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Label-free detection microarray for novel peptide ligands screening base on MS–SPRi combination

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ABSTRACT

Peptides ligands with high affinity and high specificity towards specific targets is catching a good deal of interests in biomedical field. Traditional peptide screening procedure involves selection, sequencing and characterization and each step is time-consuming and labor-intensive. The combination between different analytical methods could provide an integrated plan for efficient peptide screening. We report herein a label-free detection microarray system to facilitate the whole one-bead-one-compound (OBOC) peptide screening process. A microwell array chip with two identical units can trap the candidate peptide beads in one-well-one-bead manner. Peptides on beads were photo-released *in situ* in the well and partly transferred to two identical chips for Surface Plasmon Resonance imaging (SPRi), and peptide left in the bi-unit microwell array chip was remain for *in situ* single bead sequencing by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). Using the bi-unit imprinted chip system, affinity peptides towards AD protein were efficiently screened out both qualitatively and quantitatively from 10^4 candidates. The method provides a universal solution for high efficiency and high throughput ligands screening.

1. Introduction

Peptides are performing as excellent molecular ligands with good biocompatibility, satisfactory membrane penetrability, low immunogenicity and ease of synthesis [1–3]. Over the past few decades, novel affinity peptide probes and drugs have been screened out through high throughput combinatorial library screening strategies [4–7]. Among them, “one bead one compound” (OBOC) combinatorial peptide libraries which composed of random peptides beads are generated using chemical synthesis and have been utilized to discover specific affinity peptide ligands [8-10]. However, traditional OBOC peptide screening is time-consuming and labor intensive. Some bottlenecks constraints the screening efficiency: (a) positive hits need to be isolated manually from large amount of beads in the high-throughput library; (b) each of the positive beads should be identified and characterized singly; (c) positive peptide sequences need to be resynthesized; (d) the affinities of all the positive peptides need to be detected towards the target protein after screening. Nowadays, several methods have been developed to accelerate the peptide screening process [11]. With magnetic labeling, peptide beads could be isolated by magnetic field instead of being picked out manually [12,13]. High-throughput peptide beads could also be coded by specific molecules so that the sequence could be directly read out by color permutation, gene sequences or emission spectrum [14–17]. In the aspect of screening devices, high-throughput and high-content screening has been performed with advanced analytical instrumentation [18,19]. High-throughput peptide screening were performed integratedly by means of micro electromechanical systems (MEMS) and largescale integration (LSI) [20–23]. Peptide mapping and detection could be realized in the microchip platform [24]. Surface Plasmon Resonance imaging (SPRi) microchips provide a high-throughput approach for affinity measurements [25–29]. Furthermore, the combination of SPRi and mass spectrometry has been developed as an attractive label-free technique in analytical chemistry [30,31]. However, it is not easy to apply it to on bead ligand screening. Therefore, developing an integrated system consisting of peptide selection, sequencing and affinity characterization in a high-throughput manner is highly desirable. Our previous works have been concentrating on peptide synthesis and screening based on microfluidic chips. Affinity peptides could be synthesized and analysed on a microchip [27,32,33]. SPRi and mass spectra was also combined as an imprinted system to realize *in situ* screening [34]. Herein, an

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upgrade model of lab-on-chip system including bi-unit beads trapping, *in situ* imprinting, peptide sequencing and affinity analyses was presented. Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) and SPRi were combined for a label free detection and sequencing.

As shown in Fig. 1, the system was consisted of three microchips. One is silver coated bi-unit chip with two identical microwell arrays and the other two are gold coated microchips for peptide imprinting of which the pattern is from the bi-unit chip. The silver coated bi-unit chip was design and fabricated as a positive peptide trapper. Positive peptide beads which was isolated from the library using magnetic beads interaction were trapped in the microwells and up to 800 peptide beads could be detected simultaneously by combination with MALDI-TOF-MS. Both of the two gold coated chips were performed as the peptide array duplicators by which the peptide patterns of the two units was copied. Affinities of the peptides towards target protein could be detected and analysed on the gold coated chips by SPRi technique. Based on the microchip system, model OBOC peptide was constructed toward AD proteins. Novel peptides ligands with affinities were screening out form the high throughput library.

