-15]. Recombinant monoclonal antibodies have become important biological pharmaceuticals and are widely used as

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research reagents. SpA-based purification, as a key purification step, is the main part of the raw-material costs [16, 17]. In addition to the specific interaction with the Fc region of IgG during antibody purification, SpA may also bind to the Fab domain of the VH3-clan of IgG, IgM and IgA. Therefore, this VH3 binding potential might disturb the purification of recombinant monoclonal antibodies and lead to an inaccurate specific detection of the IgG antibody responses against pathogens by false detection of the IgM antibodies. Developing SpA-derived IgG binding proteins with preserved IgG Fc binding potential and diminished VH3 binding potential remains an interesting research topic.

Numerous novel combinations of Ig-binding domains of SpA, SpG and PpL that do not exist in natural bacterial IBPs were generated by *in vitro* molecular evolution. These molecules are named as newly evolved Ig-binding molecules (NEIBM) and exhibit novel Ig-binding properties [18-22]. For example, NEIBM LD5 and LD3 not only could interact with human Ig Fab in the VH3 and Vk regions via a double-binding method, but also show a high affinity for human IgM [19]. Additionally, the horse-radish peroxidase (HRP) conjugated LD5 has better IgM detection sensitivity in the ELISA assay for anti-HCV and improves antibody detection in anti-HIV and anti-EV71 VP1 ELISA assays [20, 23, 24]. Additionally, NEIBM D-C-G3, with potential simultaneous tri-site binding for some IgG subclasses, is generated through *in vitro* molecular evolution as well [21].

In the present study, the amino acids which are involved in the interaction with VH3 at positions 29 and 30 of SpA A domain and those at positions 36 and 37 of SpA C domain, were randomly mutated, and a combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA was constructed. A NEIBM combination, A_{L29I30}-A_{V29K30}, was obtained by *in vitro* molecular evolution using human IgG as bait, which preserved the IgG Fc binding potential and diminished the IgM and IgA binding potential.

Materials and methods

Ethical approval

All aspects of the study on the forty anti-HIV-positive human serum samples were approved

by the Ethics Committee of Beijing You An Hospital, Capital Medical University, China; the written informed consent was provided by all subjects in the study [23]. And all aspects of the study on the forty anti-HIV-negative human serum samples were approved by the Ethics Committee of Changhai Hospital, Shanghai, China; oral informed consent was provided by all subjects in the study.

Vectors, reagents and serum samples

The phagemid vector pCANTAB5S was constructed in advance in our laboratory. The prokaryotic expression plasmid, pET-32a (+), and *E. coli* host strains Top10, were purchased from Novagen Company (Darmstadt, Germany), and *E. coli* TG1 was obtained from Stratagene Company (Cambridge, England). All the primers in this study were synthesized by the Sangon Biotech (Shanghai, China). The horseradish peroxidase (HRP)-conjugated anti-M13 antibody and helper phage M13K07 were obtained from Pharmacia Biotech (Uppsala, Sweden). Human IgG (hIgG), human IgM (hIgM) and human IgA (hIgA) were purchased from Sigma (St. Louis, MO, USA).

Forty anti-HIV-positive human serum samples were collected from the AIDS high-risk cohort at YouAn Hospital in Beijing, China [23]. Forty anti-HIV-negative human serum samples were collected from healthy blood donors at Changhai Hospital, Shanghai, China. Upon receipt, all samples were aliquoted and stored at -80°C. Referring to the related study, the seropositive status of the subjects was confirmed using Western blotting (HIV Blot 2.2 WB, MP Biomedicals Asia Pacific Pte. Ltd., Singapore) and ELISA (Diagnostic Kit for Antibody to HIV (ELISA), Shanghai Kehua Bio-Engineering Co., LTD., China) [23].

Construction of combinatorial phage libraries

The combinatorial phage library displaying randomly-rearranged A domain with randomly mutated amino acids at positions 29 and 30 and C domain with randomly mutated amino acids at positions 36 and 37 of SpA, respectively, was constructed through polymerase chain reaction (PCR)-based random mutagenesis technology using primers listed in [Table S1](#). *Xba* I restriction sites were introduced at both ends of the fragments of the mutated A and C domain. Then, the combinatorial mutat-

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Table 1. The names, mutational sites and evaluation of the combinatorial phage libraries

Phage display libraries	Name	Mutational amino acid sites of SpA A domain	Mutational amino acid sites of SpA C domain	Transformation efficiency (cfu)	Titre (TU/ml)
Library 1	A ₂₉ , A ₃₀ and C ₃₆ , C ₃₇ site-directed randomly mutational phage display library	29.30	36.37	5.3*10 ⁶	1.8*10 ¹²
Library 2	A and C randomly combinatorial phage display library	None	None	3.6*10 ⁶	1.6*10 ¹²

ed phage library was constructed by digesting the PCR products with *Xba* I and ligating into the phagemid pCANTAB5S on the *Xba* I site (library 1, **Table 1**).

Gene fragments with similarly *Xba* I restriction sites that encode the SpA A and C domains were respectively synthesized by PCR with primers listed in **Table S2**. Then, the combinatorial phage library displaying various randomly-rearranged A and C domains was built with above mentioned method (library 2, **Table 1**).

