



# A new method for multilayered, site-directed immobilization of antibody on polystyrene surface



Bo Feng<sup>a,\*</sup>, Caiyun Wang<sup>a</sup>, Xiaomei Xie<sup>a</sup>, Xi Feng<sup>a</sup>, Yuqin Li<sup>a</sup>, Zhijian Cao<sup>b</sup>

<sup>a</sup> College of Chemical Engineering, Xiangtan University, Xiangtan 411105, Hunan Province, China

<sup>b</sup> College of Life Science, Wuhan University, Wuhan 430072, Hubei Province, China

## ARTICLE INFO

### Article history:

Received 23 May 2014

Available online 5 June 2014

### Keywords:

Antibody immobilization

Affinity ligand

Staphylococcal protein A

Immunoglobulin G

Polystyrene surface

## ABSTRACT

Polystyrene is a common substrate material for protein adsorption in biosensors and bioassays. Here, we present a new method for multilayered, site-directed immobilization of antibody on polystyrene surface through the linkage of a genetically engineered ligand and the assembly of staphylococcal protein A (SPA) with immunoglobulin G (IgG). In this method, antibodies were stacked on polystyrene surface layer by layer in a potential three-dimensional way and exposed the analyte-binding sites well. Enzyme-linked immunosorbent assay (ELISA) revealed that the new method showed a 32-fold higher detection sensitivity compared with the conventional one. Pull-down assay and Western blot analysis further confirmed that it is different from the ones of monolayer adsorption according to the comparison of adsorption capacity. The differentiated introduction of functional ligands, which is the key of this method, might offer a unique idea as a way to interfere with the dynamic behavior of a protein complex during the process of adsorption.

© 2014 Elsevier Inc. All rights reserved.

## 1. Introduction

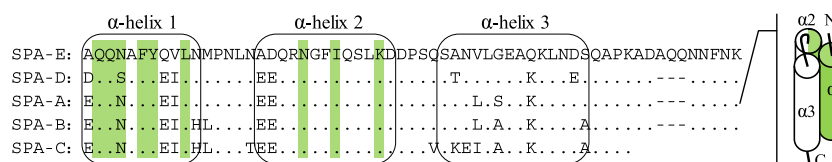
The solid phase antibody used as probe is a key component in immunosensors and immunoassays microarrays, where the molecular recognition of target analyte by immobilized antibody to form a stable antigen–antibody complex and the separation of unbound immunoreagents from the immunocomplex are required [1–4]. In conventional method, antibodies were randomly bound to a certain surface (e.g., 96-well polystyrene microtiter plate employed in enzyme-linked immunosorbent assay) physically or chemically via surface modification (e.g., biotinylated, carboxylated and histidine-tagged) [4–6]. However, it has been found that such immobilized antibodies are active at a rate of only 5–10%, the majority of resident molecules on matrix surface is useless because its Fab fragment, which is indispensable in subsequent molecular recognition, may be denaturated or blocked during the attachment process, which makes them inaccessible to analytes [6,7]. In order to overcome these defects, oriented antibody immobilization was developed via an intermediate molecule (e.g., protein A or G), which binds to Fc fragment of antibody, making the rest of the molecule including Fab fragment exposed well, thus enhancing their accessibility to analytes [8,9]. This type of immobilization method can result in a significantly higher fraction of

active antibodies, but usually suffers from a lower surface density of immobilized antibody since two immobilization steps are required [10,11]. Due to the limitation of two-dimensional (2D) space, the amount of antibody that can be immobilized on planer surface is usually low. To break through the bottleneck, porous substrates and three-dimensional (3D) polymeric networks have been proposed [9,12,13]. Undoubtedly, these could significantly increase the immobilization capacity of antibody than that on 2D substrates. In addition to the sophisticated process of creating a 3D matrix, the major disadvantage is that it is more difficult to change buffers and recover trapped molecules because of the mass transfer limitation [11,14,15].