2. Experimental

2.1. Materials and reagents

Tentagel Resin (loading 0.53 mmol/g) was from Rapp Polymere (Germany), 9-Fluorenylmethoxy carbonyl (Fmoc)-amino acids were purchased from GL Biochem (China). Trifluoroacetic acid (TFA), streptavidin coated magnetic beads (1 μm) and CHCA (α-Cyano-4-hydroxycinnamic acid) were from Sigma (USA). N-methylmorpholine (NMM), piperidine and N, N′-dimethylformamide (DMF) were all from Beijing Chemical Plant (China). Fmoc-3-Amino-3-(2-nitrophenyl) propionic acid (ANP) was from Advanced ChemTech (USA). Silicon wafer (N/1-0-0, 500 μm) was from KYKY Tech. (China) and the gold surface SPRi chip was Plexera Nanocapture bare gold chip with a gold layer of 47.5 nm thickness and size of 25 cm × 75 cm. Anti-DDK (AD protein) was from OriGene (USA), biotin-labeling kit was from Solulink (USA).

2.2. Apparatus

MALDI-TOF-MS (ULTRAFLEXTREME mass spectrometer, Bruker Daltonics, Germany). ICP etching system (Plasmalab System100 ICP180). PlexArray HT SPRi system (Plexera LLC, Bothell, WA). HPLC system consisting a Hitachi L-7610degasser (Japan), a Hitachi L-7100 pump (Japan), a Hitachi L-7420 UV–vis detector (Japan), a Hitachi L-7300 column oven (Japan), a Hitachi D-7000 interface (Japan) and a Rheodyne 7725i injection valve (USA). ALP-IP800C multi-channel syringe pump (Alcott, China).

2.3. Microarray chip fabrication

The mask of the bi-unit microwell array chip was fabricated by soft lithography techniques (Photoresist: AZ4620, 7.6 μm) [35]. Bosch techniques are utilized for dry etching (Height: 260 μm). Then the chip was sputtered with Titanium (adhesion layer, 30 μm) and Ag (conduction layer, 200 μm) in consequence. Finally, the chip was cut by wafer scriber (25 cm × 75 cm) to fit the target holder of the MS machine. More fabrication details are shown in Fig. S1 in the Supplementary information.

2.4. Solid phase synthesis of the OBOC peptide library

Synthesis of the DDK peptide: Fmoc strategy SPPS (solid phase peptide synthesis) [36,37] was employed for synthesis of the three peptides. Tentagel Resin is used as the solid phase support [38]. All the synthesis process was carried out in dehydrous DMF. In coupling step, the Fmoc-amino acid reagent was dissolved in 0.4 mol/L NMM in DMF and the coupling time was 40 min. In deprotection step, 20% (v%) piperidine was used to remove the