After amplification in *E. coli* Top10, the recombinant phagemids were transformed into the host bacterial strain TG1. Helper phages M13-K07 (approximately 1.8*10¹¹ transformation unit (TU)) were added when the optical density (OD) value at 600 nm was about 0.5, and cultured in the shaker at 230 rpm for 1 h at 37°C. Subsequently, kanamycin (15 µg/ml) was added and the cells kept culturing overnight. After the centrifugation at 5,000 g for 10 min, the supernatant was collected and the phages were harvested by a 0.22 µm filter. Then, the combinatorial phage display libraries displaying the randomly-rearranged A and C domains of SpA were obtained (**Table 1**). The primer pairs pCANTAB5S-1 (5'-CAACGTGAAAAAATTATTATTCGC-3') and pCANTAB5S-6 (5'-GTAATGAATTTTCTGTATGAGG-3') were applied for the amplification and sequencing analysis of inserted fragments for the positive phages [22].

In vitro molecular evolution of the libraries by hlgG molecule

Dilute the hlgG to the concentration of 10 µg/ml in 0.1 M NaHCO₃ (pH9.6), then add to 96-well ELISA plates for incubating at 37°C for 3 h. Then, block the plates with 0.01 M phosphate buffered saline (PBS) containing 10% of skimmed milk, 0.1% of Tween 20 and 0.2% of

mercuriothiolate (blocking buffer) for 3 h. Add the constructed phage-displaying libraries into the plates and incubate at 37°C for 2 h. Wash each well with PBS containing 0.1% of Tween 20 for 10 times to remove the unbound phages. When the OD at 600 nm was about 0.5, 100 µl of the *E. coli* TG1 was added and the plates were incubated at 37°C for 1 h. Take 10 ml infected TG1 cells to culture in tryptone-yeast extract (2X) plates containing ampicillin (100 µg/ml) and then, calculate the number of colony forming units. After growing with helper phages M13K07 (approximately 1.8*10¹¹ TU) overnight, the residual cells were cultured in 2X tryptone-yeast extract medium (8 ml) containing ampicillin (100 µg/ml). After the centrifugation at 5,000 g for 10 min, the supernatant was collected and the phages were harvested by a 0.22 µm filter for the next selection round with the hlgG as well. The selection was performed 3-6 rounds totally.

A total of 22 phage clones that randomly picked from each primary library and each round of the post-selection libraries were cultured with 2X tryptone-yeast extract medium (1 ml) at 37°C overnight. The primers pCANTAB5S-1 and pCANTAB5S-6 were applied for PCR amplification and analysis of the inserted fragments of phages. The empty plasmid pCANTAB5S was used as negative control for the evolution [22].

Phage ELISA test

Screening the hlgG binding phages in post-selection libraries by ELISA: The recombinant phagemids in the post-selection library 1 and library 2 were transformed into the *E. coli* TG1, respectively. Subsequently, select ninety clones from each library randomly and then culture them by shaking at 230 rpm at 37°C overnight. With the helper phages M13K07 (about 1.8*10¹¹ TU), monoclonal phages were selected out from the last round from each post-

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selection library and added to plates after coating with 10 µg/ml of hlgG as mentioned above, and the plates were incubated at 37°C for 2 h. After washing with the solution containing 0.25% of Tris, 0.05% of Tween 20, HRP-conjugated anti-M13 phage antibody was applied to detect the bound phages. Then, 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA) and H₂O₂ were adopted as the substrate for HRP, and the absorbance at 450 nm was measured with an ELISA Reader. Plasmid pCANTAB5S-phage was applied as a negative control [22].

Sequence analyses: A total of 10 positive phage clones with the highest OD_{450nm} were sequenced with the primers pCANTAB5S-1 and pCANTAB5S-6 by JIE LI Biology Company (Shanghai China). The sequences of target amino acid were deduced on the basis of DNA sequencing result, and the DNASTAR software package was adopted of the multiple sequence alignment analysis.

Prokaryotic expression, purification, and binding analysis of A_{L29I30}-A_{V29K30} and A-A

Expression and purification of A_{L29I30}-A_{V29K30} and A-A: With the primer pairs U-AA-BamH and D-AA-Sal, the representative phagemids, including A_{L29I30}-A_{V29K30} and A-A, were used as templates to amplify DNA fragments by PCR using primers in Table S3 respectively. After the amplification, the target DNA was inserted into the plasmid pET-32a (+) and the result was examined via DNA sequencing. The proteins expression of A_{L29I30}-A_{V29K30} and A-A was mediated by isopropyl-beta-D-thiogalactopyranoside (1 mmol/L) in *E. coli* BL21 (DE3). After sonication for 30 min, the proteins were collected and purified by a Ni-NTA column (Amersham Pharmacia Biotech).

ELISA test: The ELISA test was conducted as previously described [22]. Briefly, the immunoassay strips (Nunc, Rochester, NY, USA) were coated with the purified A_{L29I30}-A_{V29K30} and A-A at a concentration of 1 µg per well that diluted by 0.1M NaHCO₃ (pH9.6) and incubated at 37°C for 3h. After blocking with blocking buffer for 3h, a fold-dilution of biotin-labeled, IgG, IgM and IgA (0.1 mg/ml) was added and incubated at 37°C for 45 min respectively. The reactive compounds were detected with HRP-conjugated streptavidin (2 µg/ml, Sigma, St. Louis, MO,

USA), and the absorbance at 450 nm was measured with an ELISA Reader.

Application of A_{L29I30}-A_{V29K30} and A-A in anti-HIV ELISA test

HRP labeling: Using sodium periodate, HRP-labelled A_{L29I30}-A_{V29K30} (HRP-A_{L29I30}-A_{V29K30}) and HRP-labelled A-A (HRP-A-A) were prepared as follows [22]. Briefly, a mixture with 5 mg/ml of HRP and 200 µl of 0.1 M sodium periodate was stirred about 20 min in the dark surrounding and dialyzed against 1 mM sodium acetate buffer (pH4.4) at 4°C overnight. Then 1 ml of 5 mg/ml A_{L29I30}-A_{V29K30} and A-A in PBS (pH7.2) was added immediately after adding 20 µl of 0.2 M carbonate buffer (pH9.5). Keep gently stirring for 2 h in the dark surrounding, 100 µl of sodium borohydride solution (4 mg/ml) was added and reacted for 3h at 4°C. Finally, the HRP-A_{L29I30}-A_{V29K30} and HRP-A-A were dialyzed against 0.01M PBS (pH7.2) at 4°C overnight and stored at -20°C [22].

