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Porous silicon affinity chips for biomarker detection by MALDI-TOF–MS

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ABSTRACT

B-type natriuretic peptide (BNP) is an important biomarker in early diagnosis of congestive heart failure. Many efforts have been made previously to evaluate the BNP level in human plasma. We developed a porous silicon (PSi) affinity chip to detect BNP present at low concentrations in human plasma by matrix assisted laser desorption/ionization–time of flight–mass spectrometry (MALDI-TOF–MS) directly. The PSi surface immobilized with antibodies captured and concentrated BNP through antibody–antigen interaction specifically and sensitively. A detection limit as low as 10 pg/mL BNP in human plasma was demonstrated by mass analysis. This effective on-chip recognition, enrichment, and detection strategy could be employed in identification of biomarkers in complex body fluids in diagnoses.

1. Introduction

Mass spectrometry has been widely used for characterization of biomolecules, especially after the two soft ionization methods (matrix assisted laser desorption/ionization and electrospray ionization) were developed. MALDI-TOF–MS now is an effective and sensitive method in proteomics for protein identification. It is a complementary technique to immunoassay, which provides the most sensitive detection of low molecular mass proteins and peptides [1]. Many sample preparation techniques have been developed to remove impurities and to enrich biomarkers of interest in biological fluids. These include solid-phase extraction [2–4], avidin–biotin interaction [5], thin layer chromatography [6,7], immobilized metal affinity capture (IMAC) [8–10], and the capture of proteins by antibodies [11,12]. All these affinity capture techniques were applied on the MALDI target and were introduced as the so-called surface-enhanced laser desorption/ionization (SELDI) [13]. In 1999, Siuzdak first developed an ionization technique by using porous silicon (PSi) as a non-matrix target plate to analyze small molecules, the so-called desorption ionization on silicon (DIOS) [14]. The PSi surface provides high specific surface area and adaptability for modification with functional groups or biomolecules to affinitively capture target biomolecules of interest. These on-chip affinity capture methods based on DIOS facilitate a fast and high-throughput platform, including purification, characterization, and quantification, which can separate and detect the target molecules from complex body fluids in one system without additional treatment [15–20]. Nowadays the on-chip separation approach coupled with MALDI has been widely used in clinical proteomics, process proteomics, and research proteomics, especially in biomarker identification and disease diagnoses [13,21,22].

B-type natriuretic peptide (BNP) is a 32 amino acid peptide neurohormone secreted mainly by the cardiac ventricles in response to wall stretching, ventricular dilation, and/or increased ventricular pressures. Previous studies have shown that BNP is a useful biomarker in the diagnosis of congestive heart failure [23]. A BNP concentration level in human plasma higher than cutoff (100 pg/mL) is proposed as the congestive heart failure, while BNP concentration level more than 480 pg/mL means a high risk of heart failure or death [24,25]. The measurement of BNP concentration levels provides independent indication of ventricular function without the use of other invasive or expensive diagnostic tests, facilitating early diagnosis and determination of appropriate treatment modalities. Two methods are accepted nowadays to measure BNP in clinical diagnosis, radioimmunoassay [26] or rapid fluorescence immunoassay [27]. Results with fluorescence immunoassays generally correlate well with radioimmunoassay [28].

Here we report a chip fabrication procedure and an on-chip separation and enrichment of BNP from human plasma followed

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by MALDI-TOF–MS analysis. Undecylenic acid was first attached on the porous silicon surface, whose end carboxyl group was converted to NHS-ester, facilitating covalent conjugation with primary amino groups of the target antibodies. Three antibodies, 5E8, D2, and B2-3, were immobilized and their capability of capturing BNP were evaluated. 5E8 showed the best performance of capturing BNP from solutions and it was chosen to detect BNP in human plasma by means of MALDI-TOF–MS. The PSi chip surface was stable during the whole experimental procedure. This method can reach a limit of detection of BNP concentration in human plasma as low as 10 pg/mL. In addition, signals of BNP and BNP derivatives can be clearly distinguished by mass spectrometry unlike immunological methods that only provide cumulative results of all BNP compounds. Therefore, we anticipate that the on-chip affinity capture technique in conjunction with MALDI-TOF–MS could become an important tool in the diagnosis of heart failure.

