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High performance aptamer affinity chromatography for single-step selective extraction and screening of basic protein lysozyme

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ABSTRACT

A DNA aptamer based high-performance affinity chromatography is developed for selective extraction and screening of a basic protein lysozyme. First, a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic column was synthesized in situ by thermally initiated radical polymerization, and then an anti-lysozyme DNA aptamer was covalently immobilized on the surface of the monolith through a 16-atom spacer arm. The target protein lysozyme but non-target proteins can be trapped by the immobilized anti-lysozyme DNA aptamer. In contrast, lysozyme cannot be trapped by the immobilized oligodeoxynucleotide that does not contain the sequence of the anti-lysozyme DNA aptamer. The study clearly demonstrates the trapping of lysozyme by the immobilized anti-lysozyme DNA aptamer is mainly due to specific recognition rather than simple electrostatic interaction of positively charged protein and the negatively charged DNA. The inter-day precision was determined as 0.8% for migration time and 4.2% for peak area, respectively. By the use of aptamer affinity monolith, a screening strategy is developed to selectively extract lysozyme from chicken egg white, showing the advantages of high efficiency, low cost and ease-of-operation.

1. Introduction

Aptamers are single stranded DNA or RNA oligonucleotides that can be screened from a large random combinatorial nucleic acid library by systematic evolution of ligands by exponential enrichment (SELEX) according to their high affinity and specificity for various targets [1–3], such as metal ions [4], organic dyes [1], oligonucleotides [5], amino acids [6], peptides [7], proteins [8], and even cells [9]. Aptamers can fold into three-dimensional shapes with advanced structures, such as hairpin, pseudoknot, stem-loop, G-quartet, which are easily folded into "pocket" structure or allosteric sites to bind their target molecules with high affinity and specificity by the electrostatic interaction, hydrogen bonding, Van der Waals force, and base stacking [10]. Aptamers can rival classical affinity ligands (antibodies, metal ions, dyes, etc.) due to their unique properties, including good stability, non-toxicity, low cost, ease-of-synthesis and -modification, and lack of immunogenicity [11–13]. These merits bring wide applications in many fields such as protein purification [14], enantiomer recognition [15], drug development [16] and target validation [17].

Aptamers can be immobilized for the separation, pre-concentration or detection of biomolecules as aptamer affinity chromatography [18,19]. The aptamers may be immobilized on the inner surface of fused silica capillary as in open-tubular capillary liquid chromatography [20,21], or on the microparticles as in particle packed affinity chromatography, including capillary elelctrochromatography [22–26], capillary liquid chromatography [27,28], microchip [29,14] or HPLC [15,18,30–33]. The immobilization capacity of aptamer is limited in open-tubular capillary liquid chromatography, and the packing of microparticle in column in particle packed affinity chromatography although having high immobilization capacity is labor intensive and troublesome, particularly for capillary chromatography. Due to these limitations, aptamer modified polymer monolith is of choice. The monolithic capillary chromatography technique has the advantages of rapid mass transfer, low back pressure, ease-of-preparation, and larger loading capacity. In such a format, Zhao et al. reported the immobilization of a biotinylated G-quartet DNA-aptamer on a streptavidin-modified poly(trimethylolpropane trimethacrylate-co-glycidyl methacrylate) [poly(TRIM-co-GMA)] polymer monolithic capillary column. They demonstrated that cytochrome *c* and thrombin could be separated from each other and from the non-retained proteins [34]. The achieved separation of cytochrome *c* and thrombin may result from the difference in their recognition of the G-quartet structures. Based on poly(TRIM-co-GMA) monolith, they developed a highly sensitive sandwich chromatographic assay for pre-concentration and detection of

Abbreviations: EDMA, ethylene dimethacrylate; GMA, glycidyl methacrylate; Lys, lysozyme; SELEX, systematic evolution of ligands by exponential enrichment.

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thrombin using two DNA-aptamers, one for the fibrinogen-binding site of thrombin and the other for heparin-binding site, with an improved detection limit of 0.1 nM [35]. In addition, aptamers were also covalently immobilized on magnetic beads through a spacer arm for off-line purification of proteins from complex cell lysates [36,37]. The aptamer affinity supports showed long-term stability and high specificity.

By taking the advantages of ease-of-preparation, large loading capacity of HPLC and fast mass transfer and low back pressure of monolith, we attempted to develop a novel DNA-aptamer high-performance affinity chromatography for online separation and extraction of basic proteins that are easily adsorbed on the solid surface. Lysozyme (Lys), existing extensively in egg white, saliva, tear, urine and serum, was chosen as the model basic protein. Lys was found by Alexander Fleming in 1922 [38,39], which can break cell wall of pathogenic bacteria by hydrolyzing β-1,4 glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of mucopolysaccharide in cell wall. Otherwise, Lys can directly bind negatively charged virus proteins and form double salts with DNA, RNA and apoprotein, resulting in inactivation of virus. Lys can also protect us from invasion of bacterial, which presents tremendous value in pharmacy [40–42] and food preservations [43,44]. Furthermore, Lys in organism secretions correlates with many diseases, and has been used as the clinical index for diagnosis and treatment [45–47].