Test of binding activity of HRP-A_{L29I30}-A_{V29K30} and HRP-A-A: The ELISA plates were coated with hlgG, hlgM and hlgA respectively (1 µg per well) using 0.1 M NaHCO₃ (pH9.6) and were incubated at 37°C for 3 h. After blocking with skimmed milk, 100 µl of serial double-diluting solutions of HRP-A_{L29I30}-A_{V29K30} and HRP-A-A (1 mg/ml) were added to each well which was then incubated at 37°C for 45 min. The plates were developed by TMB and the absorbance at 450 nm was measured by ELISA Reader.

Detection of anti-HIV antibody: To detect the anti-HIV antibody using HRP-A_{L29I30}-A_{V29K30} and HRP-A-A, the immunoassay strips were coated with 1 µg of the recombinant HIV core antigen (CA, GenBank accession No. AAC82593.1). HIV CA was inserted into the cloning sites of a pET-32a (+) vector under the control of the T7 promoter. A His tag was added at the N-terminus of the target protein to form a fusion protein. The strips were incubated at 37°C for 3 h, and then blocked as above. Then, 100 µl of 10-fold dilutions of the forty anti-HIV-positive human serum samples were added for binding reaction at 37°C for 45 min. Meanwhile, the forty anti-HIV-negative human serum samples were set as negative control. After washing four times with washing buffer, 100 µl of a 1000-fold dilution of HRP-A_{L29I30}-A_{V29K30} (1 mg/ml) and HRP-A-A (1 mg/ml) was added and incubated at

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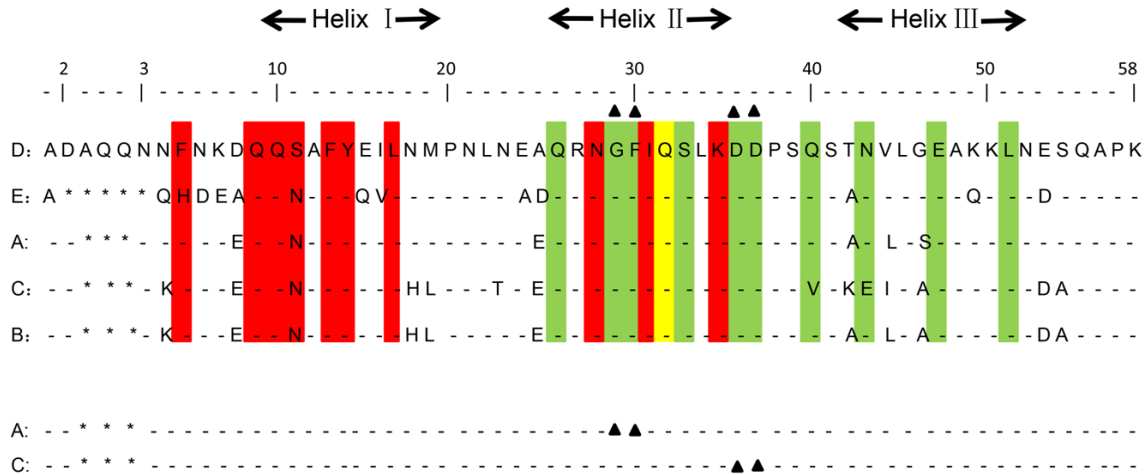


Figure 1. Alignment of the amino acid sequences of the five SpA domains and the mutation sites of SpA A domain and SpA C domain. The SpA A domain residues involved in the interaction with Fab are highlighted in green, and the A domain residues mediating Fc binding are highlighted in red with the overlapping residue Gln-32 (highlighted in yellow). The mutation sites of A and C domains are marked by solid triangles.

37°C for 45 min. The strips were developed with TMB and the absorbance at 450 nm was measured by ELISA Reader.

Biosensor analyses

The binding properties of $A_{L29I30}-A_{V29K30}$ and A-A to hIgG, hIgM, and hIgA were studied by surface plasmon resonance (SPR) using the BioRad XPR36 protein interaction array system. Briefly, hIgG, hIgM and hIgA (diluted in 10 mM sodium acetate, pH4.5) were coupled to a GLC ProteOn sensor chip using amine-coupling chemistry according to the manufacturer's instructions. The association and disassociation condition were measured with serial 1:5 dilutions of $A_{L29I30}-A_{V29K30}$ and A-A with the concentrations of 1.6 nM, 8 nM, 40 nM, 200 nM, 1 μ M and 5 μ M. The flow rate was set as 100 μ l/min using PBST (pH7.4, 0.005% Tween 20) as flow buffer. The sensor-chip surfaces were regenerated by using 10 mM Glycine-HCl (pH-2.0). K_A (affinity constant) = k_a (association rate constant)/ k_d (dissociation rate constant) [21].