In this study, we established a new method of omni-directional antibody immobilization on polystyrene surface. First, a 12-mer affinity ligand for polystyrene surface from a phage display random library [16] was genetically recombined to SPA fusion protein. Next, by mimicking the molecular mechanisms of viable cells, two SPA and five IgG molecules can assemble a pod-shaped complex in an anti-parallel model (SPA containing five homologous IgG-binding domains: E, D, A, B and C, and each domain consisting of three  $\alpha$ -helices, as illustrated in Fig. 1) [17–21]. The pod-shaped complex stands on polystyrene surface via the affinity ligand, thereby resulting in the formation of multilayered solid phase antibody in a site-directed manner. A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed for quantifying the active immobilized antibody using this novel

\* Corresponding author. Fax: +86 731 58298172.

E-mail addresses: [fengbo5460@hotmail.com](mailto:fengbo5460@hotmail.com), [fengbo@xtu.edu.cn](mailto:fengbo@xtu.edu.cn) (B. Feng).



**Fig. 1.** Amino acid sequence of SPA's five IgG-binding domains (E, D, A, B and C) and the  $\alpha$ -helix model of a single domain (on the right). The entire sequence of SPA-E is given and identical amino acids in other domains are depicted with a dot (.), while symbol (–) represents a vacancy. Positions of three  $\alpha$ -helices in the domain structure are framed by boxes. Fc-interacting amino acid residues or regions are highlighted in cyan. It is because of the asymmetric distribution of Fc-interacting residues that produce the anti-parallel binding model with IgG.

method. Pull-down assay and Western blot analysis further confirmed that it might be quite different from planar immobilization according to the comparison of adsorption capacity.

## 2. Materials and methods

### 2.1. Preparation of ligand-linked SPA

The coding sequence of SPA (GenBank accession number: J01786.1, nt 292–1209) was cloned into the prokaryotic expression vector pET-21a allowing for bacterial expression of the SPA. An available affinity ligand for polystyrene surface screened by us from a phage display random library, named Lig1 (peptide sequence: KFWLYEHVIRG) [16], was genetically fused to the N/C-terminus of SPA. To construct the plasmid encoding Lig1-SPA (Lig1 fused to the N-terminus of SPA), a *Bam*H I–*Bam*H I fragment was prepared by annealing the sense (5′-GATCC TTC AAA TTC TGG CTA TAC GAA CAT GTA ATA CGG GGG G-3′; the underlined sequence corresponding to Lig1) and the antisense (5′-GATCC CCC CCG TAT TAC ATG TTC GTA TAG CCA GAA TTT GAA G-3′). The annealing procedure was performed by incubating the pair of single-strand DNAs at 95 °C for 15 min followed by its cooling down to 30 °C within 60 min. The *Bam*H I–*Bam*H I fragment was then inserted at the *Bam*H I site of pET-SPA (the resulting plasmid named pET-Lig1-SPA). To construct plasmid encoding SPA-Lig1 (Lig1 fused to the C-terminus of SPA), a *Xho* I–*Xho* I fragment obtained by annealing 5′-TCGAG TTC AAA TTC TGG CTA TAC GAA CAT GTA ATA CGG GGG C-3′ and 5′-TCGAG CCC CCG TAT TAC ATG TTC GTA TAG CCA GAA TTT GAA C-3′ in the same way, was introduced into pET-SPA predigested with *Xho* I (the resulting plasmid named pET-SPA-Lig1). After the recombinant expression plasmids were transformed into *Escherichia coli* BL21 competent cells, the N- and C-terminal Lig1-tagged fusion proteins were induced at 37 °C by using 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG). Next, cells were harvested by centrifugation for 15 min at 5000 $\times$ g, and the pellet was suspended in an equal volume of ice-cold phosphate-buffered saline. After sonication on ice (4 times for 30 s each at 48–56 W power and 30 s pulse in between), the cell homogenate was centrifuged at 15,000 $\times$ g for 15 min. Proteins from supernatant fraction were purified by using Talon metal affinity resin (BD Biosciences) and determined by performing a Western blot.