2. Experimental

2.1. Reagents and materials

Silicon wafers (⟨1 0 0⟩, 3″, p-type, boron-doped, 5–8 Ω cm) were purchased from Huajing Microelectronics Co. Ltd. (Wuxi, China). α-Cyano-4-hydroxycinnamic acid (α-CHCA) (99%) (Bruker Daltonics) and trifluoric acid (TFA) (99%) (Fluka), undecylenic acid (Shanghai Experiment Reagent Co. Ltd.), 3′,3′,5′,5′-tetramethylbenzidine (TMB) (Fluka), 1,4-dioxane, tetrahydrofuran, N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), and other reagents were reagent grade and used without further purification. BNP (Natrecor®) was purchased from Scios Inc. (USA) and a mutant BNP (c-BNP) was prepared in one of our groups, Institute of Molecular Medicine at Nanjing University. ImmunoPure Goat Anti-Mouse IgG (H+L) (GαM IgG) was purchased from PIERCE. Monoclonal antibodies 5E8 and D2 for recognition of BNP and B2-3 for recognition of bone morphogenetic protein (BMP) as negative control for a better clarity were prepared as described previously [29].

2.2. Preparation of PSi chip

The procedure for grafting proteins on a PSi surface was described in our previous report [30]. The single-side polished, ⟨1 0 0⟩ oriented p-type Si wafers were boiled in 3:1 (v/v) concentrated H_2SO_4/30% H_2O_2 for 30 min and then rinsed extensively with Milli-Q water (18 MΩ). The silicon wafers were electrochemically etched in an ethanolic HF solution (3:1 (v/v) 40% HF/EtOH) at a constant current density of 100 mA/cm^2 for 180 s. After etching, PSi was rinsed with pure ethanol and pentane sequentially, and then dried under a stream of dry nitrogen. The freshly etched samples were immediately put into undecylenic acid in tetrahydrofuran solution (15 wt.%). The reaction was performed under reflux conditions with microwave irradiation (200 W) (Nanjing Huiyan Microwave Instrument Ltd.) in nitrogen atmosphere for 20 min. The product was rinsed with pure ethanol and 1,4-dioxane, and then was incubated in 5 mL of 1,4-dioxane containing 0.092 g of NHS (8 mmol) and 0.165 g of DCC (8 mmol) at room temperature for 1 h. The NHS-ester modified sample was rinsed with plentiful ethanol and pentane, and dried with nitrogen stream. The antibody of BNP was immobilized on the surface by incubation of the NHS-ester chip in an antibody solution (1 mg/mL) in phosphate buffered saline (PBS) (pH 9.0) for 1 h at 37 °C. After washing with PBS buffer and Milli-Q water, the wafer surface was dried with mild nitrogen stream and stored at 4 °C.

2.3. Capture of biomarker on PSi chip

The antibody-anchored PSi chip was first blocked with 1% bovine serum albumin (BSA) in PBS buffer (pH 7.4) at room temperature for 2 h. After washing with 0.05% Tween 20/PBS buffer (PBS-T) (pH 7.4), the chip was incubated in the BNP-containing (100 pg/mL and 10 pg/mL) PBS buffer (pH 7.4) or human plasma respectively at 4 °C over night. After incubation, the PSi chip was washed twice in PBS-T buffer and then three times in Milli-Q water to remove salts and physically adsorbed proteins. The chip was dried with nitrogen stream for MALDI measurement.

2.4. FTIR measurement

Fourier transform infrared (FTIR) spectra were recorded with a Bruker IFS66/S spectrometer at 0.5 cm^{-1} resolution. Typically 32 scans were acquired per spectrum. The samples were mounted in a purged chamber. A flat untreated silicon wafer was used as reference.

2.5. XPS measurement

Elemental components on modified PSi surfaces were characterized with an XP spectrometer (Thermo ESCALAB 250), which has a monochromatized Al Kα X-ray source (150 W). The X-ray spot size is 500 μm. The PSi sample was cut into 1 cm in size and was placed on a Ni substrate with double-side adhesive tapes. Survey scans (Constant Analyzer Energy (CAE) = 20 eV, step = 1.0 eV) over a binding energy range of 0–1100 eV were run for the elemental information, and followed with high-resolution scans of C 1s, O 1s, N 1s, and Si 2p (CAE = 20 eV, step = 0.05 eV) to determine binding energies and atomic concentrations. All chemical shifts of binding energies were normalized to C 1s (main peak) at 285.0 eV. Measurements were carried out with a takeoff angle of 45° with respect to the sample surface. Peak fitting was done with ESCALB MK-II software.