Affinity chromatography

The $A_{L29I30}-A_{V29K30}$ coupled-column was made by immobilizing 30 mg of the $A_{L29I30}-A_{V29K30}$ fusion protein onto 3 ml sepharose column (Amersham, Uppsala, Sweden) according to the manufacturer's instructions. Then, 2 ml of human serum was diluted in PBS (1:8) and then appli-

ed on the 3 ml $A_{L29I30}-A_{V29K30}$ coupled-column or SpA coupled-column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) respectively at room temperature. Subsequently, wash the columns with extensive PBS (pH7.0), elute the bound proteins using 100 mM sodium acetate (pH3.0) and collect the approximately 4 ml of the eluted proteins respectively, and dialyze the proteins against PBS (pH7.0). Ten-microliter of the eluted proteins were analyzed by 12% SDS-PAGE using the purchased hIgG, hIgM, and hIgA as control [21].

Data analysis

All the experiments were repeat no less than three times in each triplicate ($n = 9$) and performed independently. The data were analyzed with one-way ANOVA or Student's *t*-test. *P* value < 0.05 was considered significant.

Results

In vitro molecular evolution of the combinatorial phage library displaying randomly-rearranged mutated A and C domains of SpA

To eliminate the VH3 binding activity, the amino acids at positions 29 and 30 of SpA A domain and the amino acids at positions 36 and 37 of SpA C domain, which both interact with VH3 of Igs, were randomly mutated (**Figure 1**) [22]. A combinatorial phage library displaying the randomly-rearranged mutated A and C domains

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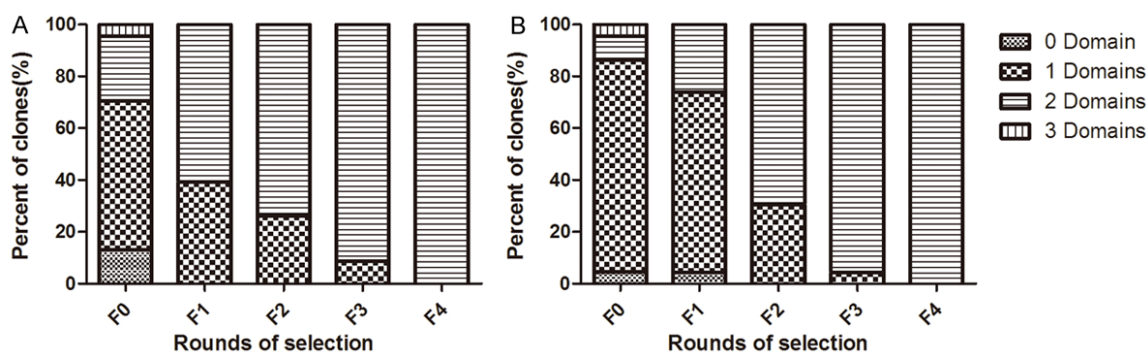


Figure 2. Proportion of phage clones with different sizes of inserted fragments from the 22 phage clones after each round of selection with hlgG. (A): Library 1; (B): Library 2.

of SpA (library 1) was constructed and subjected to *in vitro* molecular evolution with the bait hlgG. As a control, the combinatorial phage library displaying randomly-rearranged A and C domains of SpA without any mutation (library 2) was also constructed and subjected to *in vitro* molecular evolution as well. The library 1 had 5.3×10^6 members, and the titre of the phage library was 1.8×10^{12} TU/ml (**Table 1**). The library 2 had 3.6×10^6 members, and the titre of the phage library was 1.6×10^{12} TU/ml (**Table 1**). The capacity of the two established libraries satisfied the needs of the subsequent *in vitro* molecular evolution.

To check the randomness of nucleotides in the mutation sites in A and C domain of SpA from library 1, eighty clones from the original library 1 were randomly selected and primers pCANTAB5S-1 and pCANTAB5S-6 was adopted for the PCR amplification of the inserted fragments of phages [22]. The results are as follows: there were three phage clones displaying three domains, twenty phage clones displaying two domains, forty-six phage clones displaying one domain and eleven phage clones with no inserted fragment. Among them, then twenty phage clones displaying two domains were chosen for sequencing analysis. The rearrangements of A and C domain of SpA in the twenty phage clones were as follows: three A-A combinations, two A^R-A combinations (R refers to reverse complementary sequence), two A-A^R, two C-C^R combinations, two C^R-C^R combinations, two A-C combinations, two A-C^R combinations, two A^R-C combinations, one C^R-C combination, one C-A^R combination and one C^R-A^R combination. Hence, the twenty phage clones included 22 A domains and 18 C domains. In

the A domain, the sequence analyses indicating that the A:T:C:G ratio in the NNS mutations of the amino acids at both positions 29 and 30 was 11:10:11:12 for the first base N, 10:13:11:10 for the second base N, and the C:G ratio was 21:23 in the third base S. In the C domain, the A:T:C:G ratio in the NNS mutations of the amino acids at both positions 36 and 37 was 9:8:9:10 for the first base N, 7:8:11:10 for the second base N, and the C:G ratio for the NNS mutation was 19:17 in the third base S. In summary, the variety and randomness of the library 1 satisfied the needs of the subsequent *in vitro* molecular evolution.

As observed in the previous studies [18, 22], the distribution of the inserted fragment sizes showed remarkable change throughout the *in vitro* evolution of both libraries in this study (**Figure 2**), which indicated an effective evolution. As a result, the ratio of phage clones displaying two domains was less than 30% in the original library, and increased to 100% during four rounds of selection of both libraries.