In this work, we prepared poly(glycidyl methacrylate-co-ethylene dimethacrylate) [poly(GMA-co-EDMA)] affinity HPLC monolithic column with the immobilization of anti-Lys DNA aptamers for the selectively screening of Lys. In the presence of precursors (GMA and EDMA) and porogens (cyclohexanol and 1-dodecanol), the monolithic column was synthesized by thermal polymerization, and then grafted by ethylenediamine followed by a link of bi-funtional glutaraldehyde. Anti-Lys DNA aptamers were then covalently immobilized on the porous polymer monolith. The separation performance and selectivity of the column were evaluated. Subsequently, the affinity column was used to screen Lys in chicken egg white.

2. Material and methods

2.1. Chemicals

Glycidyl methacrylate (GMA, purity 97%), ethylene dimethacrylate (EDMA, purity 98%), sodium cyanoborohydride ($NaBH_3CN$, purity 95%) were obtained from Acros (NJ, USA), 2,2′-azobisisobutyronitrile (AIBN, CR), cyclohexanol (AR, purity ≥98.0%), ethylenediamine (AR, purity ≥99.0%) and tetrahydrofuran (THF, AR, purity ≥99.0%) were from Sinopharm Chemical Reagent Co., Ltd. (Beijing, PR China). AIBN was re-crystallized from alcohol before use. 1-Dodecanol (purity 98%) was prepared from Sigma–Aldrich (St. Louis, MO, USA). Glutaraldehyde solution (purity ≥50%) was from Beijing Chemical Reagents Company (Beijing, PR China). Lys (from chicken egg white, MW 14.3 kDa, p*I* 8.9, purity ≥98%), cytochrome *c* (from bovine heart, MW 14 kDa, p*I* 10.8, purity ≥95%) and transferrin (from human plasma and blood, MW 80 kDa, p*I* 5.9, purity ≥98%) were purchased form Sigma–Aldrich (St. Louis, MO, USA). Hemoglobin (from bovine blood, MW 64.5 kDa, p*I* 7.0, purity 99.0%) was from Beijing Xinjingke Biotech Co., Ltd. (Beijing, PR China). Recombinat Mut S protein (MW 95 kDa, p*I* 6.8, purity 90%; stock solution contained 20 mM HEPES pH 7.8, 150 mM NaCl, 1 mM DTT, 20% glycerol) was expressed and purified from *Escherichia coli* by our lab. Protein stock solutions were diluted to desired concentrations using a buffer of 10 mM Tris–HCl/5 mM $MgCl_2$ (pH 7.5). 5′-NH_2 labeled anti-Lys DNA aptamers [48] (5′-NH_2—$(CH_2)_6$—GCA GCT AAG CAG GCG GCT CAC AAA ACC ATT CGC ATG CGG C) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All ultrapure water was prepared from a Millipore Milli-Q system and the solutions were filtered through 0.22 μm membrane before use.

2.2. Preparation of poly(GMA-co-EDMA) monolithic column

The monolithic column was prepared by an in situ polymerization in a stainless steel tube according to the previous work [49–51] with slight modifications. Briefly, 0.3 mL GMA, 0.2 mL EDMA, 0.2 mL dodecanol, 0.8 mL cyclohexanol and 7 mg AIBN were mixed together. The mixture was sonicated to accelerate dissolution of AIBN, followed by nitrogen purge for 10 min, and then transferred into a stainless steel tube (4.6 mm I.D. × 50 mm). The stainless steel tube was put into water bath for polymerization (60 °C, 12 h) with both ends sealed by silicon rubber. After completion of polymerization, the silicon rubber was removed and the stainless steel tube was connected with HPLC pump using fittings. The column was rinsed in turn with tetrahydrofuran (THF), methanol, and deionized water to remove the residual compounds after polymerization. Finally, the poly(GMA-co-EDMA) monolithic column was prepared.

The monolithic rod synthesized in the stainless steel tube was pushed out by removing the bottom fitting and applying the pressure of water, and then dried at 60 °C. The dried rod was cut into 5 pieces of 1 cm long, and each piece could be inserted into a steel tube of 4.6 mm i.d. × 10 mm. After rinsed with deionized water, the monolithic column could be used for further modifications.

