第 27 卷第 8 期 无 机 化 学 学 报 Vol.27 No.8
2011 年 8 月 CHINESE JOURNAL OF INORGANIC CHEMISTRY 1642-1648

Ni^{II}-NTA 修饰的银纳米粒/多孔硅芯片在线分离组氨酸标记蛋白和 MALDI-TOF 质谱检测

颜 红 王 冲 周小会 肖守军*
(南京大学配位化学国家重点实验室,南京 210093)

摘要: 本文通过沉积在多孔硅表面的银纳米粒吸附对氨基苯硫酚和氨基的化学转化得到终端为 Ni^{II}-Nα,*N*α-二(羧甲基)-*L*-赖氨酸水合物–即 Ni^{II}-NTA 体系的芯片。Ni^{II}-NTA 修饰的芯片被用于从高浓度的盐和助溶剂的缓冲体系中亲和捕获组氨酸标记的融合蛋白:thioredoxin-urodilatin 和 SUMO-hu-aprotinin,并进行在线的 MALDI-TOF 质谱检测,克服了 MALDI-TOF 质谱中直接点样污染物妨碍样品与基质共结晶的问题,避免了繁琐的离线样品预处理。芯片在线分离、纯化和 MALDI-TOF 质谱分析体系有望在复杂或原始体液的溶液中分析目标分子。

关键词: 基质辅助激光解析电离时间飞行质谱; Ni^{II}-*N*α,Nα-二(羧甲基)-*L*-赖氨酸水合物;银纳米粒/多孔硅芯片; 组氨酸标记的融合蛋白
中图分类号: O614.122; O614.81+3; O657.63 **文献标识码**: A **文章编号**: 1001-4861(2011)08-1642-07

On-Chip Separation and MALDI-TOF MS Analysis of His-tagged Protein with Ni^{II}-NTA Derivatized Ag Nanoparticles/Porous Silicon Chip

YAN Hong WANG Chong ZHOU Xiao-Hui XIAO Shou-Jun*
(*State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing*, 210093, *China*)

Abstract: An affinity chip was developed via self-assembly of 4-aminothiophenol onto silver nanoparticles (AgNPs)/porous silicon (PSi) chip and chemical conversion of amino groups to Ni^{II}-nitrilotriacetic acid (Ni^{II}-NTA) termini. The Ni^{II}-NTA modified chip was applied to separate and preconcentrate histidine-tagged (his-tagged) fusion proteins, thioredoxin-urodilatin and the lysate of small ubiquitin-related modifier (SUMO)-hu-aprotinin, in a buffer solution containing high levels of salts and solubilizing agents. The on-chip system overcomes the interruption problem of co-crystallization between analytes and matrix molecules due to contaminants from direct injection. It also avoids the complicated off-line pre-treatment of samples. This on-chip separation, purification, and MALDI-TOF MS analysis system is possible to detect target molecules from a complex or an original body fluid solution.

Key words: MALDI-TOF MS; Ni^{II}-NTA; AgNPs/PSi chip; his-tagged fusion proteins

Proteomics has spread into every field of life science and medicine as a key part of post-genomics era research. As one of the most important analysis tools, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been widely used for analyzing peptides and proteins in relatively complicated biological systems[1-2]. Its an effective and sensitive method in proteomics, particularly suited for high-throughput analysis. However, complex biological materials such as blood, serum, plasma, lymph,

收稿日期:2011-02-27。收修改稿日期:2011-04-06。
国家自然科学基金(No.20827001)资助项目。
*通讯联系人。E-mail:sjxiao@nju.edu.cn

interstitial fluid, urine, exudates, whole cells, cell lysates, and cellular secretion products typically contain hundreds of biological molecules plus organic and inorganic salts which would disrupt the co-crystallization of analytes and matrix molecules. Thus, sample preparation and purification are necessary prior to MS investigation[3].