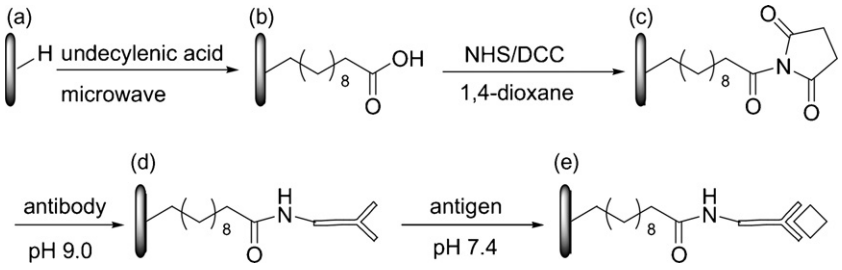
2.6. MALDI-TOF–MS analysis

All mass spectra were acquired by using a MALDI-TOF–MS spectrometer AutoflexII ToF/ToF (Bruker Daltonics, Germany) equipped with a 337-nm nitrogen laser. The PSi chips were mounted onto a stainless steel MALDI target plate using the double-side conductive adhesive tape. Mass analysis was performed after careful addition of 2 μL α-CHCA matrix solution consisting of a saturated α-CHCA in 50/50 (v/v) ACN/0.1% TFA in water. The spectra were recorded in the linear mode using an accelerating voltage of 20 kV, 100 ns delay time, and a low-mass gate of 600 Da. External mass calibration is a mixture of Peptide Calibration mono, ACTHclip (1–24) $[M+H]^+$(2932.58) and (7–38) $[M+H]^+$(3657.93), from Bruker Daltonics, which covers the m/z range of $[BNP+H]^+$(3463.71). A typical mass spectrum was obtained by collecting a cumulative signal of 50 or 100 laser shots followed by Gaussian smoothing and baseline correction using Bruker Daltonics FlexAnalysis 2.4.

3. Results and discussion

3.1. Design of PSi chip

Analysis of the human plasma proteome, especially for biomarkers, has great potential for diagnosis of human disease. However, to identify a specific biomarker for its corresponding disease in early stages is not easy because the biomarker concentration is very low in the early stage of the disease. In human plasma, even the total concentration of proteins is only 60–80 mg/mL, in addition to various small molecules including amino acids, lipids, salts, and sugars



Scheme 1. The reaction route for preparation of porous silicon chips for detection of biomarkers. Surface 'a' is a freshly etched porous silicon; 'b' represents the undecylenic acid monolayer; 'c' is terminated by NHS-ester; 'd' indicates the antibody covered surface; 'e' is the antigen–antibody complex captured on the chip.

[31]. With regard to the complexity of the human proteome, it is necessary to pretreat samples prior to biomarker identification by various analytical techniques. New trends in human plasma proteome are directed toward the development of affinity capture mass spectrometry. Several on-chip affinity capture approaches coupled with MALDI have been tried [21]. The on-chip affinity capture can be designed according to hydrophobic, electrostatic, coordination bond, or Lewis acid–base interactions. It may also be done due to antibody–antigen, receptor–enzyme, or DNA–protein interactions. Our experiment demonstrated an approach that the antibody-modified PSi chip could be used to selectively capture biomarkers in body liquids through antibody–antigen interactions. Scheme 1 illustrated our experimental procedure: the PSi surface 'a' was first covered with an undecylenic acid monolayer (surface 'b') and then its end carboxyl group was activated to NHS-ester (surface 'c'). Surface 'c' was reacted with free amino groups of antibody to achieve surface 'd'. The antibody–antigen interaction was utilized to capture biomarker of interest from complex body fluids and the biomarker-enriched chip (surface 'e') was analyzed by a MALDI-TOF–MS instrument.

3.2. Characterization of monolayer by FTIR

The advantages of FTIR for PSi-supported molecular monolayers are rapid, sensitive, easy to handle, and non-destructive. We applied FTIR to monitor each step of surface reactions. All recorded spectra were shown in Fig. 1. Fig. 1(a) presented the spectrum of surface 'a', where a typical tri-partite band presented three SiH_x ($x = 1$–3) species (2089 cm^{-1} for νSiH_1, 2116 cm^{-1} for νSiH_2, and 2139 cm^{-1} for νSiH_3). After SiH_x species were reacted with undecylenic acid under microwave irradiation, Fig. 1(b) for surface 'b' illustrated the attenuation of SiH_x bands and the new appearance of COOH bands (1710 cm^{-1}). The strong bands of asymmetric and symmetric stretching vibrations of CH_2 were easily detected at 2924 and 2853 cm^{-1} respectively. After activation of 'b' with NHS/DCC to surface 'c', the disappearance of COOH at 1710 cm^{-1} and a new tri-partite band of NHS-ester at 1742, 1786, and 1816 cm^{-1} indicated the full conversion of COOH to NHS-ester [32]. The antibody 5E8 was anchored on the PSi chip (surface 'd') through the coupling of primary amines to NHS-ester. As shown in Fig. 1(d), the characteristic vibrations of amide I and II from the abundant peptide bonds of 5E8 occurred at 1653 and 1552 cm^{-1} respectively, and the NHS-ester bands almost disappeared. These FTIR spectra validated that 5E8 had been immobilized successfully. An optimum reaction time was evaluated as 1 h at this step because the residual SiH_x species on PSi were metastable and were oxidized easily in an alkaline solution.