2.3. Aptamer modification of monolithic column

The mixture of ethylenediamine and THF (1:1, v/v) was continuously pumped through the column at a flow rate of 0.2 mL/min to allow the reaction of epoxy groups distributed on the surface of poly(GMA-co-EDMA) monolith with amine groups of ethylenediamine for 12 h at 60 °C. Then, the column was washed in sequence with THF, methanol, deionized water, and 25 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8). Glutaraldehyde solution (20%, 25 mM Na_2HPO_4/NaH_2PO_4 diluted) was introduced to react with amine groups modified on monolith at 4 °C for 6 h, then the unreacted compounds were wash out using 25 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8). 5′-NH_2 labeled anti-Lys DNA aptamers (4 nmol, dissolved in 200 μL 25 mM Na_2HPO_4/NaH_2PO_4) were injected into the column to statically graft on the monolith through the 12 h reaction of aldehyde groups and amine groups at ambient temperature. At last, sodium cyanoborohydride (2 mg/mL) was used to reduce the residual reactive imines groups. After all the above steps, the anti-Lys DNA aptamers were immobilized on the monolithic column.

2.4. Characterization of aptamer modified monolithic column

The morphology and pore sizes of monolithic column were visualized by scanning electron microscopy (SEM, Hitachi S-3000N, Tokyo, Japan). The modified aptamers on the monolithic column were determined by elemental analysis (Flash EA-1112A, Thermo Electron Corporation). The covalent immobilization of anti-Lys DNA aptamers on the surface of monolith was characterized with FT-IR spectra (Irtron Infrared Microscope, Jasco IRT-5000, Japan).

2.5. Extraction of Lys from chicken egg white

Chicken egg white was roughly extracted from fresh egg according to Ref. [52] with modifications. A certain volume of chicken

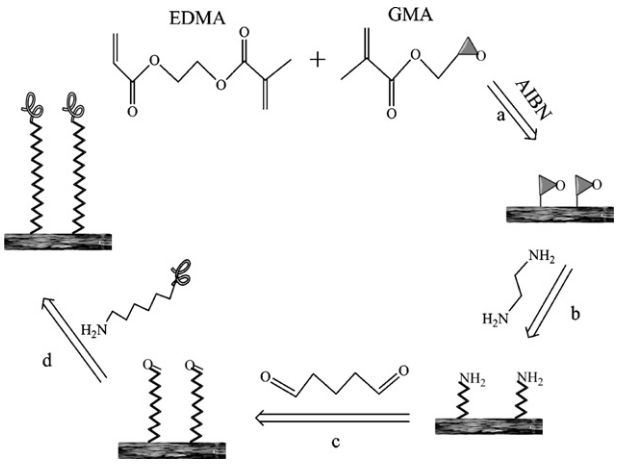

Fig. 1. Preparation procedure for DNA-aptamer immobilized poly(GMA-co-EDMA) affinity monolithic column. (a) Poly(GMA-co-EDMA) monolithic column preparation; (b) ethylenediamine grafting; (c) glutaraldehyde grafting; (d) aptamer immobilization.

egg white was diluted by 2-fold with 10 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. The diluted egg white was homogenized by sonication in an ice bath, followed by centrifugation at 4 °C, 10,000 rpm for 30 min. The 2-fold dilution of the supernatant was set as the stock solution, and the 4-fold dilution (1.1 mg/mL, nucleic acid/protein analyzer, Beckman DU800) of the stock solution as the direct injection sample.

High performance liquid chromatography system (Shimadzu, Kyoto, Japan), consisting of binary 10 ADvp pumps, SPD-20A/SPD-20AV UV–vis detector (detection at 280 nm, 12 μL flow cell) and Shimadzu CBM-20A/20Alite system controller with LC solution software, was used to carry out the chromatography analysis. The data were acquired and processed with LC solution software (Shimadzu, Kyoto, Japan). All chromatography experiments were carried out at ambient temperature. The mobile phase A for equilibrium, sample loading and enrichment was 10 mM Tris–HCl/5 mM $MgCl_2$ (pH 7.5); the eluting solution B was 1 M $NaClO_4$ (pH 6.4). The flow rate of mobile phase was 0.6 mL/min. All executive runs were equilibrated with mobile phase A for at least 20 min prior to injection. The aptamer modified monolithic column was stored in 10 mM Tris–HCl/5 mM $MgCl_2$ (pH 7.5) at 4 °C.

3. Results and discussion

3.1. Preparation and characterization of the aptamer immobilized monolith

The aptamer immobilized poly(GMA-co-EDMA) monolith was prepared using in situ polymerization followed by reactive groups grafting and aptamer immobilization (Fig. 1). The porous structure, permeability and mechanical strength of the monolith can be controlled by adjusting the proportion of the porogens accounted in polymer solution and the ratio of the two porogens (cyclohexanol to dodecanol). The ratios of GMA to EDMA (3:2, v/v) and monomer to porogen (1:2, v/v) were selected to afford enough content of epoxy groups for subsequent modifications, meanwhile, high mechanical strength and good permeability were maintained. To reduce the possible interaction of immobilized DNA aptamers with the monolithic surface and to keep the native conformation of DNA aptamers, ethylenediamine/glutaraldehyde graft procedure was selected. The aptamers were immobilized through the reaction of NH_2 groups labeled at the 5′ end of the aptamers with the aldehyde groups grafted on the surface of the monolith, providing a low cost immobilization procedure.