Traditional purification methods, such as liquid chromatography, membrane dialysis and electrophoresis, are often labor-intensive and demand a preponderance of analyte molecules, while suffering from analyte and minor component loss due to nonspecific binding and dilution effects. Many new sample preparation techniques have been developed to remove impurities and to enrich target molecules in biological fluids before MS detection, such as solid-phase extraction[4-6], avidin-biotin interaction[7], immobilized metal affinity capture (IMAC)[8-9] and antibody-antigen interaction [10-11]. Among these strategies, the probe-modified surface plays an active role in extraction, separation, enrichment, and signal amplification of the target molecule. This technique is defined as surface-enhanced laser desorption/ionization (SELDI), which incorporates the straightforward sample preparation and detection via a simple on-chip handling [12]. Compared to the conventional MALDI, SELDI simplifies sample extraction, facilitates effective on-probe investigation of target molecules and promotes direct and facile mass spectrometric detection of both major and minor components in heterogeneous samples. Many exciting SELDI applications have been described from numerous laboratories [13-18]. As one of the most powerful affinity purification methods, the IMAC approach has been widely used for enrichment of phosphopeptides and proteins [19-20], which is important for understanding post translational modifications in mammalian cells.

Nanoparticles are used in many fields because of their extremely small size, high surface area to volume ratio and ease of chemical modification[21]. Many kinds of tailored nanoparticle surfaces have been used in SELDI for the detection of clinical samples, providing much valuable information for the related pathology[22-24]. In this paper, an IMAC chip, Ni^{II}-NTA derivatized silver nanoparticles/porous silicon (AgNPs/PSi), was developed to purify his-tagged proteins from the sample solution with high concentration salts and denaturants. The *in-situ* stepwise reactions on the chip to prepare the Ni^{II}-NTA derivatized surface were characterized by FTIR and the affinity performance was demonstrated with two hexa-his-tagged proteins dissolved in aqueous buffer containing high levels of nonvolatile salts and solubilizing agents.

1 Experimental

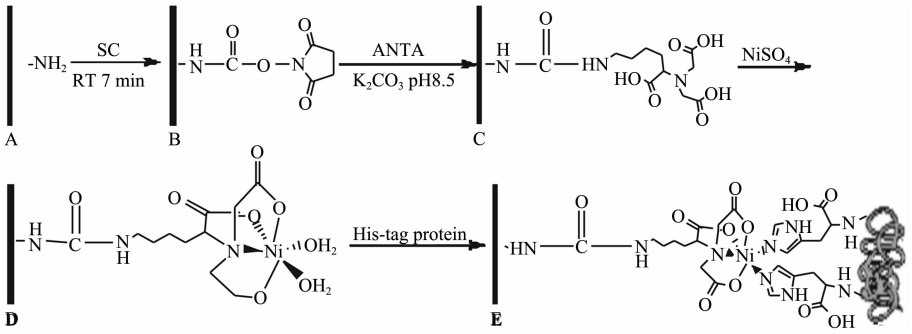
1.1 Chemicals and reagents

Silicon wafers ((100), p-type, boron-doped, 5.0~8.0 Ω·cm, 500 μm thick) were purchased from Guangwei Electronic Material Co., Ltd (Shanghai, China). Silver nitrate (A.R.) was from Boshen Chemical Co., Ltd. (Shanghai, China), di-(N-succinimidyl) carbonate (SC) from Medpep Co., Ltd. (Shanghai, China) and 4-aminothiophenol (4-ATP) from Zhejiang Shou & Fu Chemical Co., Ltd. (Zhejiang, China). Nα, Nα-bis (carboxymethyl)-*L*-lysine hydrate (ANTA) was obtained from Fluka and sinapinic acid (SA) from Bruker Daltonic GmbH (Bremen, Germany). Acetonitrile was refluxed over CaH_2 for 8 h prior to distillation to exclude moisture. The fusion proteins of histidine-tagged thioredoxin-urodilatin and the lysate of small ubiquitin-related modifier (SUMO)-hu-aprotinin were kindly provided by Prof. Liu and Prof. Tang in the Institute of Molecular Medicine in Nanjing University. Other reagents were reagent grade or higher and used as received unless otherwise specified. Milli Q water was used for all experiments.