### 2.2. Antibody immobilization and Hepatitis B surface antigen (HBsAg) ELISA

Two types of specific anti-HBsAg antibodies are employed in HBsAg ELISA: monoclonal antibody for immobilization, and the polyclonal one conjugated to horseradish peroxidase (HRP). Anti-HBsAg monoclonal antibody, and N- and C-terminal Lig1-tagged SPA, at a molar ratio of 5:1:1, were added to a siliconized eppendorf tube with a binding buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% BSA, 1 mM dithiothreitol, 0.05% Nonidet P-40, plus antiproteases) and incubated for 1 h at 4 °C

on a turntable. After resting for another 1 h, this binding solution was added to polystyrene 96-well microplate (Nunc, Denmark) with 100  $\mu$ L per well for protein coating. Plates were incubated for 8 h at 4 °C, the solution was removed and replaced with a washing buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). After rinsing extensively, the plate was ready for detection of target analytes according to the routine protocols.

Load 100  $\mu$ L per well of serially diluted HBsAg into the coated plate, followed by incubation at 37 °C for 0.5 h. After washes with 300  $\mu$ L per well of washing buffer, 100  $\mu$ L of HRP-conjugated anti-HBsAg polyclonal antibody at 1:3600 dilution, was transferred into each well and incubated for another 0.5 h. During the assay, monoclonal and polyclonal antibodies reacted with the existing HBsAg to form an “antibody–HBsAg–antibody–HRP” immune complex. After the unbound conjugates were washed off, tetramethyl benzidine (TMB) was applied to indicate the test result by measuring the absorbance value at 450 nm ( $A_{450}$ ) using a model 550 microplate reader (Bio-Rad, USA). All tests were repeated four times and the arithmetic mean of  $A_{450}$  was calculated.

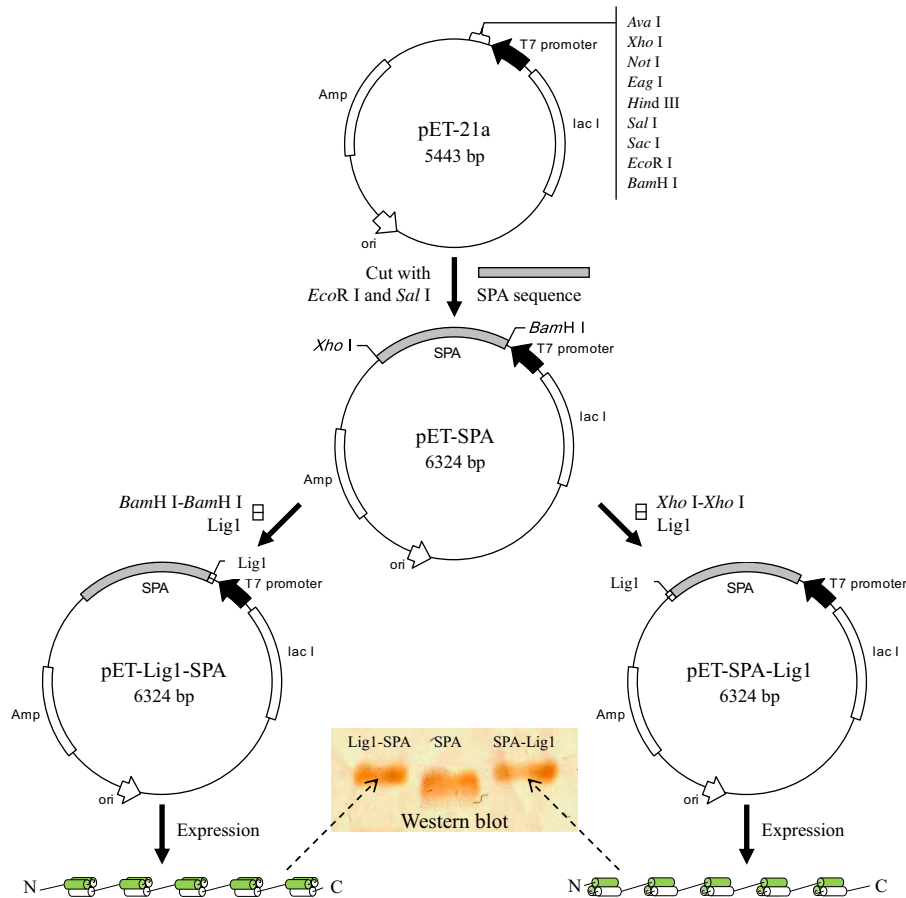
### 2.3. Pull-down assay with polystyrene beads