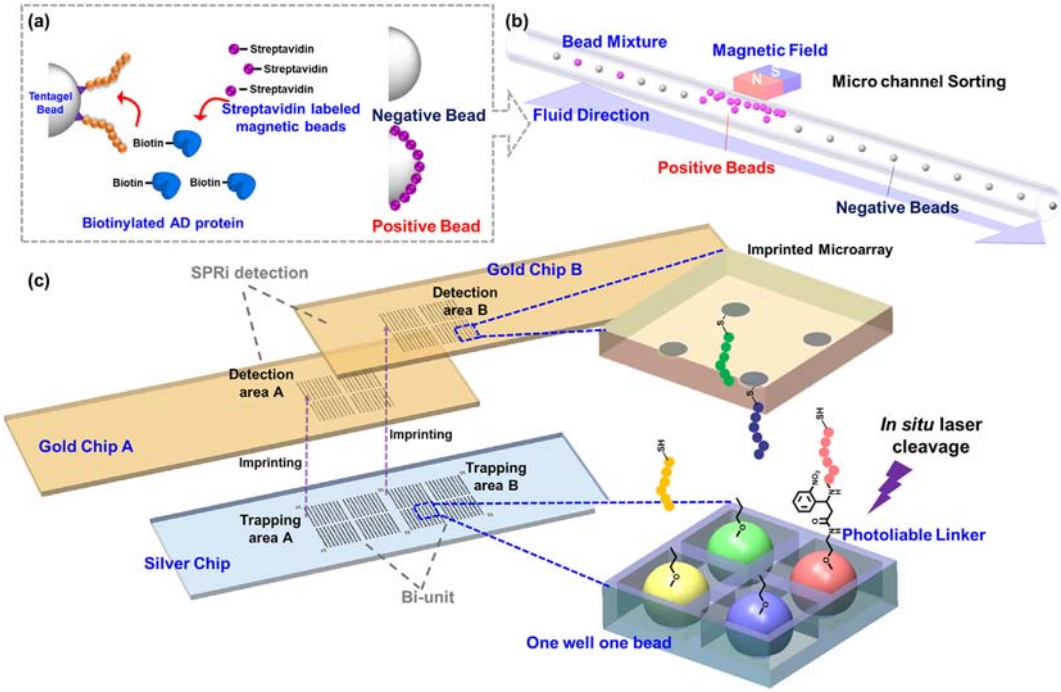

Fig. 1. The principle of the integrated peptide screening system consisting of peptide selection, sequencing and affinity characterization in a high-throughput manner: (a) positive peptide beads were selected out by magnetic trapping from the OBOC library; (b) all the beads were sorting in a Teflon tube; and (c) the positive beads were trapping in the bi-unit microarray, photocleaved and imprinted on two identical SPRi chips.

Fmoc group and the deprotection time was 10 min. Synthesis of the OBOC library: Fmoc SPPS was also employed. Three kind of amino acid reagents: Fmoc-Asp(OtBu)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Lys(Boc)-OH were employed in each synthesis cycle for the elongation for each peptide in the library. All the above experiment was carried out in the solid phase peptide synthesis vessels with sieves in it.

2.5. Positive peptide trapping and screening

Schools of peptide beads were incubated with biotinylated AD and streptavidin coated immunomagnetic beads successively. Then peptide beads were introduced into a Teflon tube (diameter: 1 mm, flow rate 600 μL/min) with a magnet closely next to the outer wall of the tube. Finally, magnet was removed and trapped beads were flushed out and collected. The mixture of positive peptide beads and streptavidin coated magnetic beads were suspended in a tube, and centrifuged for a very short time to collect the beads at the bottom.

2.6. Chip imprinting and SPRi detection of the microarray

CHCA was dissolved in Solvent TA30 (30:70 [v/v]) Acetonitrile: TFA 0.1% in water to prepare a matrix solution. Peptide beads was suspended in matrix solution and loaded into the wells of both the two units. One gold-coated chip was pressed on one unit with the gold surface down onto the microarray chip and the other one was pressed on the other unit correspondingly. The chip was exposed to the UV light (36 W, 365 nm) for 20 min and then incubated in 4 °C overnight in water box. The two gold coated chip was blocked by 5% (m/v) non-fat milk. The SPRi analysis procedure follows the following cycle of injections: running buffer (PBST, baseline stabilization); sample (five concentration of the protein, binding); running buffer (PBST, washing); and 0.5% (vol/vol) H_3PO_4 in deionized water (regeneration). Protein AD were diluted into 10 μg/mL with PBST and multiple proportion diluted into 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL and 0.625 μg/mL. Real-time binding signal were recorded and analysed by SPRi system. The PlexArray® Analyzer was used to process the data.