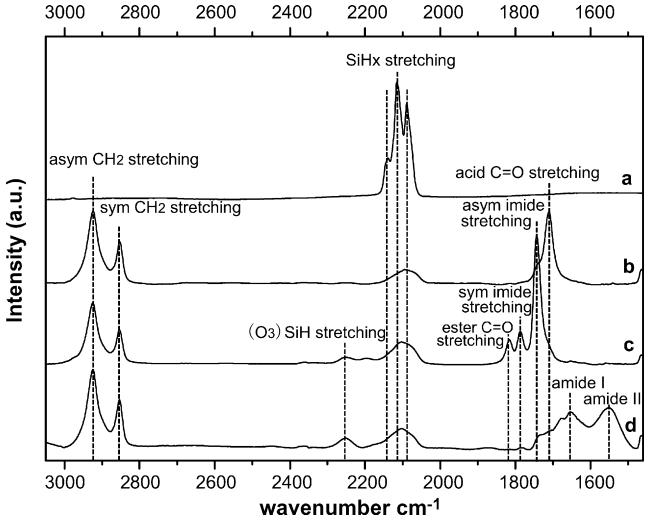


Fig. 1. Absorbance FTIR spectra of freshly prepared PSi (a), carboxyl terminated surface (b), NHS-ester terminated surface (c), and 5E8 terminated surface (d). For clarity, the wavenumber range was plotted from 3050 to 1500 cm^{-1}. sym, symmetric; asym, asymmetric.

3.3. XPS measurement

X-ray photoelectron spectroscopy (XPS) as a surface analysis technique has been most widely used, since Siegbahn and his research group developed the instrumentation and theory in the mid 1960s [33]. XPS provides an energy spectrum of the core electron bands of elements present in the top nanometers of a sample surface. The information of elements on PSi can be given from the wide scan spectra. To quantitatively evaluate the derivatized surfaces, detailed scans of C 1s, O 1s, Si 2p, and N 1s were executed on surfaces 'b'–'d', respectively. Fig. 2 showed the survey spectra from

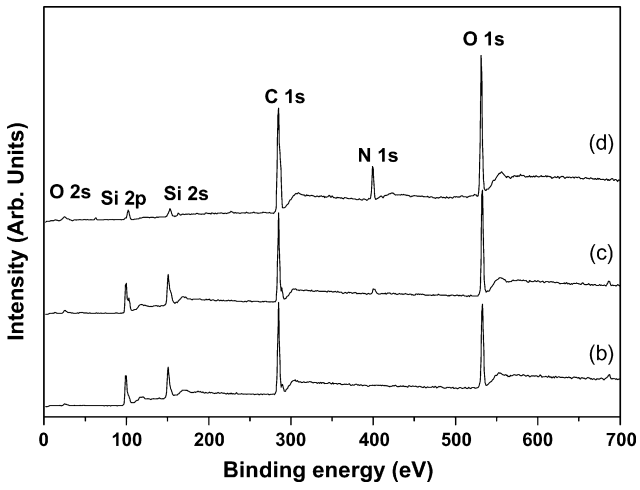


Fig. 2. XPS survey of surfaces 'b'–'d' with a scanning range from 0 to 700.0 eV.

Table 1
Atomic concentrations of elements C, N, O, and Si, and the deconvolution of C 1s on surfaces 'b'–'d'.