1.2 Preparation and characterization of Ni^{II}-NTA derivatized AgNPs/PSi chip

Fig.1 described the schematic work-flow for preparation of Ni^{II}-NTA derivatized surface. The silicon chips were porosified by anodic dissolution of p-type silicon wafers in HF/EtOH (3:1, *V*/*V*) electrolyte. The detailed porosification protocol has been reported previously [13,25]. The amine functionalized AgNPs/PSi surface was prepared by incubating the freshly etched PSi in the mixture of 5 mmol·L^{-1} silver nitrate in water

($AgNO_3/H_2O$), 10 mmol·L^{-1} 4-ATP in ethanol (4-ATP/EtOH) and 1% trifluoroacetic acid (TFA) with volume ratios of 10:5:4 (pH=1~2) for 100 min[25]. The chip was then activated with SC to NHS ester by immersing it in the solution of 5 mol·L^{-1} SC dry acetonitrile at room temperature for 10 min. Rinsed with dry acetonitrile for three times and dried under a stream of dry nitrogen, the NHS ester chip was exposed to an aqueous solution containing 100 mmol·L^{-1} aminobutyl NTA (ANTA) in K_2CO_3 buffer at pH 8.5 for 1 h, resulting NTA termini via amide linkages. Excess ANTA was removed with copious water. The NTA-terminated chip was then immersed in 100 mmol·L^{-1} $NiSO_4$ solution for 30 min to ligate the $Ni^{Ⅱ}$ ion via the three carboxylates and the tertiary amine of NTA. Finally, a 25 μL protein solution was added onto the $Ni^{Ⅱ}$-NTA chip. Incubated for 1 h at 25 ℃, the chip was rinsed with phosphate buffer saline (PBS), water, and finally dried under a mild stream of nitrogen.



(A) amine-functionalized AgNPs/PSi; (B) NHS ester activated surface; (C) NTA-grafted surface; (D) $Ni^{Ⅱ}$-NTA functionalized surface; (E) his-tagged protein affinity-captured surface.

Fig.1 Schematic work-flow for preparation of the $Ni^{Ⅱ}$-NTA derivatized AgNPs/PSi chip

The *in situ* stepwise reactions on the chip to prepare the $Ni^{Ⅱ}$-NTA derivatized surface were characterized by Fourier-transform infrared spectroscopy (FTIR). The spectra were obtained with a Bruker VERTEX80V vacuum spectrometer at 4 cm^{-1} resolution. The chips were mounted in a dry-air purged chamber. Background spectra were recorded by using a flat untreated and cleaned Si (100) wafer. Typically 32 scans were required for each spectrum. The absorbance mode was used for recording the spectra.

Bruker Reflex Ⅱ MALDI-TOF/TOF (Bruker Daltonics, Germany) mass spectrometer equipped with a 337 nm nitrogen laser was used for MW analysis of proteins. The AgNPs/PSi chips were mounted onto a stainless steel MALDI target plate with double-side conductive adhesive tape. The matrix solution of 1.5 μL SA was carefully added on the chip surface, air-dried, and then the target plate was introduced immediately into the mass spectrometer for detection. All spectra were recorded in linear and positive ion mode with an accelerating voltage of 20 kV and a delay time of 400 ns. A typical mass spectrum was obtained by collecting 100 laser shots.

1.3 Preparation and characterization of protein solutions

The stock solutions of his-tagged proteins were prepared as follows: 1.8 mg of the fusion protein of his-tagged thioredoxin-urodilatin was dissolved in 2 mL of 50 mmol·L^{-1} PBS buffer (pH 7.4); the product of the lysate of small ubiquitin-related modifier (SUMO)-huaprotinin was prepared by dissolving 1.4 mg of such substance in 2 mL of 50 mmol·L^{-1} PBS buffer (pH 7.4) consisting 4 mol·L^{-1} guanidine hydrochloride. Both of them were diluted and processed to what required for evaluating the affinity performance of the $Ni^{Ⅱ}$-NTA modified AgNPs/PSi chip. The saturated SA matrix solution was prepared in 50% ACN/0.1%TFA and used for the mass detection.