2.7. MALDI-TOF identification of the peptide

MALDI-TOF mass spectrometer was equipped with a nitrogen laser (wavelength 337 nm, laser pulse duration 3 ns) with reflectron and positive-ion modes. The laser power energy was adjusted between 0% and 100% to provide laser pulse energy between 0 and 100 μJ per pulse. The mass spectra were typically recorded at an accelerating voltage of 19 kV, a reflection voltage of 20 kV, and with laser pulse energy of 60 μJ. Each mass spectrum was acquired as an average of 500 laser shots.

3. Results and discussion

3.1. Design and fabrication of microarray system

Traditionally, it was very difficult to isolate one single bead in the OBOC library during the peptide ligands screening process. Beads were usually picked out one by one manually with the aid of micro tweezers and microscope. We there up on think about that arrays of microwells with a suitable size could act as an automate separator. In order to achieve the *in situ* single bead trapping and sequencing, a silicon chip with bi-unit microwell arrays was fabricated. As shown in Fig. 2a, two identical microwell array units was designed on the chip symmetrically. Each of the microwell array unit was divided into four regularly arranged subarrays with 10 × 10 wells. The microwells with vertical sides were prepared by a conventional soft lithography procedure. In order to realize an accurately capture of each bead, several size of the microwells was chosen and optimized. As shown in Fig. S2, 150 μm, 200 μm, 250 μm and 300 μm was designed as the side length of the microwell. We considered the diameter of the Tentagel beads after swollen in the solution and also we avoided more than one beads dropped into the wells. 250 μm was chosen as the optimized size (Fig. 2b). Therefore, each well was a cube shape

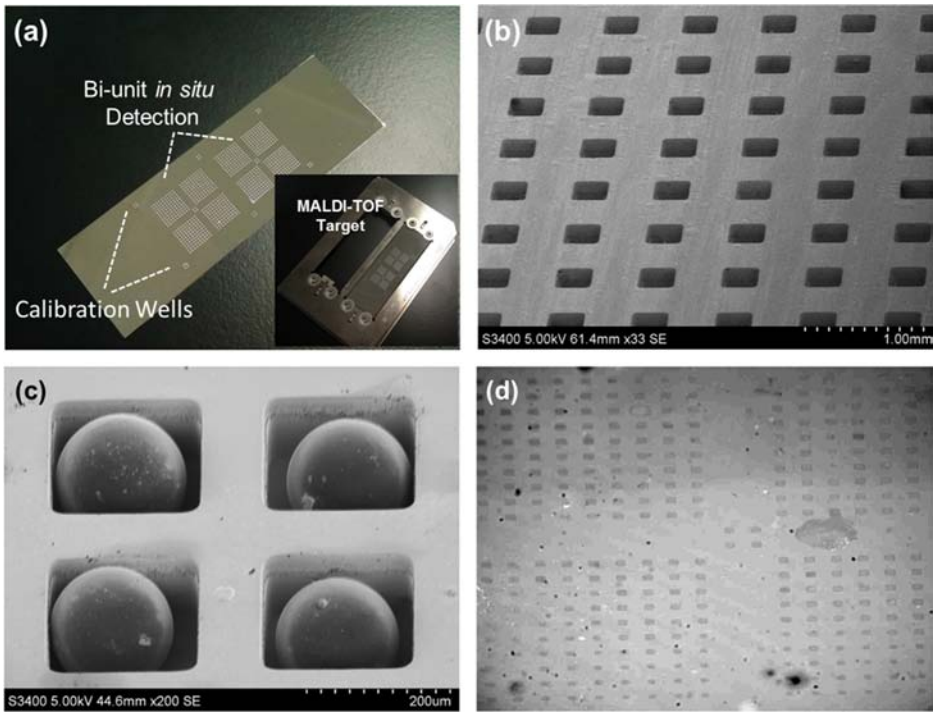

Fig. 2. (a) Bi-unit microwell array chip (inserted graph shows the chip in MALDI target), (b) SEM image of the microwell array, (c) SEM image of the one-well-one-bead trapping, and (d) The SPR image of the imprinted microarray on the gold surface chip.

with the dimension of 250 μm (L) × 250 μm (W) × 200 μm (D) (Fig. 2c). According to the principle of MALDI-TOF-MS, the distance between the target plate and the detector is the key parameter for the time of flight. For accurate measurement, extra external calibration wells with the same 3D dimension were designed and fabricated in the adjacent positions of the two units.