Surface	Atomic concentration (atom%)				Binding energy (eV) (relative peak area of C 1s %)			
	C	O	N	Si	C–C, C–H	C–O, C–N	HN–C=O	Imide-, carboxy-C
'b'	42.8	21.5	0.0	35.7	285.0 (81.4)	286.4 (13.2)		289.3 (5.4)
'c'	43.0	22.9	1.6	32.5	285.0 (76.6)	286.5 (14.2)		289.3 (9.2)
'd'	52.5	29.6	8.7	9.2	285.0 (60.8)	286.5 (20.0)	288.2 (17.4)	289.3 (2.8)

0 to 700 eV. The elemental concentrations were listed in Table 1. Obviously, C 1s, O 1s, and Si 2p were the main peaks on three PSi surfaces. Surface 'c' terminated with NHS-ester gave minor changes of the atomic concentration of C, O, and Si from 'b', but a new N 1s peak appeared. As a consequence, for surface 'd' immobilized with antibody 5E8, the Si 2p peak declined obviously from 32.5% to 9.2%, and the overall carbon intensity increased, leading to an increase of the carbon concentration from 43.0% on 'c' to 52.5% on 'd'. The significant increase of N 1s from 'c' to 'd' also indicated a major coverage of the surface with antibody 5E8.

Fig. 3 showed the evolution of the high-resolution XPS spectra of C 1s, N 1s, O 1s, and Si 2p respectively, which gave both chemical shift and intensity changes. In general, the subsistent C 1s peaks correspond to alkyl carbon (285.0 eV), aryl carbon (284.5 eV), C–O, C–N bonds (286.5 eV), carbonyl carbon C=O in aldehyde, ketone, and amide (288.0 eV), and the imido and carboxyl carbons (289.3). The main C 1s peak on surfaces 'b' and 'c' at 285.0 eV was originated from alkyl carbons of the undecylenic chain, while the peak at 286.5 eV on 'b' and 'c' owed to side products of some oxidized carbons. After the carboxylic acid of 'b' was converted into NHS-ester of 'c', the C 1s at 289.3 eV was enhanced due to the addition of imide-C in NHS-ester. On surface 'd', a new peak of amide C 1s at 288.0 eV came up obviously (17.4%) because of the high abundance of amide carbons (HN–C=O) in 5E8. Many C–N bonds in antibody led to the obvious increase of the C 1s peak at 286.5 eV.