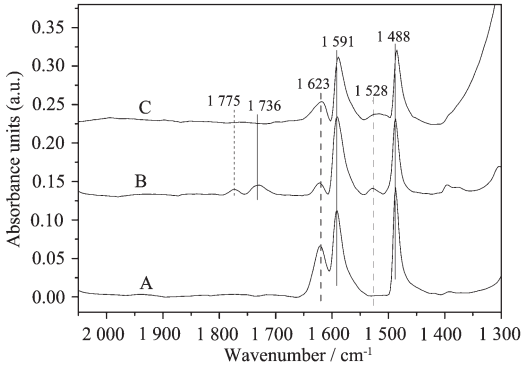
For the direct MALDI-TOF MS detection, 1 μL of the protein solution was deposited on the standard stainless steel MALDI target (Bruker Daltonics, Germany) first and then the same volume of SA solution

was deposited and mixed with the protein solution for co-crystallization. After being dried in air, the standard target was introduced immediately into the mass spectrometer for detection. The same desorption/ionization conditions with the chip detection were used for the protein detection on the standard target.

2 Results and discussion

2.1 Monitoring stepwise surface modifications by FTIR

The effective chemical modifications on the chip surface were recorded by FTIR. Fig.2A shows the spectrum of 4-ATP-covered AgNPs/PSi (surface A). Three prominent absorption bands are observed at 1 488, 1 591, and 1 623 cm^{-1}, which are assigned to the benzene ring vibration bands (1 488: CC stretching+CH bending; 1 591: CC stretching) and the NH bending band (1 623 cm^{-1}), respectively[26]. After reaction with SC, the NHS ester activated surface (Fig.2B) exhibits two significant peaks at 1 736 and 1 775 cm^{-1}, attributable to the antisymmetric and symmetric stretching bands of succinimidyl ester, respectively[27]. Meanwhile, the peak at 1623 cm^{-1} demonstrates that the unreact primary amines and 1 528 cm^{-1} (amide Ⅱ) exhibit a red shift to the bending bands of amines on surface B. Fig.2C demonstrates the IR spectrum of the surface after the attachment of ANTA to the NHS ester activated surface. Two significant peaks of NHS ester at 1 736 and 1 775 cm^{-1} disappear and the amide band around 1 623 cm^{-1} increases and broadens, which likely included contributions from the remaining N-H bending and the terminal COO^- groups of NTA (asymmetric stretching modes, expected at around 1 625 cm^{-1}). The peak of 1 528 cm^{-1} is readily assigned to amide Ⅱ. The appearance of all these bands provides unequivocal evidence for the covalent coupling of ANTA. Coordination of Ni^{II} via exposure of surface C to 100 mmol·L^{-1} $NiSO_4$ solution does not significantly change its IR spectrum (data not shown), probably due to the spectral similarity of the Ni^{2+} and K^+ salts of carboxylic acids from ANTA.



(A) 4-ATP AgNPs/PSi surface; (B) NHS ester activated surface; (C) NTA-funtionalized surface

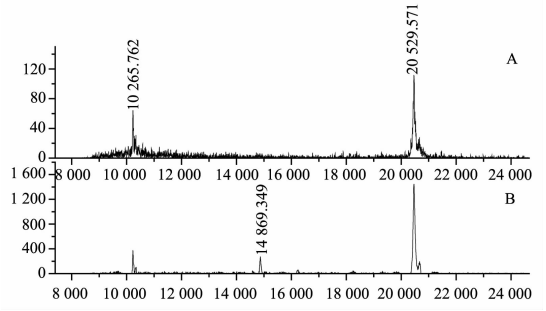
Fig.2 FTIR spectra for surface modifications