Analysis of IgG binding activity of phage clones in the post-selection populations

Ninety monoclonal phages from each of the two post-selection libraries were prepared respectively to evaluate the binding activity with hlgG via ELISA. As shown in **Figure 3**, phage clones exhibited different binding activities, and ten phage clones with the highest hlgG binding from each of the two post-selection libraries were chosen for sequencing analysis. All ten phage clones from the library 1 that displayed randomly-rearranged mutated A and C domains showed the same NEIBM combination

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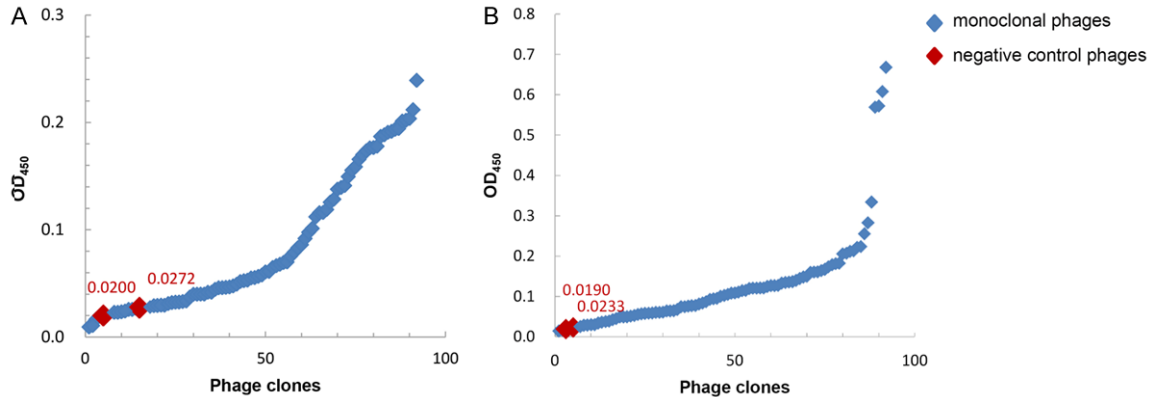


Figure 3. Detection of IgG binding activities of the phages clones in post-selection population through phage ELISA. (A): Library 1; (B): Library 2.

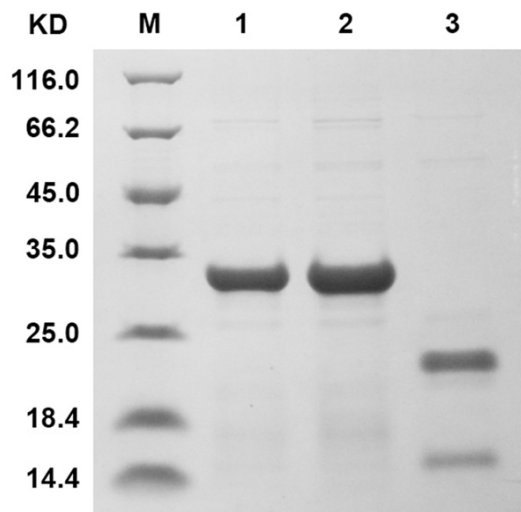


Figure 4. The SDS-PAGE of purified protein. M: protein molecular weight marker, including 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa markers; lane 1: A-A; lane 2: $A_{L29I30}-A_{V29K30}$; lane 3: pET-32a (+).

of $A_{L29I30}-A_{V29K30}$ and all ten phage clones from the library 2 also showed the same NEIBM combination of A-A.

The Ig binding properties of NEIBM $A_{L29I30}-A_{V29K30}$

$A_{L29I30}-A_{V29K30}$ and A-A were expressed and purified as fusion proteins using pET-32a (+) expression vector to compare their Ig binding activities (Figure 4). ELISA analysis showed that $A_{L29I30}-A_{V29K30}$ and A-A exhibited comparable binding activities with hIgG. In contrast to the remarkable binding activities of A-A with hIgM and hIgA, $A_{L29I30}-A_{V29K30}$ presented no hIgM and

hIgA binding activities (Figure 5). Consistent with these results, the SPR data (Table 2) also demonstrated that $A_{L29I30}-A_{V29K30}$ showed hIgG binding potential which was comparable to that of A-A but its hIgM and hIgA binding potential was diminished.

Improved hIgG binding potential and anti-HIV detection effect of HRP- $A_{L29I30}-A_{V29K30}$

The conjugates of HRP- $A_{L29I30}-A_{V29K30}$ and HRP-A-A were produced, and their binding activities with hIgG, hIgM, and hIgA were compared. To our surprise, HRP- $A_{L29I30}-A_{V29K30}$ exhibited significantly enhanced binding activities with hIgG compared with those of HRP-A-A (Figure 6). Then, we compared the detection effects of the HRP- $A_{L29I30}-A_{V29K30}$ and HRP-A-A in a panel comprising of forty anti-HIV-positive human serum samples from HIV patients and forty anti-HIV-negative serum samples from healthy blood donors, respectively. As shown in Figure 7, both HRP- $A_{L29I30}-A_{V29K30}$ and HRP-A-A showed the same detection effects for negative serum samples, whereas the HRP- $A_{L29I30}-A_{V29K30}$ -based assay presented significantly improved detection effects for positive serum samples compared with HRP-A-A ($P < 0.001$).