Polystyrene beads (another form of polystyrene surface, 1  $\mu$ m in diameter; Sigma–Aldrich, USA) were transferred into the binding solution (containing N- and C-terminal Lig1-tagged SPAs, and Monoclonal antibody at a molar ratio of 1:1:5), mixed, and incubated with tumbling for 2 h at 4 °C. After being washed with washing buffer, proteins bound to polystyrene beads were released by boiling in SDS sample buffer and analyzed by a Western blot. For Western blot, proteins were separated in a 10% non-reducing SDS–polyacrylamide gel and then transferred by electroblotting to a polyvinylidene difluoride (PVDF) membrane. The membrane was pre-blocked with dried milk in TBS for 20 min, hybridized with HRP-conjugated anti-mouse IgG antibody (1:4000 diluted in the milk block) for 2 h at room temperature, washed in TBST (TBS containing 0.1% v/v Tween 20) and developed with 3,3′-diaminobenzidine (DAB). Densitometric analysis of the hybridizing bands was done with the software Gel-Pro Analyzer (Media Cybernetics).

## 3. Results and discussion

For the expression of N/C-terminal Lig1-tagged SPA, both pET-Lig1-SPA (encoding Lig1-SPA) and pET-SPA-Lig1 (encoding SPA-Lig1) were constructed from the original plasmid pET-SPA, as illustrated in Fig. 2. Subsequently, the resultant plasmids and the original one (as a control) were transformed into *E. coli* BL21 for protein expression. After purification, expression products were confirmed by Western blot using a monoclonal anti-SPA antibody that can specifically recognize SPA (Fig. 2). We may notice that, compared to the original SPA, the molecular weights of Lig1-SPA and SPA-Lig1 obviously increased with the insertion of Lig1.

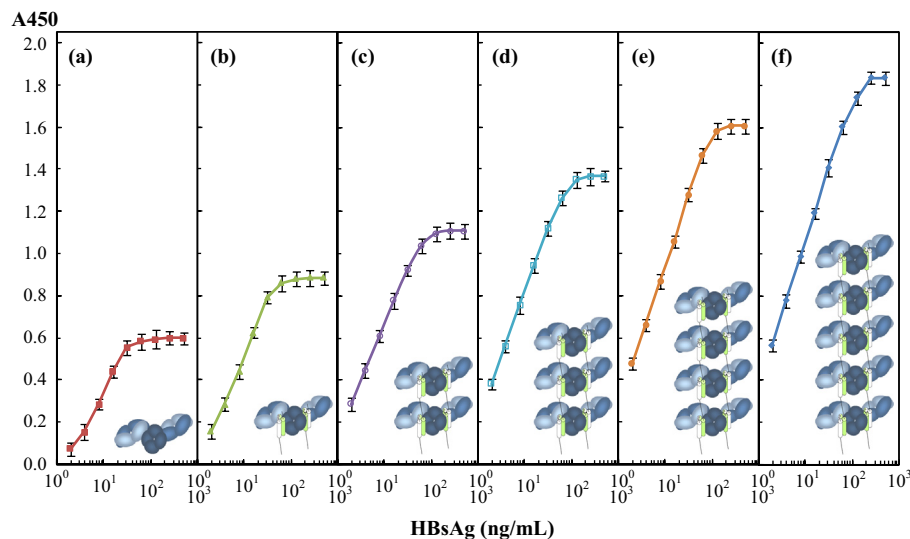
As shown in Fig. 3,  $A_{450}$  values of ELISA detection with the conventional immobilization method (Fig. 3A), in which monoclonal IgG antibodies specific for HBsAg were directly immobilized on



