Immobilization of Aminophenylboronic Acid on Magnetic Beads for the Direct Determination of Glycoproteins by Matrix Assisted Laser Desorption Ionization Mass Spectrometry

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Aminophenylboronic acid (APBA) has been immobilized on magnetic beads for the direct determination of glycoprotein by matrix assisted laser desorption/ionizaton time of flight mass spectrometry (MALDI-TOF-MS). An APBA layer was formed on the surface of carboxylic acid terminated magnetic beads by coupling with carbodiimide and subsequently reacted with an N-hydroxysuccinimide moiety. The immobilized APBA was identified by MALDI-TOF-MS without a matrix. Glycoproteins, such as HbA1c, fibrinogen, or RNase B were separated and desalted using APBA magnetic beads by simply washing the magnetic beads and then separating them by external magnet. Proteins can be identified by direct determination of proteins on beads on MALDI plate and confirmed again by peptide mass finger printing after digestion of proteins on magnetic beads by trypsin. Fluorescence image with a FITC tagging protein using confocal laser microscopy showed the difference of immobilization efficiency between glycoproteins and nonglycoproteins. The methods developed within this work allow the simple treatment and enrichment of glycoproteins as well as direct determination of proteins on beads by MALDI-TOF-MS. (J Am Soc Mass Spectrom 2005, 16, 1456–1460) © 2005 American Society for Mass Spectrometry

Covalent addition of sugar chains to proteins through glycosylation is a common co- and post-translational modification in eukaryotic cells. N-glycans are attached to asparagines, O-glycans to serine or threonine [1]. These glycans play important roles in protein folding, cell–cell recognition, cancer metastasis, and the immune system [2].

Especially, the particular structures play an essential role in fertilization and embryogenesis. The large discrepancy between the extreme diversity of the glycoforms found in nature and thus, the development of a sensitive and specific technique for their elucidation, was required. Mass spectrometry has been proven to be particularly useful for the analysis of protein glycosylation [3–5]. Glycosylation site analysis is usually performed following proteolytic cleavage of the glycoprotein, such that each glycosylation site is located within a separate peptide. However, this technique is often hampered by poor ionization of glycoproteins or glycopeptides compared with their unmodified forms, thereby limiting the sensitivity of the assay [5, 6]. A full characterization of glycoprotein component in complex protein mixtures by ESI and MALDI/MS is a challenging task.

Recently, magnetic beads have been used for the immobilization of protein because they provide a simple procedure of separating reacted protein from other reaction mixture using an external magnet [7]. Several methods that employ magnetic beads exist for the recovery of nucleic acid or protein from solution [8, 9]. These particles tend to bind the target molecules in the presence of chaotropic salts. Several kinds of magnetic beads with affinity tags such as Histidine or GST,

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immobilized antibody, or streptavidin-conjugated are commercially available (Sigma and Dynal Biotech). However, separation of proteins from magnetic beads for further use of protein or identification usually leads to complications depending on the characteristics of interaction between protein and the ligand on the surface [8]. By spotting protein immobilized magnetic beads on a MALDI sample plate with matrix, proteins can be identified without further steps for separation. The intensity may be low and the contamination of beam line by sputtered beads could also be possible. However, by carefully regulating the matrix concentration and laser intensity, direct determination of protein on beads could be achievable. Further identification could be possible by peptide mass finger printing analysis of digested protein on beads by enzyme.

The key objective of this study is to find a higher sensitive method for glycoprotein analysis by testing the ability of magnetic beads to enrich the glycoprotein and applying these beads for direct determination by MALDI-TOF-MS.

To do this, aminophenylboronic acid was immobilized on magnetic beads and applied for the specific binding of glycoprotein or glycated proteins. Because boronates react reversibly with hydroxyl groups oriented in proper arrangements, and particularly with cis-1,2-diols of diol-containing substances such as nucleotides or glycoproteins, they have been used for separation of glycoproteins by HPLC [10]. Since the magnetic beads can be washed and separated easily with an external magnetic field, the use of PBA immobilized magnetic beads is a very efficient method for the preparation of glycoprotein for mass spectrometry. Glycoproteins such as HbA1c, fibrinogen, and RNase B have been studied and compared with the results of albumin and myoglobin. The immobilization of glycoprotein on APBA magnetic beads was confirmed by confocal laser microscopy.