2.2 Affinity performance: detection of his-tagged proteins with MALDI-TOF MS read-out

Polyhistidine-tags are often used for affinity purification of polyhistidine-tagged recombinant proteins expressed in *E. coli* and other prokaryotic expression systems. The principle of the purification method is that the Ni^{II}-NTA complex can be used to chelate multiple imidazole groups of histidine (Fig.1). The chelate group of NTA bound strongly to the nickel ion via four binding sites, leaving two vacant sites of Ni^{II} to be coordinated with imidazole groups of histidine units on the his-tagged protein. The affinity performance of the modified AgNPs/PSi chip was tested with two hexa-tagged proteins dissolved in aqueous buffer containing high levels of nonvolatile salts, solubilizing agents and denaturants. The two proteins in this work were thioredoxin-urodilatin and the insoluble part of the lysate of small ubiquitin-related modifier (SUMO)-hu-aprotinin.

Urodilatin is a 32-amino acid peptide hormone, synthesized in kidney to regulate natriuresis and diuresis. Clinical studies have demonstrated that it is useful for the treatment of acute decompensated heart failure. Thioredoxin-urodilatin, consisting of a hexa-histidine and an enterokinase cleavage site, is a fusion protein linking urodilatin at its N-terminus to a thioredoxin (Trx) carrier protein[28]. In this work, the thioredoxin-urodilatin was dissolved in 50 mmol·L^{-1} PBS buffer, a commonly adopted buffer in a very high level of saline concentration. Its MS traces were compared via two approaches: direct MALDI-TOF MS analysis on the standard target and on the modified AgNPs/PSi chip. Fig.3A demonstrates the direct

analysis result of thioredoxin-urodilatin (0.09 mg·mL^{-1}) in the PBS buffer. Peaks of *m/z* 20529.5 ($[M]^+$) and *m/z* 10265.7 ($[M]^{2+}$) dominates the mass spectrum with visible noises because of the high concentration of salts in PBS buffer.



(A) MALDI read-out for direct sampling; (B) MALDI read-out of the same sample processed on the Ni^{II}-NTA derivatized AgNPs/PSi chip

Fig.3 Mass spectra of thioredoxin-urodilatin (0.09 mg·mL^{-1}) in 50 mmol·L^{-1} PBS buffer

In Fig.3B, the spectrum was acquired after the pretreatment of the same sample on the Ni^{II}-NTA derivatived AgNPs/PSi chip. Both $[M]^+$ and $[M]^{2+}$ peaks were obtained with much higher resolutions than those in Fig.3A. Many investigations have demonstrated the contaminants, such as salts or surfactants, in solution would strongly suppress ionization of analyte molecules in MALDI-TOF MS, because these contaminants probably disrupt the co-crystallization of analytes and matrix molecules[3,15]. For the on-chip affinity detection, the target molecules are selectively trapped on the surface from the saline solution first and then the salts are removed during the washing step with buffer and water, so the high resolution mass spectrum is obtained finally. Meanwhile, for the AgNPs/PSi chip in Fig.3B, AgNPs also provides assistance in desorption/ionization of analytes into their gas phase. Addition of SA matrix to the AgNPs/PSi surface overcomes its limitation in analyzing high molecular weight compounds [25]. The peak at *m/z* 14 869.3 observed from the AgNPs/PSi surface, may be a by-product with histidine as the tag from overexpression in *E. coli*. It is obvious that the intensity and the resolution of the peaks are improved dramatically after sample pretreatment on the chip. The S/N values of the peaks from the above two methods are listed and compared in Table 1. The value of S/N is defined as 3 when the smallest quantity of the sample could be detected.

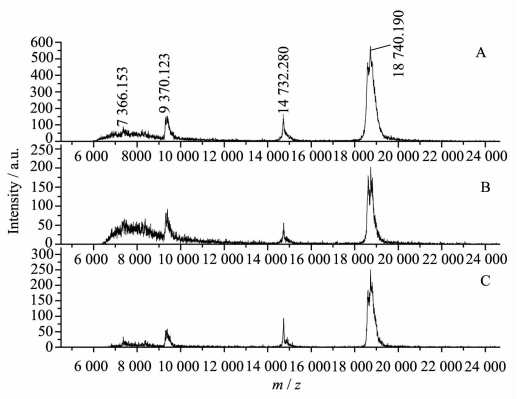
Table1 S/N value of peaks for Trx-urodilatin (0.09 mg·mL^{-1}) in 50 mmol·L^{-1} PBS buffer