**Fig. 2.** Plasmid construction for N/C-terminal Lig1-tagged SPA (Lig1-SPA/SPA-Lig1) and the Western blot analysis of its expression product. Lig1-SPA and SPA-Lig1 migrate more slowly than the original SPA owing to the insertion of Lig1.

polystyrene microplate, were low, although IgG adsorption was saturated. The new method with SPA (Fig. 3F) showed up to 7.5-fold increased sensitivity over a wide range of HBsAg concentrations (from 2 to 2<sup>9</sup> ng/mL), indicating a much higher analytical

signal strength compared with the conventional method. After a steady rise, the curve in Fig. 3F appeared to level off at an HBsAg concentration of 2<sup>8</sup> ng/mL, while in Fig. 3A about at 2<sup>5</sup> ng/mL, which reflects the amount difference of capture antibody. It must



**Fig. 3.** Performance comparisons of immobilization methods through ELISA signal detection. (A) Monoclonal IgG immobilized on polystyrene microplate with the conventional method. (B–F) Monoclonal IgG immobilized on polystyrene microplate with SPA-E, SPA-ED, SPA-EDA, SPA-EDAB, SPA, respectively. The optimum molar ratio of monoclonal IgG, N- and C-terminal Lig1-tagged SPAs was determined to be 5:1:1. 10 µg/mL of anti-HBsAg monoclonal IgG antibody (served as capture antibody) and 1:3600 diluted HRP-conjugated anti-HBsAg polyclonal antibody (as secondary antibody) were used in (A), (B), (C), (D), (E) and (F). Absorbance values of negative and blank controls (no capture antibody) suggest that (A), (B), (C), (D), (E) and (F) were kept at the same specificity level ( $A_{450} < 0.05$ , data not shown). Mean  $A_{450} \pm SD$  is given ( $n = 4$ ). Schematic representation at the lower right of (A), (B), (C), (D), (E) and (F) provides the deduced immobilization model on polystyrene surface.

be because not enough immobilized IgG was present to capture the excess HBSAg, and the unbound HBSAg was washed away before the HRP conjugate was added. Furthermore, N- and C-terminal Lig1-tagged SPAs with different numbers of IgG-binding domains (SPA-E, SPA-ED, SPA-EDA, SPA-EDAB) were also tested in the same way. As shown in Fig. 3B–F, ELISA signal strength was gradually increased with the increasing number of domains it contains, from one to five. These results indicated that the pod-shaped complex formed by IgG and Lig1-tagged SPA was probably immobilized on polystyrene surface in a multilayered manner as illustrated in Fig. 3. In this case, five IgG molecules were stacked up one by one by the grip of two anti-parallel SPA (the asymmetric distribution of SPA Fc-interacting residues determining its anti-parallel binding model with IgG, as illustrated in Fig. 1). All the active sites located in Fab were uniformly exposed and directed to analytes, rendering them all accessible to target antigenic epitopes, which resulted in a substantial enhancement of detection sensitivity.

Pull-down assay is a well-established approach to confirm direct binding in protein–protein interactions. N/C-terminal Lig1-tagged SPAs containing different numbers of IgG-binding domains (SPA-E, SPA-ED, SPA-EDA, SPA-EDAB and SPA) were used to perform pull-down experiments in the same condition. Fig. 4 showed that all the SPAs can bind to monoclonal IgG. The chromogenic signals of five hybridizing bands (formulated as IOD: 105.7, 342.4, 564.9, 772.7 and 1063.8, respectively), each stronger than its predecessor, indicate the amount of monoclonal IgG pulled down by polystyrene beads via SPAs was gradually increased with the increasing length of SPAs. These results coincide well with those obtained from ELISA.