3.2. Model peptide ligand and receptor preparation

To evaluate the microarray system, proof-of-principle experiments were carried out. The DDK peptide and its antibody AD (Anti-DDK) protein were chosen as the model ligand-receptor pair. DDK is an octapeptide that can be conjugated to a protein using recombinant DNA technology. DDK peptide has been extensively used in genetic engineering as a protein tag [39]. The sequence of the wild type DDK peptide is DYKDDDDK which was known with a high binding affinity toward AD. DDK peptide was synthesized on monodisperse Tentagel solid support through the Fmoc solid phase peptide synthesis strategy. In order to achieve the *in situ* cleavage, photocleavable linker ANP at C-terminal was attached the using $-NH_2$ and COOH conjugation with the coupling reagent of HBTU in the C-terminal. Furthermore, a cysteine was introduced in the N-terminal of the DDK peptide in order to realize the transference onto the gold surface of the SPRi chip through the thiol group.

3.3. Magnetic trapping of the model positive beads

DDK peptide beads and control blank beads were mixed together in the ratio of 1:5. The total number of the beads is about 6000, which means that there was about 1000 positive beads in the artificial library. At the beginning, AD protein was biotinylated with the biotin/protein ratio of 2.5. After the incubation between the mixed beads and biotinylated AD, the mixture was introduced into the sorting micro channel (Fig. 1b). Positive peptide beads were enriched and isolated by a magnetic field through a bridge joining between peptide, biotinylated AD, and streptavidin coated magnetic beads. So that positive beads were

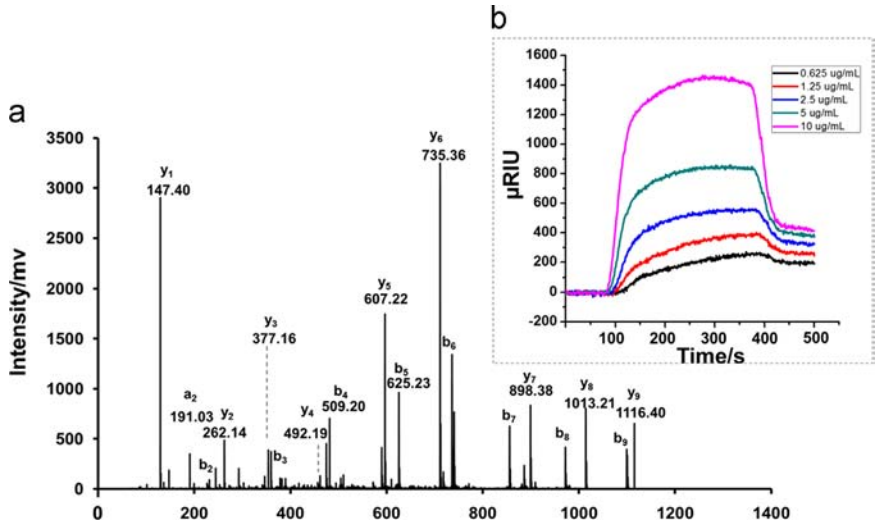

Fig. 3. A scheme of the identification of the peptide: (a) Ms/ms spectrum of the positive peptide (AD) and the (b) corresponding SPRi curves (inset).