Peak	S/N	
	A	B
20 529.5	2 656.3	12 319.4
14 869.3	/	2 357.5
10 265.7	1 496.9	3 227.5

(A) direct sampling; (B) on-chip sampling

Aprotinin, known as basic pancreatic trypsin inhibitor, has been demonstrated useful clinically to prevent excessive loss of blood in cardiac surgery and organ transplantation. As its analogue, human aprotinin (hu-aprotinin) was cloned from a human cDNA library. Only two out of 58 amino acids are different between aprotinin and hu-aprotinin, with the Arg 17-Ile18 in aprotinin being substituted by Lys17-Met18 in hu-aprotinin. SUMO-hu-aprotinin, overexpressed in *E. coli*, is a fusion protein consisting of a hexa-histidine tag, SUMO and hu-aprotinin [29]. For the insoluble protein, chemical denaturant like urea or guanidinium (Gua) is commonly used to increase the solubility of the protein by unfolding protein from 3D structure to 2D or linear type. However, the high abundance of denaturant prevents the proteins from being desorbed/ionized in MALDI-TOF MS analysis. SUMO-hu-aprotinin with different concentrations of guanidine hydrochloride at 2 mol·L^{-1}, 6 mol·L^{-1} and 8 mol·L^{-1} in 50 mmol·L^{-1} PBS were investigated for the affinity performance of the Ni^{II}-NTA modified AgNPs/PSi chip as shown in Fig. 4. The lowest concentration of guanidine hydrochloride needed for dissolving 0.7 mg SUMO-hu-aprotinin in 1 mL PBS, is 2 mol·L^{-1}. The high concentrated guanidine hydrochloride buffer disrupts the co-crystallization process between protein sample and matrix and forms a transparent thin film on the standard target. No signal was detected for SUMO-hu-aprotinin by direct MALDI-TOF MS with the traditional matrix, SA. However, after the same sample solution being treated on the Ni^{II}-NTA modified AgNPs/PSi surface and finally co-

crystallized by addition of a SA matrix solution, the spectra of the protein are easily acquired in Fig.4. The peaks at *m/z* 18740.2 and 9370.1 are attributed to $[M]^+$ and $[M]^{2+}$, respectively, while the other two peaks of *m/z* 14 732.3 and 7 366.2 are from a by-product because of error expression in *E. coli*. There is no big difference for the three different concentrations of guanidine hydrochloride, which means that the affinity performance of the surface is stable and repeatable, since the amount of the target molecule is the same (17.5 μg) for the three different solutions.



(A) 2 mol·L^{-1}; (B) 6 mol·L^{-1}; and (C) 8 mol·L^{-1} guanidine hydrochloride in 50 mmol·L^{-1} PBS. No signal was detected by direct MALDI-TOF MS detection (data was not shown)

Fig.4　On-chip mass spectra of SUMO-hu-aprotinin (0.7 mg·mL^{-1}) in 50 mmol·L^{-1} PBS buffer with different concentrations of guanidine hydrochloride

The limit of detection (LOD) of both thioredoxin-urodilatin and SUMO-hu-aprotinin for the on-chip detection is tens to hundreds of nanograms, which indicates that the $Ni^{Ⅱ}$-NTA derivatized surface could be used for isolating his-tagged protein from a solution containing high concentration of contaminants effectively. More important is the integration of binding, enrichment and detection on chip in a much easy and cheap way, even with the crude protein solutions.

3　Conclusions

The $Ni^{Ⅱ}$-NTA modified AgNPs/PSi hybrid surface was designed for the capture and purification of his-tagged proteins from the buffer with high levels of inorganic salts and other solubilizing agents, which makes the desorption/ionization of the samples in MALDI MS analysis much more effective. We have proved that this simple sample pretreatment is successful.

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