$A_{L29I30}-A_{V29K30}$ affinity chromatography recovered pure IgG from human serum

To investigate whether $A_{L29I30}-A_{V29K30}$ has an application advantage in IgG purification, the purification efficiency of affinity chromatography columns made from $A_{L29I30}-A_{V29K30}$ or SpA was compared. As shown in Figure 8, the $A_{L29I30}-A_{V29K30}$ affinity column recovered the compara-

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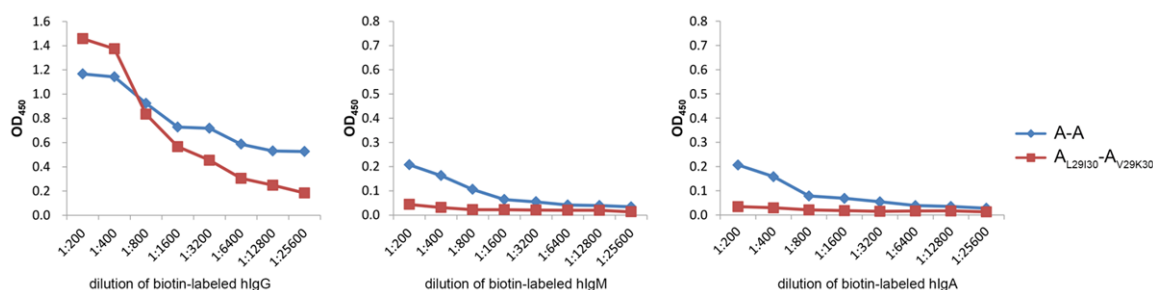


Figure 5. Binding activities of $A_{L29I30}-A_{V29K30}$ to hIgG, hIgM and hIgA compared to A-A according to ELISA analysis. The plates were coated with purified $A_{L29I30}-A_{V29K30}$ and A-A, and 1:2 serial dilutions of biotin-labeled hIgG, hIgM and hIgA were incubated in the wells. The reactive complexes were detected using horseradish peroxidase (HRP)-conjugated streptavidin.

Table 2. Surface plasmon resonance analysis of the interactions between $A_{L29I30}-A_{V29K30}$ or A-A and IgG, IgM or IgA

Ligand	Constant	Analyte	
		$A_{L29I30}-A_{V29K30}$	A-A
IgG	k_a ($M^{-1}s^{-1}$)	1.00×10^5	1.53×10^5
	k_d (s^{-1})	2.86×10^{-4}	3.61×10^{-4}
	K_A (M^{-1})	3.50×10^8	4.24×10^8
IgM	k_a ($M^{-1}s^{-1}$)	6.56×10^{-3}	1.71×10^3
	k_d (s^{-1})	5.91×10^{-5}	1.99×10^{-5}
	K_A (M^{-1})	1.11×10^2	8.59×10^7
IgA	k_a ($M^{-1}s^{-1}$)	10.8	1.08×10^4
	k_d (s^{-1})	1.51	2.34×10^{-4}
	K_A (M^{-1})	7.15	4.62×10^7

ble amount of IgG antibodies from human serum as that recovered by SpA affinity chromatography. Additionally, no IgM and IgA antibodies were recovered by the $A_{L29I30}-A_{V29K30}$ affinity column, whereas they were recovered by SpA affinity chromatography. The data indicated that $A_{L29I30}-A_{V29K30}$ had the obvious advantage in IgG purification.

Discussion

SpA, which is a natural Igs binding protein, especially for IgG, has fundamental applications in IgG antibody diagnostic detection, antibody purification, immunoprecipitation assays and immunoabsorption therapy. Crystal studies have shown that each SpA binding domain has two different binding interfaces: one is the Fc binding interface, which is located in helix I and helix II and interacts with one of Fc sites in two IgG heavy chains; the other one is the VH3 Fab binding interface, which is located in helix II and

helix III and interacts with the VH3 chain in one Fab of IgG, IgM and IgA [10, 11]. Meanwhile, SpA contains a tandem repeat of five highly homologous Ig-binding domains and thus presents a number of binding avidity models by using some of the five Fc binding interfaces and the five VH3 binding interfaces. Among these potential binding models, the two-site Fc binding mode (Fc-Fc, two Fc binding interfaces from two Ig-binding domains of SpA simultaneously bind with two Fc binding sites on one IgG molecule) is the key binding model for IgG and is utilized for the purification and specific detection of IgG. The VH3 binding interfaces may produce a low affinity for Fab of IgG, IgM and IgA, and may thus complicate the applications that are specific for IgG. For example, when a natural SpA chromatography was used for the purification of recombinant IgG consisting of VH3, besides the Fc-Fc binding model, it provided a number of other binding models for a single IgG molecule, such as VH3-Fc, VH3-Fc-Fc, VH3-Fc-Fc-VH3, VH3-VH3 and VH3-VH3-Fc. Namely, these models could recover IgG with different conformations along with that recovered by the Fc-Fc binding model, to generate heterologous IgG conformations, and thus to complicate IgG purification. Based on this consideration, the residues involved in VH3 binding, at positions 29 and 30 of SpA A domain and at positions 36 and 37 of the C domain, were chosen to randomly mutate to diminish the VH3 binding potential and preserve the Fc binding potential.

In theory, proper combinations of the two SpA binding domains are necessary to produce the Fc-Fc binding avidity, and all four types of combinations, A-A, A-C, C-C and C-A, may produce

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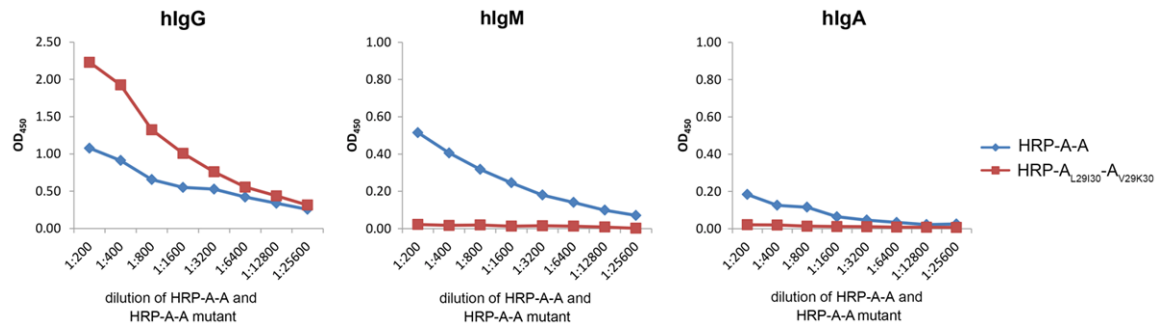


Figure 6. Detection of the binding activities of HRP-A_{L29I30}-A_{V29K30} and HRP-A-A with hlgG, hlgM and hlgA. The plates were coated with hlgG, hlgM and hlgA. 1:2 serial dilutions of A_{L29I30}-A_{V29K30} and HRP-A-A were incubated in each well. Binding was detected by the addition of TMB.