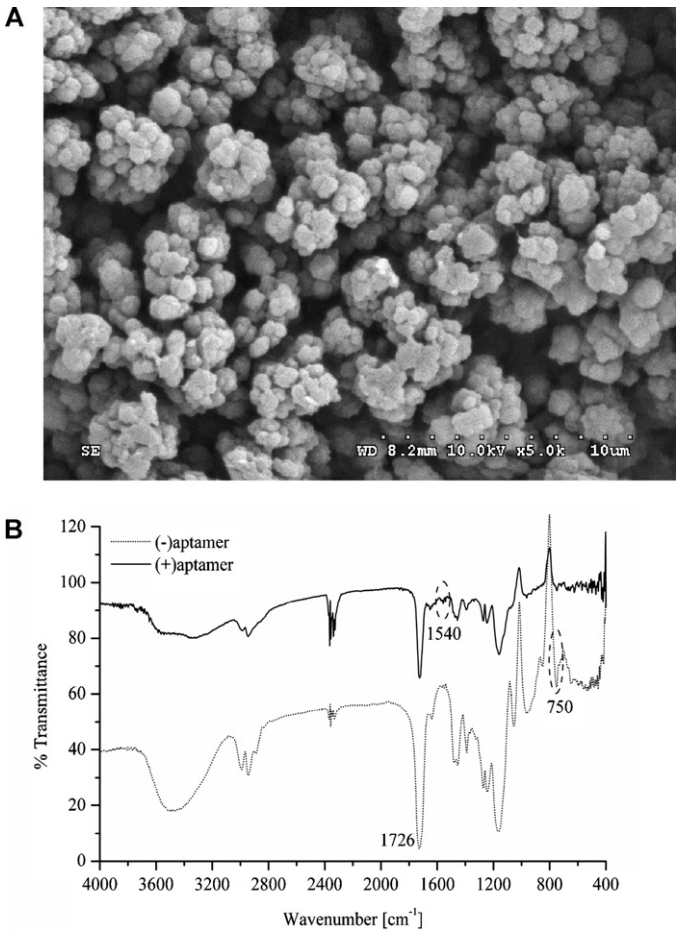

Fig. 2. Characterization of aptamer affinity monolithic column. (A) SEM image of the porous structure (×5000); (B) infrared spectrum of the aptamer affinity column.

The porous structure of the polymer rod was characterized by SEM shown in Fig. 2a. The transverse section image of polymer rod by SEM was magnified 5000 times. The pore size of the monolith was approximately 1–2 μm, enabling the high permeability and good mass transfer. The back pressure was low (only 1.0 MPa at a flow rate of 0.8 mL/min). On the other hand, the large pores may accelerate the molecular transfer of tested proteins and provide enough space for the immobilized aptamers forming appropriate three-dimensional structures for the target protein binding.

To confirm the covalent immobilization of anti-Lys DNA aptamers on the poly(GMA-co-EDMA) monolith, FT-IR spectra of tritured monolith material was measured. As shown in Fig. 2b, a peak at 750 cm^{-1} (real curve) could be assigned to the presence of epoxy group, which existed in poly(GMA-co-EDMA) monolith column before the immobilization of aptamers. After the immobilization of anti-Lys DNA aptamers on the monolith, significant change was observed in the IR spectrum. The strong absorption displayed at 1726 cm^{-1} was caused by C=O of the polymer matrix, and an obvious change appeared after the introduction of ethylenediamine/glutaraldehyde linker. As can be seen from Fig. 2b, a new peak at 1540 cm^{-1} (dashed curve), which corresponded to heterocycle, indicating the presence of nucleotide immobilized on the monolith. These results confirm that the surface of poly(GMA-co-EDMA) monolith column was successfully functionalized by DNA-aptamers. The coverage density of modified aptamers on monolith determined by elemental analysis was 290 pmol/μL, which is higher than that obtained for the microbeads packed capillary column (204 pmol/μL) [27].