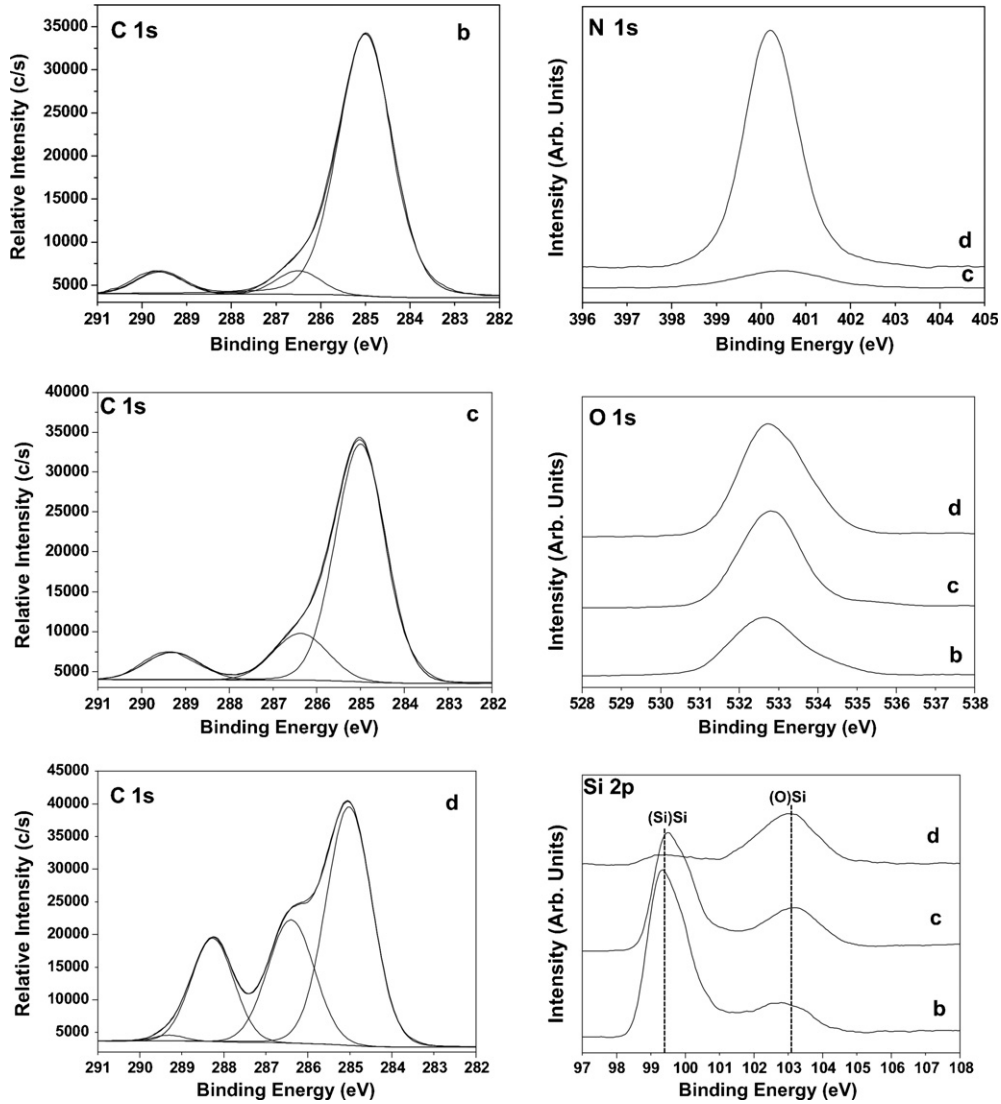


Fig. 3. Evolution of binding energies of C 1s, N 1s, O 1s and Si 2p on surfaces 'b'–'d'.

It is well known that the N 1s binding energies in common organic compounds are limited in the range of 399–402 eV. On surface 'b', the N1s peak was not detected. A minor N 1s peak appeared on surface 'c' because of an N atom present on NHS-ester. A large number of N atoms in antibody 5E8 resulted in the higher N 1s concentration on 'd'.

The binding energies of O 1s from most organic functionalities fall within a narrow range of 532.5 ± 2.0 eV. The O 1s spectra presented the increase of oxygen content following the chemical conversions. A minor change of O 1s from 'b' to 'c' owed to one more O atom on NHS-ester than on COOH. Since more oxygen atoms presented in the immobilized antibody 5E8, a higher signal of O 1s was detected on 'd'.

The Si 2p peak signal indicated the coverage and thickness of molecular films. Two peaks of Si 2p appeared at 103.1 and 99.4 eV. The former was attributed to the signal of silicon oxide (O)Si 2p, and the latter to the signal of silicon metal (Si)Si 2p. XPS can only achieve element's information on the topmost nanometer scale of surfaces. The evolution of the decreasing silicon metal (Si)Si 2p and increasing silicon oxide (O)Si 2p showed that the PSi surface was covered with more and more soft materials during stepwise reactions. Surfaces 'b' and 'c' were just covered with an organic monolayer, therefore stronger metal Si 2p signals were detected. After PSi was covered with a few nanometer antibody films, the total Si 2p signal on 'd' became much lower. However, the strength of (O)Si 2p was relatively stronger, due to oxidation of SiH_x species under an alkaline solution at pH 9.0. These results confirmed the data obtained by FTIR spectrometry.

3.4. Antibody selection

Since the affinity chip for MALDI-TOF–MS analysis is based on the antibody–antigen interaction, the efficiency and specificity of anti-BNP antibody are very important. Two monoclonal antibodies, 5E8 and D2 were used to recognize BNP, while another B2-3 was used as a negative control. Three antibodies were immobilized on PSi respectively, and then the chips were incubated in 1 ng/mL BNP solution in PBS buffer to capture BNP. Their MALDI-TOF–MS results were shown in Fig. 4. Fig. 4(1) showed no signal due to the negative control with antibody B2-3. It suggested that the PSi surface blocked by BSA had no unspecific adsorption of BNP. BNP signals $[BNP+H]^+$ (3464.17) were detected on D2 (Fig. 4(2)) and 5E8 (Fig. 4(3)) grafted samples clearly. Free amino groups in antibodies may locate at F_{ab} fragments (antigen binding site) or at F_c fragment. The antibody activity may lose or decrease when free amino groups locating at F_{ab} fragments reacted with NHS-ester. Our mass spectra suggested that 5E8 and D2 still maintained their bioactivities well after conjugation. To investigate whether multi-step antibody–antigen interactions can be employed for on-chip affinity applications, we first immobilized a goat-anti-mouse immunoglobulin (GαM IgG) on PSi chips, and then the chips were presented to 5E8 and BNP PBS-buffer (pH 7.4) subsequently. However, this approach exhibited a decreased mass signal (Fig. 4(4)) compared to that of directly immobilized 5E8 for capturing BNP (Fig. 4(3)), indication of less binding of 5E8 to GαM IgG or less activity sites on 5E8 for binding BNP. From the above screening experiments on antibodies, we conclude that the directly grafted 5E8 is the best candidate for detection of BNP in solutions.