Experimental

Materials

Carboxylic acid terminated magnetic beads (2.8 μm) were purchased from Dynal Biotech (Oslo, Norway) and HbA1c from Fitzgerald (Concord, MA). A number of other reagents were purchased from Sigma (St. Louis, MO), including the following: hemisulfate aminophenylboronic acid, 1-ethyl-3-(dimetylaminopropyl)carbodiimide (EDAC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethane sulfonic acid (MES), bovine serum albumin, horse myoglobin, bovine RNase B, bovine fibrinogen, FITC dye (for confocal laser microscopy). All other chemicals and solvents used were of reagent grade. Solutions, unless otherwise stated, were prepared using deionized (Milli-Q, Millipore, Bedford, MA) water. Matrices for MALDI-TOF experiment, a-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid were supplied by Bruker standard kit (Bruker Daltonics, Leipzig, Germany).

Preparation of APBA Magnetic Beads

Immobilization process of amino PBA on magnetic beads was stated elsewhere [11]. Briefly, carboxylic acid on the surface of magnetic beads was activated by a carbodiimide and subsequently reacted with N-hydroxysuccinimide moiety to provide a reactive site for covalent attachment of PBA through the amino group [12]. One hundred μl (about 3 mg) of carboxylic acid terminated magnetic beads were cleaned with 0.01 M NaOH twice for ten min and washed twice with deionized water to remove the excess liquid. A mixture of 50 μl EDAC (50 mg/ml) and 50 μl NHS (50 mg/ml) in 25 mM MES (pH 5.0) was added to the magnetic beads. After 30 min of incubation with slow vortexing at room temperature, the beads were washed twice with 25 mM MES (pH 5). APBA in 60 μl of 25 mM MES (pH 5.0) was added to the activated magnetic beads in 40 μl of 25 mM MES (pH 5.0). After incubation overnight at 4 °C with slow vortexing for the immobilization of APBA, incubation for 60 min at room temperature with slow vortexing was required in 0.05 M ethanolamine in PBS (pH 8.0) to quench the nonreacted group. The excess APBA was removed by washing three times with PBS.

The immobilized APBA on magnetic beads was identified by direct determination of beads by MALDI-TOF-MS and confirmed with the molecular peak of APBA ions at *m/z* 136.98. The fluorescence image of FITC-tagged proteins by confocal laser microscopy (Leica, Heisenberg, German) on APBA beads gave another indication of APBA immobilization on magnetic beads. To do this, FITC was tagged to the proteins following a standard procedure. Briefly, FITC in DMSO (10 mg/ml) was added to protein solution (10 mg/ml) in PBS at 4:1 (vol/vol) of protein:dye ratio and incubated for 1 h at room temperature while being stirred. The tagged proteins were separated using a Centricon filter (Millipore).

Enrichment of Glycoproteins Using APBA Immobilized Magnetic Beads

Twenty μl of glycoproteins (50 μM, with the exception of 14 μM for HbA1c) and nonglycoproteins (50 μM) reacted with PBA immobilized magnetic beads (0.6 mg per 20 μl) in deionized water (PBS was used for fibrinogen) and incubated for 90 min at room temperature. Then, the reacted beads were washed with deionized water several times using an external magnet for efficient collection of beads.

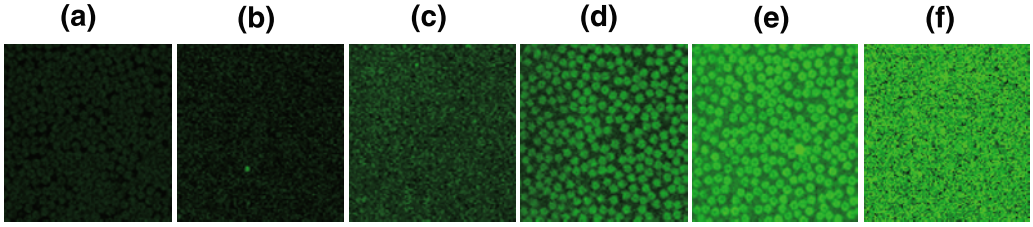


Figure 1. Images of (**a**) bare beads, (**b**) myoglobin, (**c**) bovine serum albumin, (**d**) hemoglobin, (**e**) HbA1c, and (**f**) RNase on APBA magnetic beads with FITC by confocal laser microscopy.