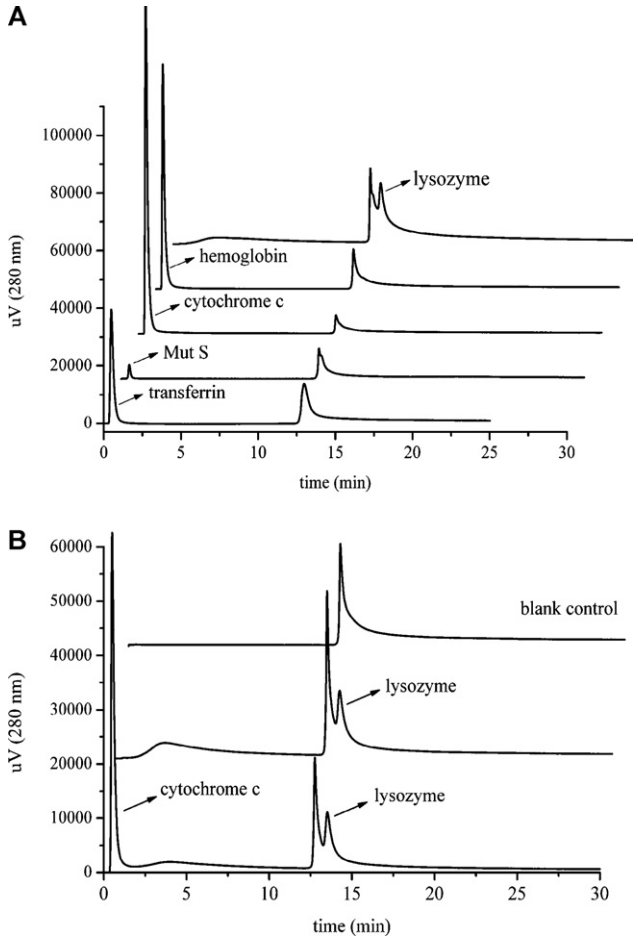

Fig. 3. The retention of target and non-target proteins on the aptamer affinity monolithic column. (A) The retention of each non-target protein; (B) the retention of target protein and non-target protein mixture. Monolithic column: 10 mm × 4.6 mm I.D., sample injection: 5 μL, and flow rate: 0.6 mL/min.

3.2. The specificity of the aptamer monolithic column to Lys

To evaluate the specificity of the aptamer modified monolithic column to Lys, non-target proteins with different isoelectric points were introduced, and the corresponding chromatographic retention behaviors were tested, including hemoglobin (Hb, p*I* 7.0), cytochrome *c* (p*I* 10.6), Mut S protein (p*I* 6.8) and transferrin (p*I* 5.9). As Fig. 3a shows, cytochrome *c* was eluted at 0.46 min, while Hb eluted at 0.45 min, Mut S at 0.52 min and transferrin at 0.48 min, showing that none of the four non-target proteins can be retained on the column. Indeed, the target protein Lys (p*I* 8.9) can be selectively trapped on the column (13.43 min). All non-target proteins with basic p*I*, acidic p*I*, and neutral p*I* eluted off at similar devoid time (~0.5 min) after sample injection. These results proved the capture specificity of the affinity column to Lys even though cytochrome *c* has a similar basic p*I* with Lys, and confirmed that affinity interaction played an important role in this retention rather than the electrostatic interaction. To investigate whether the retention of Lys on the column was influenced by another non-target basic protein, a mixture of two basic proteins with the same concentration, Lys and cytochrome *c*, was used as the injection sample (Fig. 3b). Two distinct protein peaks were observed with different retention times. After the elution of cytochrome *c* (0.50 min), the mobile phase shifted to $NaClO_4$ at 10 min and Lys was eluted out at approximately 13.5 min. It shows that the aptamer affinity column can successfully separate two similar basic proteins. Compared with Lys, the four non-target proteins have no

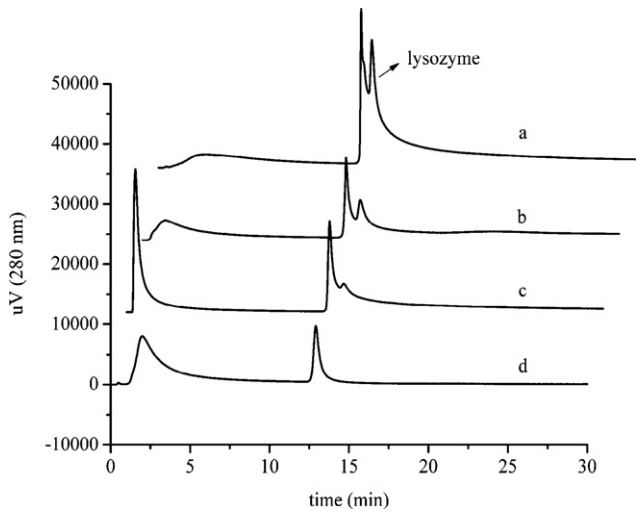

Fig. 4. The specific interaction between target protein lysozyme and aptamer with secondary structure. (a) Aptamer affinity monolithic column with ethylenediamine/glutaraldehyde linker; (b) poly T_{40} modified monolithic column with ethylenediamine/glutaraldehyde linker; (c) monolithic column with ethylenediamine/glutaraldehyde linker only; (d) monolithic column treated by acid hydrolyzation. Monolithic column, 10 mm × 4.6 mm I.D.

retention appeared on the anti-Lys DNA aptamer affinity column. The dissociation of Lys from the column can be attributed to the reduction of affinity interactions between aptamers and target protein under the high concentration of $NaClO_4$ salt.