In this method, the differentiated introduction of Lig1 to SPA, or rather, to N- and C-terminus of SPA respectively, is very important. As illustrated in Fig. 3, Lig1, whether at the N- or C-terminus of SPA, would be located on the same side of the pod-shaped complex due to the anti-parallel binding model. The ideal state, where five IgG molecules are stacked up one by one and all active sites in Fab are uniformly directed to analyte, cannot be achieved when a single type of N- or C-terminal Lig1-tagged SPA is employed (data not shown). Further tests indicated that the immobilization of IgG–SPA may be a dynamic process coordinated by matching activities and binding capacities, involving isochronous interaction of SPA and

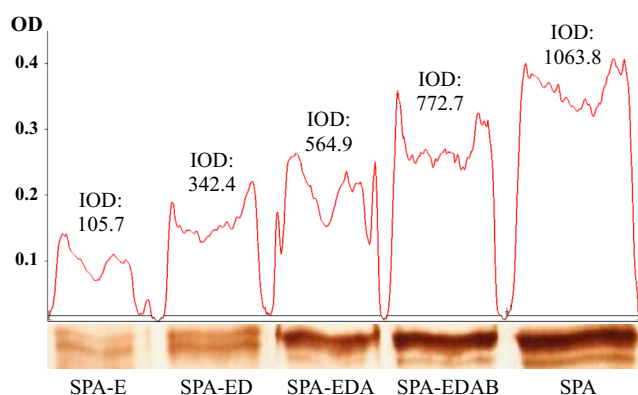
IgG antibodies against the affinity interface (data not shown). As far as we consider, the differentiated design on the introduction of functional ligands, which is the key of this method, can offer a unique idea as a way to interfere with the dynamic behavior of a protein complex during the process of adsorption.

## Acknowledgments

This work is supported by National Natural Science Foundation of China (Nos. 21105085, 31270988), Scientific Research Fund of Hunan Provincial Education Department (No. 13B120) and Doctorial Foundation of Xiangtan University (13QDZ06).