Mass Spectrometry

Linear and reflectron MALDI-TOF mass spectra were acquired on an Ultraflex mass spectrometer (Bruker Daltonics). Positive ion spectra were recorded using aluminum sample holders with standard parameter; a nitrogen laser (λ = 337 nm), 39 ns pulse duration and 20 kV for accelerating voltage. No matrix was used for PBA determination on beads, a-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA: acetonitrile (vol/vol = 70:30), solutions were used for digested peptide analysis, and sinapinic acid was used for the direct determination of proteins on beads. Standard calibration peptides from Bruker were used for the mass calibration of the system. The peaks of standard peptides from solution and mixture of solution and beads correspond with each other within a 1/23,000 difference.

Proteins on APBA magnetic beads were digested directly from beads by trypsin. Tryptic digests of proteins eluted from the beads were cleaned with desalting tips with Poros R2 (Applied Biosystems, Foster City, CA) resins. The proteins were confirmed by peptide mass finger printing.

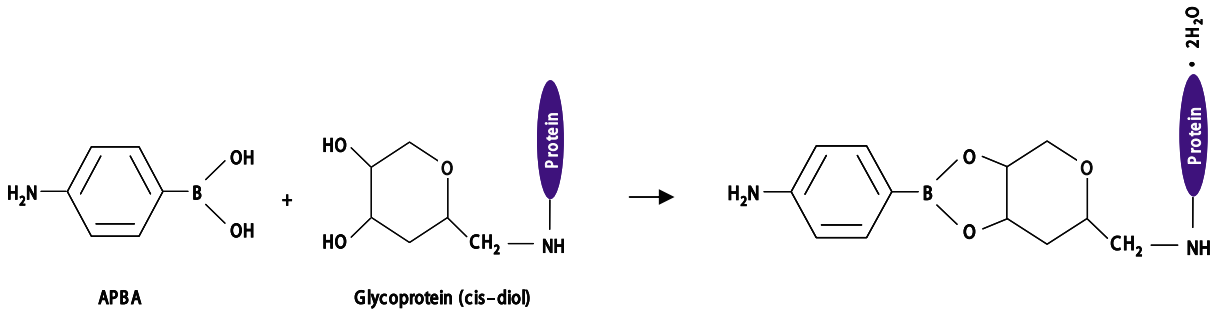
Results and Discussion

Protein Immobilization on APBA Magnetic Beads

The efficiency of immobilization of glycoproteins on APBA modified magnetic beads was confirmed by confocal laser microscopy. BSA, hemoglobin, and myoglobin were chosen as control, and ribonuclease B, HbA1c, and fibrinogen were selected as glycosylated or glycated proteins.

Figure 1 shows images of the beads prepared with fluorescent tagged proteins. The differences between the nonglycated or glycosylated proteins and glycated or glycosylated proteins are clearly distinguished. Nonspecific binding of proteins was observed in case of Hb and BSA. However, the amount was not critical compared with glycosylated or glycated proteins (Scheme **1**).

Figure 2 shows the MALDI-TOF-MS spectra of HbA1c, fibrinogen, and ribonuclease B, which are directly obtained from immobilized magnetic beads. HbA1c is glycated hemoglobin and used as a marker protein in diabetes mellitus [13]. Hb exists as a quaternary form in blood. However, the most stable form of this protein is a dimer of α and β subunits. In Figure 2a, the dimer was dissociated and determined into each subunit. Hb α subunit produced a peak at *m/z* 15,130.37, which is common with nonglycated Hb, as well as the glycated Hb β subunit at *m/z* 16,034.60. Ribonuclease B is a glycoprotein having N-linked glycan with the heterogeneous structure $GlcNAc_2Man_{5-9}$ attached to $asparagine_{60}$ [4]. All spectra in Figure 2b show the five glycosylated forms of ribonuclease B corresponding to $GlcNAc_2Man_5$–$GlcNAc_2Man_9$, with the mass difference between each adjacent ion corresponding to the molecule weight of a mannose unit (162 Da). The different glycoform was clearly identified. The peak at *m/z* 13,688 was most likely caused by the loss of the entire glycan by in-source decay [3]. Fibrinogen is a high molecular weight blood glycoprotein. It usually needs high PBS buffer concentration for solvation because of the high molecular weight and the attached glycan [14]. Therefore, the ionization of a whole protein is usually difficult. However, by immobilizing the above protein on magnetic beads, the molecular ion was determined, as shown in Figure 2c. Here again, direct determination of



Scheme 1. Interaction of PBA with glycoprotein.