3.3. The affinity of the aptamer monolithic column to Lys

The interaction of Lys and its aptamers depends on the secondary structure of the aptamers. Here, three control experiments without anti-Lys DNA aptamers were conducted to examine the specific interaction of Lys and the aptamers (Fig. 4). The poly(GMA-co-EDMA) monolithic column was modified with poly T_{40} through ethylenediamine/glutaraldehyde linker (Fig. 4b), or treated by ethylenediamine/glutaraldehyde grafting reaction to introduce the 10-atom spacer arm (Fig. 4c), or hydrolyzed by 0.05 M H_2SO_4 at 70 °C for 6 h enabling the original epoxy groups become vicinal diol functionality (Fig. 4d). The results showed that Lys had no retention on the column treated by acidic hydrolysis; while with the prolonged 10 atoms spacer, Lys retention occurred. On the column modified with poly T_{40}, there was an obvious Lys peak when eluted with $NaClO_4$. This may be due to the electrostatic interaction between Lys and the poly T_{40}. Although Lys had a weak retention on the poly T_{40}-modified column and the ethylenediamine/glutaraldehyde grafted column, Lys had a stronger retention on the aptamer modified column, demonstrating that the specific interaction between Lys and aptamers played a dominant role in the high intensity of protein retention.

3.4. High affinity interaction between Lys and its aptamers

To test the high affinity interaction between Lys and its aptamers, different concentrations of eluting mobile phase were conducted. It was found that by increasing the concentrations of $NaClO_4$ from 0.4 M to 1.0 M, the amount of Lys eluted increased simultaneously with improvement of peak shape. $NaClO_4$ of low concentration could not elute out the retained Lys thoroughly under the same condition. The results indicated that $NaClO_4$ of low concentration could not destroy the high affinity interaction between Lys and its aptamers, and $NaClO_4$ of high concentration should be used to elute the trapped Lys on affinity column.

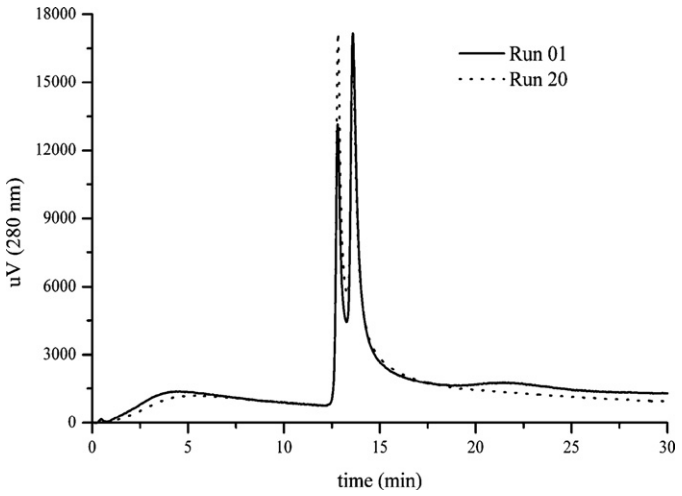

Fig. 5. The stability of the aptamer-modified monolithic column after 20 continuous executive runs by comparing Run 1 and Run 20.

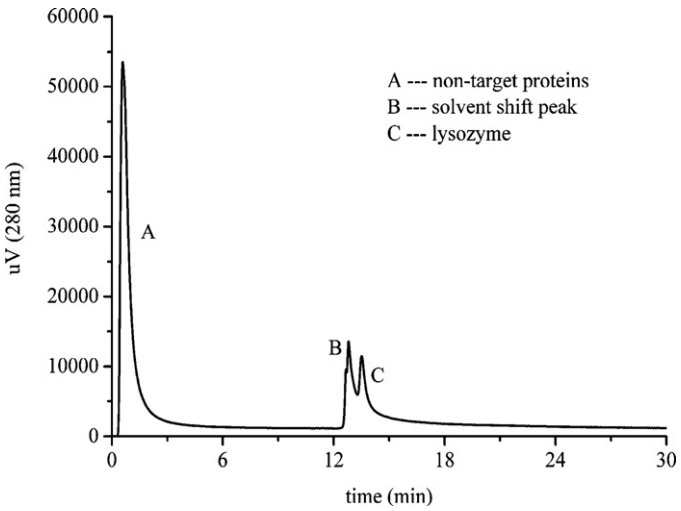

Fig. 6. The retention of non-target proteins and target lysozyme from chicken egg white on the DNA-aptamer affinity monolithic column.