## References

- [1] G. MacBeath, Protein microarrays and proteomics, *Nat. Genet.* 32 (2002) 526–532.
- [2] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces, *Adv. Colloid Interface Sci.* 162 (2011) 87–106.
- [3] H. Zhu, M. Snyder, Protein arrays and microarrays, *Curr. Opin. Chem. Biol.* 5 (2001) 40–45.
- [4] C.K. Dixit, A. Kumar, A. Kaushik, Nanosphere lithography-based platform for developing rapid and high sensitivity microarray systems, *Biochem. Biophys. Res. Commun.* 423 (2012) 473–477.
- [5] J. Lu, G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, T.R. Golub, MicroRNA expression profiles classify human cancers, *Nature* 435 (2005) 834–838.
- [6] S. Chebil, A. Miodek, V. Ambike, H. Sauriat-Dorizon, C. Policar, H. Korri-Youssoufi, Polypyrrole functionalized with new copper complex as platform for His-tag antibody immobilization and direct antigen detection, *Sens. Actuators B* 185 (2013) 762–770.
- [7] I.H. Cho, E.H. Paek, H. Lee, J.Y. Kang, T.S. Kim, S.H. Paek, Site-directed biotinylation of antibodies for controlled immobilization on solid surfaces, *Anal. Biochem.* 365 (2007) 14–23.
- [8] H.Y. Song, X. Zhou, J. Holey, X. Su, Comparative study of random and oriented antibody immobilization as measured by dual polarization interferometry and surface plasmon resonance spectroscopy, *Langmuir* 28 (2012) 997–1004.
- [9] G. Shen, C. Cai, K. Wang, J. Lu, Improvement of antibody immobilization using hyperbranched polymer and protein A, *Anal. Biochem.* 409 (2011) 22–27.
- [10] A. Kausaite-Minkstiene, A. Ramanaviciene, J. Kirlyte, A. Ramanavicius, Comparative study of random and oriented antibody immobilization techniques on the binding capacity of immunosensor, *Anal. Chem.* 82 (2010) 6401–6408.
- [11] K. Nakanishi, T. Sakiyama, Y. Kumada, K. Imamura, H. Imanaka, Recent advances in controlled immobilization of proteins onto the surface of the solid substrate and its possible application to proteomics, *Curr. Proteomics* 5 (2008) 161–175.
- [12] M. Naddaf, A. Al-Mariri, Porous silicon as a platform for immobilization of biotin anti-human IL-6; rat IgG2a antibody onto p-type porous silicon via physical absorption method, *Sens. Actuators B* 160 (2011) 835–839.
- [13] H. Tanaka, T. Isojima, M. Hanasaki, Y. Ifuku, H. Takeuchi, T. Shiroya, H. Kawaguchi, Enhancement of sensitivity of SPR protein microarray using a novel 3D protein immobilization, *Colloids Surf. B* 70 (2009) 259–265.
- [14] H. Zhu, M. Snyder, Protein chip technology, *Curr. Opin. Chem. Biol.* 7 (2003) 55–63.
- [15] V. Romanov, S.N. Davidoff, A.R. Miles, D.W. Grainger, B.K. Gale, B.D. Brooks, A critical comparison of protein microarray fabrication technologies, *Analyst* 139 (2014) 1303–1326.
- [16] B. Feng, Y. Dai, L. Wang, N. Tao, S. Huang, H. Zeng, A novel affinity ligand for polystyrene surface from a phage display random library and its application in anti-HIV-1 ELISA system, *Biologicals* 37 (2009) 48–54.
- [17] D.C. Hanson, V.N. Schumaker, A model for the formation and interconversion of protein A-immunoglobulin G soluble complexes, *J. Immunol.* 132 (1984) 1397–1409.
- [18] K.L. Atkins, J.D. Burman, E.S. Chamberlain, J.E. Cooper, B. Poutrel, S. Bagby, A.T. Jenkins, E.J. Feil, J.M. van den Elsen, S. aureus IgG-binding proteins SpA and Sbi host specificity and mechanisms of immune complex formation, *Mol. Immunol.* 45 (2008) 1600–1611.
- [19] T. Bratkovic, A. Berlec, T. Popovic, M. Lunder, S. Kreft, U. Urleb, B. Strukelj, Engineered staphylococcal protein A's IgG-binding domain with cathepsin L inhibitory activity, *Biochem. Biophys. Res. Commun.* 349 (2006) 441–445.
- [20] C. Aybay, Differential binding characteristics of protein G and protein A for Fc fragments of papain-digested mouse IgG, *Immunol. Lett.* 85 (2003) 231–235.
- [21] T. Kikuchi, Analysis of 3D structural differences in the IgG-binding domains based on the interresidue average-distance statistics, *Amino Acids* 35 (2008) 541–549.



**Fig. 4.** Pull-down assays of SPAs containing different numbers of IgG-binding domains (from left to right: SPA-E, SPA-ED, SPA-EDA, SPA-EDAB, and SPA). N- and C-terminal Lig1-tagged SPAs, and monoclonal IgG antibody at a molar ratio of 1:1:5, were incubated with polystyrene beads in the same condition. Captured proteins were separated by 10% non-reducing SDS–PAGE, hybridized with HRP-conjugated anti-mouse IgG antibody (1:4000 diluted) and stained with DAB. Densitometric analysis of the hybridizing bands was performed with the software Gel-Pro Analyzer. Quantitative data based on the measurement of optical density (OD) and integrated optical density (IOD, representing the average intensity of each band within the range) was illustrated above correspondingly.