3.5. Capture and detection of BNP from PBS buffer and human plasma

A convenient method to identify and quantify BNP in patient's plasma will be very helpful for clinical diagnoses. Our study made an effort to use the MALDI-TOF–MS technique for detection of BNP

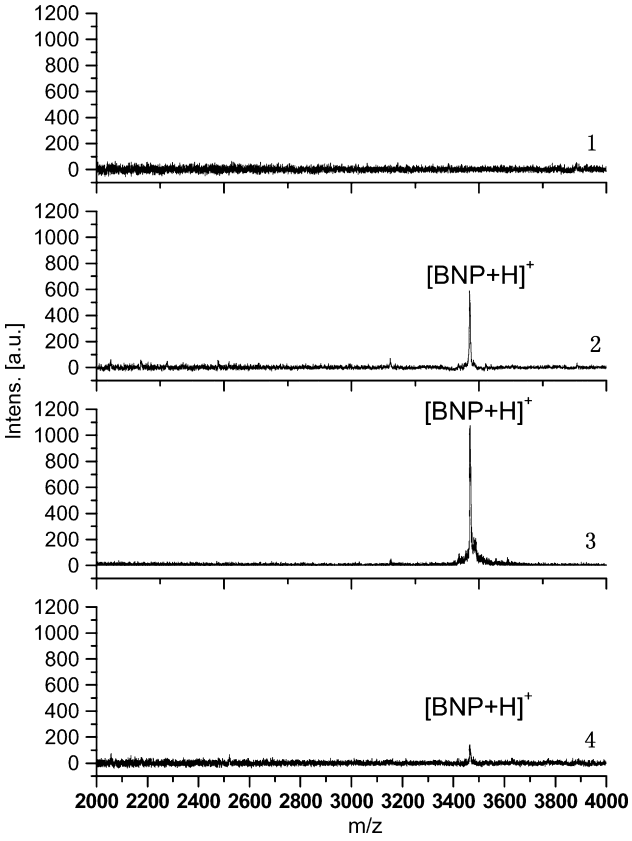


Fig. 4. MALDI-TOF–MS spectra of BNP from antibody-immobilized chips. Linear mode and 50 shots per spectrum were used to collect data. All chips were incubated in 1 ng/mL BNP solutions. The mass signals of BNP in spectra 1, 2, and 3 were from B2-3, D2, and 5E8 anchored PSi chips respectively. Spectrum 4 showed a much weaker BNP signal from the chip, which was first modified with GαM IgG, and then presented to 5E8 and BNP solutions subsequently.

in human plasma. 5E8 grafted PSi chips were incubated in different BNP concentration solutions prepared in PBS buffer and in human plasma, and also in a blank control buffer respectively. And then they were analyzed by MALDI-TOF–MS. The typical mass spectra are shown in Fig. 5. The BNP captured from the same concentration solution in human plasma presents weaker mass signal and stronger noise than from that in PBS buffer. Small molecules in human plasma were not easy to be removed from the PSi chip completely, which might suppress the BNP signal and led to a high noise value [31,34]. To reduce noise signals, the PSi surface covered with antibody was first blocked with BSA to eliminate unspecific adsorption of proteins and small molecules (Fig. 5(1)). After the PSi chip captured BNP, it was rinsed extensively with PBS-T buffer and pure water to remove contaminants physically adsorbed on the surface. On-chip separation and characterization of biomarkers reduce the analysis time and obtain accurate information of BNP species. Through the affinity PSi chip to separate and concentrate BNP from the human plasma solution, the lowest BNP concentration of 10 pg/mL can be detected in both PBS buffer (Fig. 5(3)) and human plasma (Fig. 5(5)).

3.6. Detection of mixed BNP and c-BNP in solution

With the on-chip affinity binding, separation, and detection with MALDI-TOF–MS, the different mass signals of BNP and its analogues could be easily separated. Unlike enzyme-linked immunosorbent assay (ELISA), where the summed amount of all BNP species are detected, individual peptides can be separated in