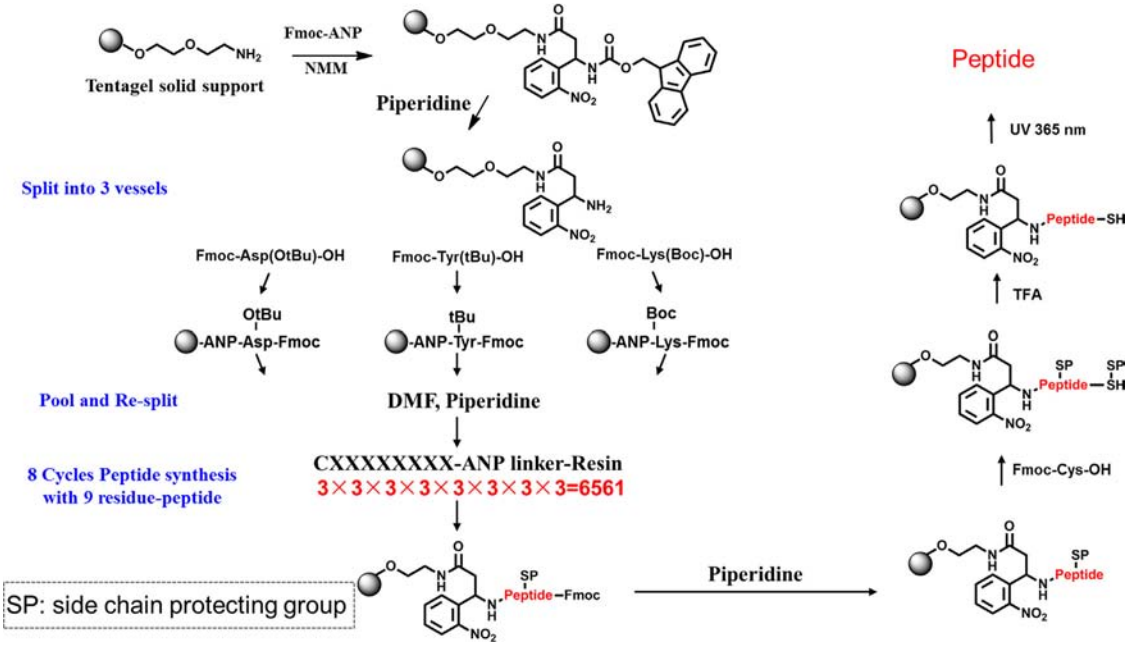

Fig. 4. Synthesis process of the OBOC peptide library towards AD protein.

trapped and adhere to one side of the tube while negative beads were flushed out. The positive beads were then collected and the streptavidin coated magnetic beads were removed from the beads system by centrifuge process as the literature reported [11]. The Tentagel beads would be spun down at the bottom of the tube immediately while the magnetic beads remain in suspension. When the supernatant were aspirated immediately, streptavidin coated magnetic beads were removed. The remaining magnetic beads bound to the Tentagel beads would not affect the following procedure.

3.4. Bi-unit imprinting and detection of the microarray

In the model experiment, about 800 positive beads were isolated from the fabricated library. Positive peptide beads suspension were dropped on the surface of the microarray, then a cover slide was used to scrape the chip gently to make the beads drop and settle into both the two units of the microwells. More than 80% success rate were achieved and beads were trapped in the two unit in a one-well-one-bead manner. For the SPRi chip, the size of the detection area is 1.4 cm × 1.4 cm, which was identically the size of the square with one single unit of the microwell array chip. Therefore, the bi-unit microarray chip was designed for the imprinting of both the two SPRi chips. Compare to the single unit chip, the screening throughput was multiplied. 800 beads in 800 wells could be detected simultaneously. The detection area of the SPRi chip was limited (1.4 cm × 1.4 cm) which was suitable for only one unit duplication. However, through the multi-fabrication of the microwell array, more SPRi gold chip array could be prepared simultaneously. Because the SPRi chip was semi-transparent, peptides in each well was *in situ* photocleaved from the beads by the ANP linker exposure under the UV light. In this condition, partial of each peptide could be cleave form the bead and label-free detection as well as sequencing could be realized. Then the free peptide with a Cys tail in each well was released into each well. After flipping the two SPRi chips and the microwell array chip together, the released peptides covalently bound to the gold surface of the SPRi chip with the through the thiol group of cysteine at the N-terminal. Therefore, a portion of the peptide in each well was transferred and imprinted in the SPRi chip (Fig. 1c). Each single spot was addressed on both chips. Finally, the microwell array was utilized for single bead MALDI-TOF-MS detection, and the imprinted spot array was for SPRi detection. In this way, peptides were patterned for both qualitative and quantitative