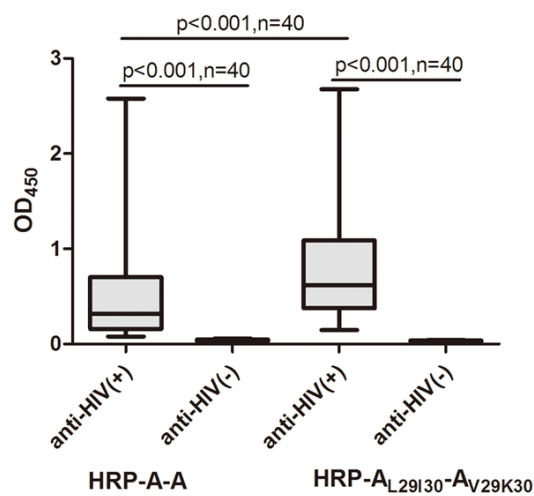


Figure 7. Comparison of the detection effects of HRP-A_{L29I30}-A_{V29K30} and HRP-A-A in anti-HIV ELISA. The strips were coated with HIV core antigen recombinant protein. One hundred-microliter of 10-fold dilutions of the forty anti-HIV-positive human serum samples and forty anti-HIV-negative human serum samples were separately added. One hundred-microliter of 1000-fold dilutions of HRP-A_{L29I30}-A_{V29K30} and HRP-A-A were added, and the plates were incubated. The strips were developed upon the addition of TMB and detected at 450 nm on an ELISA Reader.

Fc-Fc binding avidity. To our surprise, in our study, only the A-A combinations (A_{L29I30}-A_{V29K30} and A-A) were selected by *in vitro* molecular evolution of two phage libraries, which indicated that the A-A combinations possess some advantages in generating the perfect Fc-Fc binding avidity compared to other combinations. Interestingly, only the mutated A-A combination, A_{L29I30}-A_{V29K30}, was selected from the phage library displaying the randomly-rearranged mutated A and C domain, which should contain the A-A combination, indicating that the

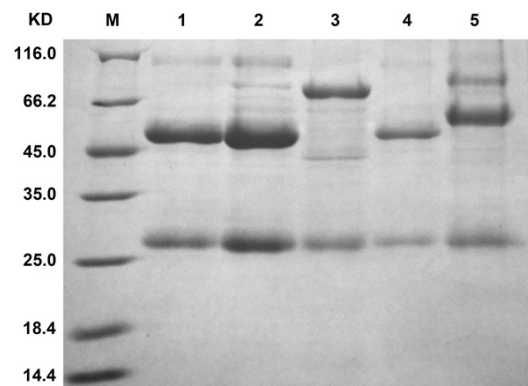


Figure 8. Comparison of IgG purification effects of human serum with A_{L29I30}-A_{V29K30} and SpA affinity chromatography through SDS-PAGE analysis. M: protein molecular weight marker; lane 1: human serum purified by A_{L29I30}-A_{V29K30} affinity chromatography; lane 2: human serum purified by SpA affinity chromatography; lane 3: hlgM; lane 4: hlgG; lane 5: hlgA.

mutations at positions 29 and 30 of A_{L29I30}-A_{V29K30} should favor Fc binding. In the present study, our strategy of *in vitro* molecular evolution only guaranteed the selection of phage clones with the strongest Fc binding potential but did not guarantee the selection of phage clones with eliminated VH3 binding potential. Hence, the elimination of VH3 binding potential of A_{L29I30}-A_{V29K30} is not the result of *in vitro* molecular evolution but is an incidental consequence of the mutations that favor IgG binding. A fine-resolution map of the sequence function landscape of computational designed IgG binding protein FcB6 revealed that substitution of any of the core residues involving Fc binding are usually depleted and that most of the substitutions of any of the other residues are allowed or not allowed, part of them are depleted, and only few are favored [25]. In this

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study, considering the randomly mutated residues at positions 29 and 30 are adjacent to the residues at positions 28, 31, and 32, which are involved in Fc binding, the substitutions at positions 29 or 30 are likely not allowed, or depleted, and few are favored. The combined substitutions at both positions should have less chance to generate the mutants that favor Fc binding. However, on the other hand, the simultaneous substitutions at positions 29 and 30 could possibly compensate for their individual negative effects, and increased the chance to generate the mutants which benefit Fc binding. In this study, only one mutant, $A_{L29I30}-A_{V29K30}$, which has combined substitutions at positions 29 and 30 both in A domain, was obligatorily selected and favor Fc binding. This finding should be helpful for designing mutations of targeted amino acids of functional domains or proteins to achieve successful protein engineering via *in vitro* molecular evolution.