Furthermore, the precision (expressed as % relative standard deviation, RSD) was determined under the optimized conditions using standard Lys (1 mg/mL). Inter-day precision was determined by repetitive analysis on three consecutive days, while intra-day precision was determined by analyzing replicate sample with same concentration ($n = 3$) in one day. The RSD values of migration time for intra-day and inter-day experiments were 0.03% and 0.8% respectively, and the peak area RSD values for intra-day and inter-day precision were 2.9% and 4.2% respectively. The results show good repeatability.

Another essential feature of chromatographic supports is related to the long-term stability of the matrix. As shown in Fig. 5, the column after 20 runs displayed similar performance as that achieved at the first run, suggesting that the aptamer-modified polymer rod could maintain good functionality and presented good stability over 20 runs. Moreover, the monolithic column were stored at 4 °C in binding buffer up to 5 months without a loss of binding capacity, consistently demonstrating the stability of the polymer rod comparable to the previous work [37].

3.5. The screening of Lys from rough extraction of chicken egg white

In order to confirm the practical application, the prepared DNA-aptamer affinity column was used to screen Lys from rough extraction of chicken egg white. The total protein concentration was diluted to 1.1 mg/mL, and the diluted sample solution of 5 μL was injected into the HPLC system. Most proteins in chicken egg white had no retention on the affinity column, and eluted out as the first peak at void time. When the mobile phase shifted to 1 M $NaClO_4$, another protein peak designated to lysozyme appeared after the peak of solvent exchange (Fig. 6). These results demonstrate that the basic protein Lys can be separated and purified from protein mixtures by taking advantage of the specific recognition of the monolithic column.

The repeatability of the monolithic column was assessed through the RSD of the retention time and the peak area of Lys from chicken egg white. The RSD values ($n = 3$) for retention time of the eluent were determined as 0.04%, and for peak area 1.5%. The good repeatability further confirms that the aptamer affinity column can be used for the practical applications.

4. Conclusions

An affinity high-performance chromatography with DNA-aptamer against Lys modified poly(GMA-co-EDMA) monolithic rod for selectively screening Lys was presented. The coverage density of immobilized aptamers on monolith was 290 pmol/μL, which is higher than that used for the micro-beads packed capillary column (204 pmol/μL). Using this method, Lys can be selectively extracted and screened with a high precision and reproducibility. Trapping of Lys from chicken egg white rough extraction was achieved with a good specificity and stability. The study shows that chromatographic retention mainly results from the specific interaction between DNA-aptamer and Lys. This strategy for selectively screening of biomolecules is facile, cost-effective, and time-saving, and is promising for extensive usage of extraction of specific target biomolecules. This method holds a great promise for the sensitive and selective quantification of various disease-related proteins.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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References

[1] A.D. Ellington, J.W. Szostak, Nature 346 (1990) 818.
[2] C. Tuerk, L. Gold, Science 249 (1990) 505.
[3] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Nature 355 (1992) 564.
[4] B. Cuenoud, J.W. Szostak, Nature 375 (1995) 611.
[5] L. Aldaz-Carroll, B. Tallet, E. Dausse, L. Yurchenko, J.J. Toulmé, Biochemistry 41 (2002) 5883.
[6] A. Geiger, P. Burgstaller, H. von der Eltz, A. Roeder, M. Famulok, Nucleic Acids Res. 24 (1996) 1029.
[7] B.A. Gilbert, M. Sha, S.T. Wathen, R.R. Rando, Bioorg. Med. Chem. 5 (1997) 1115.
[8] S.D. Mendonsa, M.T. Bowser, Anal. Chem. 76 (2004) 5387.
[9] A.T. Bayrac, K. Sefah, P. Parekh, C. Bayrac, B. Gulbakan, H.A. Oktem, W.H. Tan, ACS Chem. Neurosci. 2 (2011) 175.
[10] Y. Yang, D.L. Yang, H.J. Schluesener, Z.R. Zhang, Biomol. Eng. 24 (2007) 583.
[11] J.W. Liu, Z.H. Cao, Y. Lu, Chem. Rev. 109 (2009) 1948.
[12] X.H. Fang, W.H. Tan, Acc. Chem. Res. 43 (2010) 48.
[13] W. Sun, L.P. Du, M.Y. Li, Curr. Pharm. Des. 16 (2010) 2269.