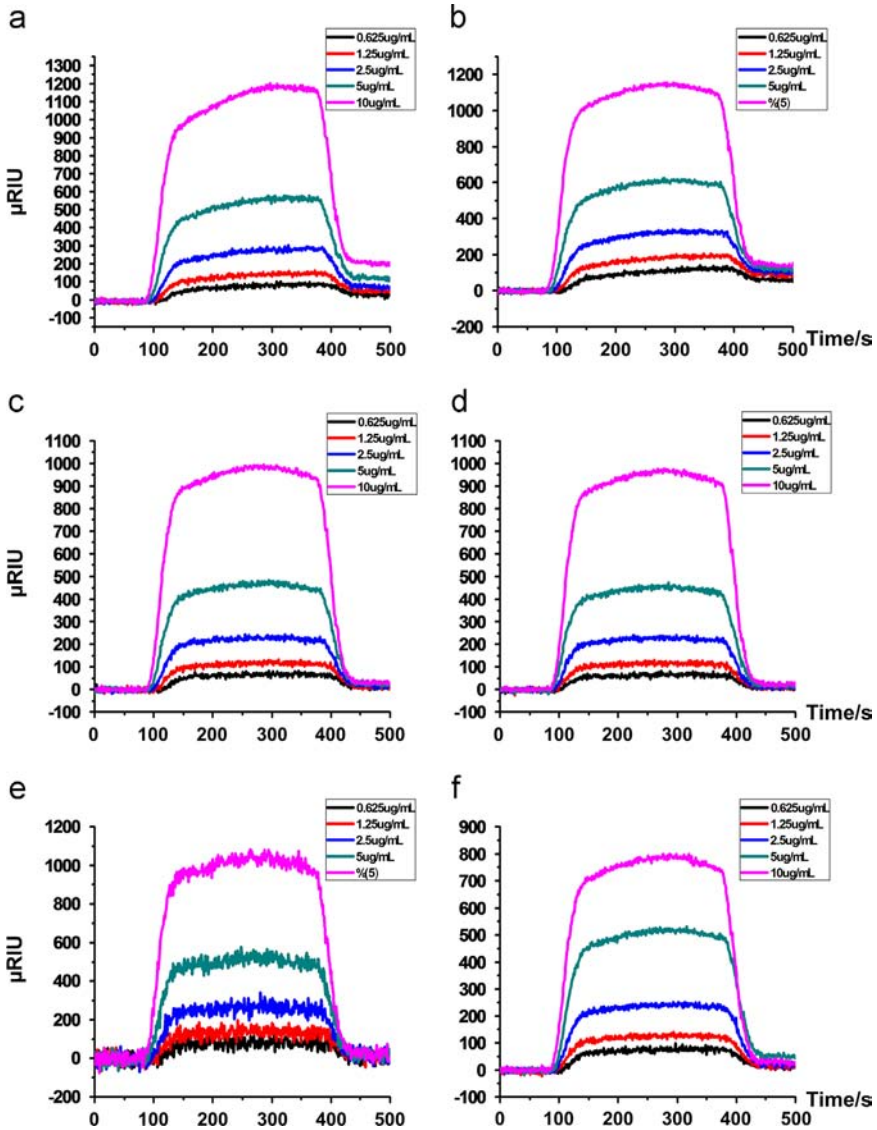

Fig. 5. SPRi curves of six peptides sorted from the OBOC library including the original ligand DDK: (a) spot 1Qb; (b) spot 1Fp; (c) spot 2Hk; (d) spot 1Np; (e) 1Oj; and (f) spot 2Sj. The "y" axis indicates the change of reflective index (μRIU) and the "x" axis indicate the time (s).

detection on two different chips. Among all the 800 wells, more than 90% was imprinted onto the gold chip, each of which show a spot in the chip image (Fig. 2d). Additionally, more than 50% of the microwell shows good mass signal, which were identified as Cys-DDK after sequencing. (Fig. 3a). The corresponding SPRi curves are shown in Fig. 3b and the dissociation constant (K_D) betweem DDK peptide and AD protein was caculated as 1.01×10^{-9} mol L^{-1}.