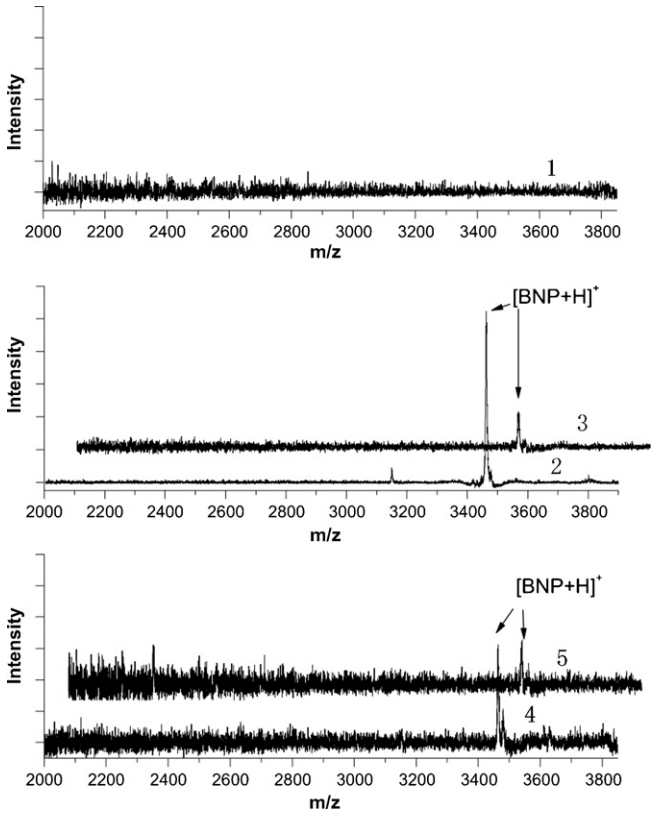


Fig. 5. MALDI-TOF–MS spectra on chips prepared from different BNP concentrations. Linear mode and 100 shots per spectrum were used to collect the data. Affinity chips were terminated with 5E8. Five spectra correspond to blank (1), 100 pg/mL and 10 pg/mL BNP in PBS buffer (2 and 3), and 100 pg/mL and 10 pg/mL BNP in human plasma (4 and 5) respectively.

the mass analyzer. To prove this concept, a mixed solution of equivalent BNP (200 pg/mL) and c-BNP (200 pg/mL) was chosen. BNP and c-BNP have the same active binding site to antibody 5E8. After incubation over night, both mass signals of $[BNP+H]^+$ (3463.02) and $[c\text{-}BNP+H]^+$ (3939.17) were achieved simultaneously in Fig. 6. It is a specific advantage of mass analysis over ELISA, where only an integral signal of BNP and its analogues can be obtained but no clue about the exact components. In practice, many analogues of

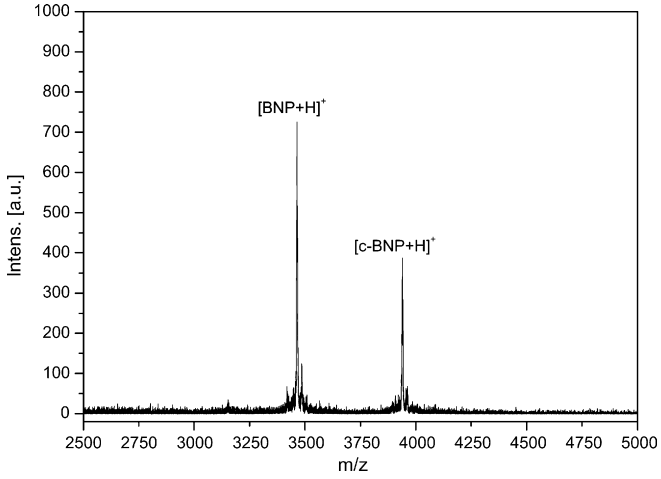


Fig. 6. MALDI-TOF–MS spectrum of BNP and c-BNP captured by PSi chip from their mixture solution in PBS buffer. The concentration of both BNP and c-BNP is 200 pg/mL.

BNP are present in human plasma, and the standard ELISA method cannot distinguish individual BNP species but the combination of MALDI-TOF–MS and the affinity chip could provide such information.

4. Conclusions

We developed a surface chemistry to realize the on-chip affinity enrichment, separation, and detection of a cardiac biomarker, BNP, in PBS buffer and in human plasma. This approach presents the feasibility of porous silicon affinity chips for detecting biomarkers in standard solutions and in human plasma. Porous silicon affinity chips have the following advantages: (1) the porous structure and conductivity of PSi makes it a perfect matrix for MALDI-TOF–MS analysis; (2) porous silicon is easy to be derivatized with organic species through the covalent bond linkage, and therefore subsequent covalent attachment of a variety of biological ligands is possible; (3) the robust covalent linkage can realize the enrichment and separation of target proteins in solutions. As an example we investigated the specificity and sensitivity of immobilized 5E8 for capturing BNP from complex solutions. A detection limit of 10 pg/mL BNP in human plasma was detected by MALDI-TOF–MS. By combining the sample dispensing robotic system and MALDI-TOF–MS, the chemically and biologically derivatized PSi chip has potential to be used in clinical diagnoses, prognoses and diagnostic screening during health assessment.

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