[14] W.J. Chung, M.S. Kim, S. Cho, S.S. Park, J.H. Kim, Y.K. Kim, B.G. Kim, Y.S. Lee, Electrophoresis 26 (2005) 694.
[15] M. Michaud, E. Jourdan, C. Ravelet, A. Villet, A. Ravel, C. Grosset, E. Peyrin, Anal. Chem. 76 (2004) 1015.
[16] C.P. Rusconi, E. Scardino, J. Layzer, G.A. Pitoc, T.L. Ortel, D. Monroe, B.A. Sullenger, Nature 419 (2002) 90.
[17] P. Burgstaller, A. Girod, M. Blind, Drug Discov. Today 7 (2002) 1221.
[18] T.S. Romig, C. Bell, D.W. Drolet, J. Chromatogr. B 731 (1999) 275.
[19] C. Ravelet, C. Grosset, E. Peyrin, J. Chromatogr. A 1117 (2006) 1.
[20] S.L. Clark, V.T. Remcho, J. Sep. Sci. 26 (2003) 1451.
[21] A.C. Connor, L.B. McGown, J. Chromatogr. A 1111 (2006) 115.
[22] M.A. Rehder, L.B. McGown, Electrophoresis 22 (2001) 3759.
[23] S.L. Clark, V.T. Remcho, Anal. Chem. 75 (2003) 5692.
[24] T.U. Vo, L.B. McGown, Electrophoresis 25 (2004) 1230.
[25] L.W. Dick, B.J. Swinteck, L.B. McGown, Anal. Chim. Acta 519 (2004) 197.
[26] T.U. Vo, L.B. McGown, Electrophoresis 27 (2006) 749.
[27] Q. Deng, I. German, D. Buchanan, R.T. Kennedy, Anal. Chem. 73 (2001) 5415.
[28] Q. Deng, C.J. Watson, R.T. Kennedy, J. Chromatogr. A 1005 (2003) 123.
[29] S. Cho, S.H. Lee, W.J. Chung, Y.K. Kim, Y.S. Lee, B.G. Kim, Electrophoresis 25 (2004) 3730.
[30] M. Michaud, E. Jourdan, A. Villet, A. Ravel, C. Grosset, E. Peyrin, J. Am. Chem. Soc. 125 (2003) 8672.
[31] A. Brumbt, C. Ravelet, C. Grosset, A. Ravel, A. Villet, E. Peyrin, Anal. Chem. 77 (2005) 1993.
[32] C. Ravelet, R. Boulkedid, A. Ravel, C. Grosset, A. Villet, J. Fize, E. Peyrin, J. Chromatogr. A 1076 (2005) 62.
[33] J. Ruta, C. Grosset, C. Ravelet, J. Fize, A. Villet, A. Ravel, E. Peyrin, J. Chromatogr. B 845 (2007) 186.
[34] Q. Zhao, X.F. Li, X.C. Le, Anal. Chem. 80 (2008) 3915.
[35] Q. Zhao, X.F. Li, Y.H. Shao, X.C. Le, Anal. Chem. 80 (2008) 7586.
[36] H.A. Oktem, G. Bayramoglu, V.C. Ozalp, M.Y. Arica, Biotechnol. Prog. 23 (2007) 146.
[37] Ö. Kökpinar, J.G. Walter, Y. Shoham, F. Stahl, T. Scheper, Biotechnol. Bioeng. 108 (2011) 2371.
[38] A. Fleming, Proc. R. Soc. Lond. B 93 (1922) 306.
[39] A. Fleming, V.D. Allison, Proc. R. Soc. Lond. B 94 (1922) 142.
[40] S. Lee-Huang, P.L. Huang, Y.T. Sun, P.L. Huang, H.F. Kung, D.L. Blithe, H.C. Chen, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 2678.
[41] V.L. Hughey, E.A. Johnson, Appl. Environ. Microb. 53 (1987) 2165.
[42] M.K. Kajla, L. Shi, B. Li, S. Luckhart, J.Y. Li, S.M. Paskewitz, PLoS One 6 (2011) e19649.
[43] İ.U. Ünalan, F. Korel, A. Yemenicioğlu, Int. J. Food Sci. Technol. 46 (2011) 1289.
[44] P. Weber, H. Kratzin, K. Brockow, J. Ring, H. Steinhart, A. Paschke, Mol. Nutr. Food Res. 53 (2009) 1469.
[45] J.F. Harrison, G.S. Lunt, P. Scott, J.D. Blainey, Lancet 291 (1968) 371.
[46] S.S. Levinson, R.J. Elin, L. Yam, Clin. Chem. 48 (2002) 1131.
[47] W.G. Guder, W. Hofmann, Scand. J. Clin. Lab Inv. 68 (2008) 95.
[48] D.T. Tran, K.P.F. Janssen, J. Pollet, E. Lammertyn, J. Anné, A.V. Schepdael, J. Lammertyn, Molecules 15 (2010) 1127.
[49] F. Svec, J.M.J. Fréchet, J. Chromatogr. A 702 (1995) 89.
[50] Y.M. Wei, X.D. Huang, R.X. Liu, Y.H. Shen, X.D. Geng, J. Sep. Sci. 29 (2006) 5.
[51] X.Y. Wei, J.F. Yin, G.L. Yang, C.X. He, Y. Chen, J. Sep. Sci. 30 (2007) 2851.
[52] G.Q. Zhang, Q. Cao, N. Li, K. Li, F. Liu, Talanta 83 (2011) 1515.