3.5. OBOC peptide library design and construction

An OBOC library was designed and synthesized. Peptide screening from varies of peptides was carried out on the bi-unit imprinted chips. DDK and AD were still chosen as the model system. A peptide library was constructed with the sequence of CXXXXXXXX with X standing for D, Y and K randomly so that the capacity of the peptide library was 6561 (3^8). In order to prevent the information loss of the positive sequence, redundancy of each random sequence was set as six during the synthesis process. Therefore, high-throughput peptide screening was carried out from 10^4 candidate beads. Fig. 4 shows the synthesis process. Affinity peptide screening was performed using the magnetic trapping procedure mentioned above. About 600 positive beads were selected out from the library. Positive beads were dropped into the two silver-sputtered chip wells to form a $2 \times 4 \times 10 \times 10$ microarray. The peptide beads were photocleaved and consequently imprinted to achieve a SPRi array. Data of each imprinted spot was obtained. Among them, the original ligand DDK was searched out and five novel peptides with decent binding affinity toward AD were sorted out. The SPRi curves of the five new peptides are shown in Fig. 5. The original ligand DDK (Fig. 5a) was identified with the highest affinity (8.79×10^{-9} mol L^{-1}) through fitting the SPRi curves indifferent concentration. The other five peptides have affinity ranging from 10^{-6} to 10^{-8} mol L^{-1}.

3.6. New affinity peptide identification by MALDI-TOF-MS analysis

Because all the spots in the bi-unit imprinted chips were addressed as a specific pixel (the coding principle is shown in Fig. S3), the sequences of the five peptides in the corresponding wells of the silver-sputtered bi-unit chip were detected through the *in situ* TOF-TOF-MS sequencing. For *in situ* "one well one bead" analysis, the laser beam was directed to the bottom of the microwell with the light spot area of 0.02 mm^2. With the system, single bead trapping and identification could be achieved automatically. The absolute amount of peptide on one single bead is as low as picomole and thanks to the *in situ* method, high sensitivity identification rate is achieved. The MALDI-TOF-MS (TOF-TOF) spectra of the 5 novel peptides screened out from the high throughput library is shown in Figs. S4–S8 in the Supplementary information. The list of the peptide sequences and the corresponding dissociation constant are lain out in Table 1.

Table 1
The affinities and the sequences of the peptides screened out of the high-throughput library.

Spot code	Affinity	K_D (mol L^{-1})	Sequence
1Qb	Original ligand	8.79×10^{-8}	CDYKDDDDK
1Fp	High	4.32×10^{-8}	CDDDYKKYY
2Hk	Middle	5.60×10^{-7}	CKKKDDYYY
1Np	Middle	7.15×10^{-7}	CDDDYYYYY
1Oj	Low	1.03×10^{-6}	CDDYYDYYY
2Sj	Low	8.51×10^{-6}	CDYYYKYYY

4. Conclusions

With the label-free detection microarray chip, the sequences and the affinities of multiple peptides could be obtained simultaneously and affinity peptides toward the target protein were screened out from the 10^4 high-throughput candidates. 800 positive candidates could be detected both qualitatively and quantitatively in the same time. The incorporation of beads trapping, photocleavage, *in situ* MALDI-TOF-MS and SPRi detection were integrated for peptide ligands screening. Our work provides a new insight into the establishment of effective and universal strategy for screening peptide probes for different ligand-receptor depended system.

Acknowledgments

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.012.

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