Unexpectedly, HRP-labeled $A_{L29I30}-A_{V29K30}$ showed obviously improved IgG binding activity compared to HRP-labeled A-A (**Figure 6**), whereas the binding assays clearly demonstrated that $A_{L29I30}-A_{V29K30}$ and A-A showed comparable IgG binding activities (**Figure 5**). Our explanation for these results is as follows. The SpA binding domains adopt different residues to interact with Fc and VH3, respectively. The residues involved in Fc binding are primarily located in helix I, and less involves in helix II [10, 11], whereas the residues involved in VH3 binding are located in helix II and helix III [5]. The substitutions of residues at positions 29 and 30 in $A_{L29I30}-A_{V29K30}$, which are involved in VH3 binding and located in the second and third turns of helix II, respectively, possibly induce conformation adjustment, and the neighboring residues at positions 28, 31 and 32 in helix II, which are involved in Fc binding, could be affected. Interestingly, the residue at position 29 in wild type A domain is G, which is conservative in all five domains of SpA and has a strong propensity for breaking the α -helical structure. In contrast, all the substituted amino acids in $A_{L29I30}-A_{V29K30}$, V, K, L and I, have a strong or medium propensity for forming α -helical structure. These substitutions may contribute to the stability of helix II and therefore benefit the Fc binding of neighboring residues at positions 28, 31 and 32 in helix II. The reinforced helix II in $A_{L29I30}-A_{V29K30}$ could also confer more resis-

tance to the HRP labelling than wild-type SpA and may thus contribute to the improved IgG binding. This result revealed that *in vitro* molecular evolution may have more substantial effects on protein property than those that we designed only according to their binding property. This finding, together with our previous finding [22], might have a significant impact on protein engineering via *in vitro* molecular evolution to improve binding activity and application potential.

The NEIBM, $A_{L29I30}-A_{V29K30}$, with preserved IgG binding potential and diminished IgM and IgA binding potential, demonstrated some application advantages. In contrast to natural SpA, the $A_{L29I30}-A_{V29K30}$ affinity column recovered pure IgG without the contamination of IgM and IgA from human serum. This provides a novel affinity chromatographic medium with a simple IgG binding mode which could favor the purification of IgG antibodies. How $A_{L29I30}-A_{V29K30}$ acts in the purification of recombinant IgG drug production remains an interesting question. With enhanced IgG binding potential, the HRP- $A_{L29I30}-A_{V29K30}$ -based ELISA exhibited a much better detection effect for anti-HIV core antigen than HRP-A-A-based ELISA, which implied the former's application advantage in the detection of specific IgG antibodies, for the diagnosis of infections by pathogenic organisms. Moreover, $A_{L29I30}-A_{V29K30}$ may contribute to the improvement of detection of specific IgM antibody responses against various pathogens by absorbing IgG from serum without any loss of IgM, which is usually absorbed by natural SpA, and eliminating competitive antigen binding between IgG and IgM.

In this study, a new NEIBM, $A_{L29I30}-A_{V29K30}$, with preserved IgG binding potential and diminished IgM and IgA binding potential was obtained through *in vitro* phage-based molecular evolution, and it showed substantial application advantages in IgG purification and detection. This study demonstrates a successful example of functional protein engineering via *in vitro* molecular evolution and provides a useful approach to remold the Ig binding property of SpA for application purposes.

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Disclosure of conflict of interest

None.

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Table S1. Primers for the amplification of DNA fragments encoding the mutants of A and C domains of SpA

Name	Description	Sequence (5'-3')
UA1-AX-1	Forward amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a GCTGACAACAATTTCAAC
DA1-A1-1	Reverse amplifying primer	ATCTTTTAAGCTTTGGAT SNNSNN ^b ATTGCGTTGTTCTTCGTT
UA2-A1-2	Forward amplifying primer	ATCCAAAGCTTAAAAGATGACCCAAGTCAAAGT
DA2-AX-2	Reverse amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a TTTCGGTGCTTGAGATTC
UC1-CX-1	Forward amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a GCTGACAACAATTTCAAC
DC1-C1-1	Reverse amplifying primer	TTCTTTGCTCACTGAAG SNNSNN ^b TTTAAGGCTTTGGATGAA
UC2-C1-2	Forward amplifying primer	CCTTCAGTGAGCAAAGAAATTTTAGCAGAAGCT
DC2-CX-2	Reverse amplifying primer	TCCTCAGACGCCGTACCTGCTCTAGA ^a TTTTGGTGCTTGAGCATC

Note: ^aThe restriction sites are underlined, *Xba* I cutting site is "TCTAGA"; ^bNucleotide sequences of randomly mutational peptide are in bold (N = A/T/C/G, S = G/C).

Table S2. Primers for the amplification of DNA fragments encoding the A and C domains of SpA

Name	Description	Sequence (5'-3')
UA1-AX-1	Forward amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a GCTGACAACAATTTCAAC
DA2-AX-2	Reverse amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a TTTCGGTGCTTGAGATTC
UC1-CX-1	Forward amplifying primer	TCGTCAGACGCCGTACCTGCTCTAGA ^a GCTGACAACAATTTCAAC
DC2-CX-2	Reverse amplifying primer	TCCTCAGACGCCGTACCTGCTCTAGA ^a TTTTGGTGCTTGAGCATC

Note: ^aThe restriction sites are underlined, *Xba* I cutting site is "TCTAGA".

Table S3. Primers for the DNA sequences amplification of A_{L29I30}-A_{V29K30} and A-A

Name	Description	Sequence (5'-3')
U-AA-BamH	Forward amplifying primer	CGCTCGGGATCC [*] GCCCAGCCGGCCTCT
D-AA-Sal	Reverse amplifying primer	GTGGGCGTCGAC [*] CTAAGGCCTGAGCTCTCT

Note: ^{*}The restriction sites are underlined, *Bam*H I cutting site is "GGATCC", *Sal* I cutting site is "GTCGAC".