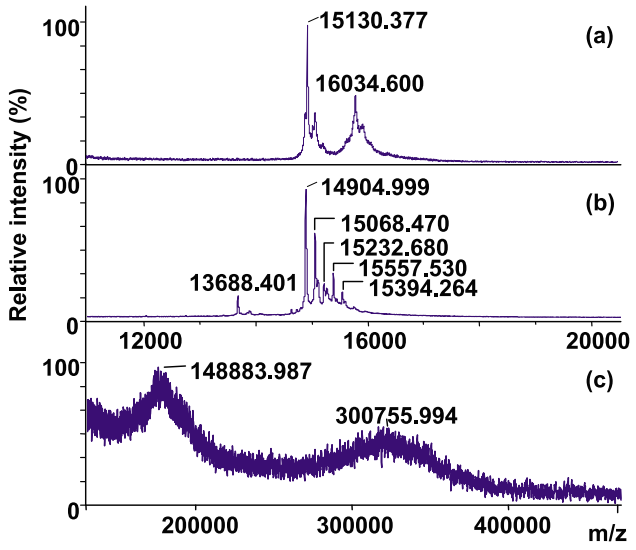


Figure 2. MALDI spectra of intact proteins of (**a**) HbA1c, (**b**) RNAse B, (**c**) fibrinogen, (**d**) myoglobin, and (**e**) myoglobin and RNase B immobilized on APBA magnetic beads. The spectra were obtained by directly spotting magnetic beads on MALDI plate with sinapinic acid. Around 6 fmol of proteins was sampled with 10 μg of beads if the immobilization efficiency is 60% in case of RNase B which is confirmed by UV spectroscopy by measuring the difference between the immobilization.

proteins from the beads on the sample plate was performed. The molecular peaks of fibrinogen were clearly identified.

To show the selective enrichment of a glycoprotein by APBA magnetic beads, the same amount of RNase B and myoglobin was mixed and treated with APBA magnetic beads. MALDI-TOF-MS spectrum of this mixture is compared with the one directly obtained from the treated APBA beads (Figure 3). As expected, myoglobin gives higher peak intensity (Figure 3a), which is usually known to have high ionization yield in mass spectrometry. However, the dramatic decrease of myoglobin peak intensity in Figure 3b compared with RNase B indicates the selective enrichment of a glycoprotein by APBA magnetic beads. Moreover, the ratio of the peaks at *m/z* 13,681.3 to 14,897.7 that is assigned as RNase A and RNase B is similar in both cases, (Figure 3a and b), while the intensity of myoglobin decreases substantially. Therefore, the peak more likely represents "in-source fragmentation" than nonspecific binding of RNase A.

Protein identification, which is immobilized on magnetic beads, is also possible through peptide mass finger printing by digesting the proteins with beads. After treating the beads with trypsin, the supernatant was collected and analyzed by MALDI-TOF-MS. By washing the beads several times with deionized water before treating with trypsin, uncontaminated and clear peptide peaks were obtained with CHCA matrix without any further treatment, such as with zip-tip. Peptide peaks obtained from RNase B and HbA1c cover 82.5% of amino for the HbA1c β chain and 66.7% for the RNnase B. Glycosylated or glycated peptides were not found in this supernatant peptide mixture. This is because they are still bound to the beads and the MALDI matrix was not adequate for glycopeptides in this case. Therefore, DHB or a mixture of DHB and CHCA [15], which is a better matrix for carbohydrate analysis, was used and only one small peak at *m/z* 1691.017 was observed. This is regarded as a glycopeptide NLTK with $GlcNAc_2$–Man_5. However, the intensity is really low; more experiments for the clear identification of glycopeptides from the digested proteins are planned in future studies, using a variety of matrices and additives for better ionization of immobilized glycopeptides from the beads.

Direct Determination of Glycoprotein from Magnetic Beads

Direct determination of glycoprotein on magnetic beads has a lot of advantages over eluting the proteins or peptides from the magnetic beads before MALDI-TOF-MS. It can save several sample preparation steps, and the sample is less susceptible to contamination. Moreover, more a sensitive spectrum was obtained because of the denaturing effects of protein bound on beads. Recently, magnetic beads with various functional surface modifications have been commercially available for cleaning samples and enriching functional peptides. The direct determination of proteins from these beads will make sample preparation steps more simple and contamination-free.

Conclusions

APBA has been immobilized on magnetic beads and used for the direct determination of glycoprotein by MALDI-TOF-MS. The sample preparation step for washing and desalting was simplified by using magnetic beads. Direct determination of proteins on beads on MALDI plate was possible and confirmed again by peptide mass finger printing after digestion of proteins

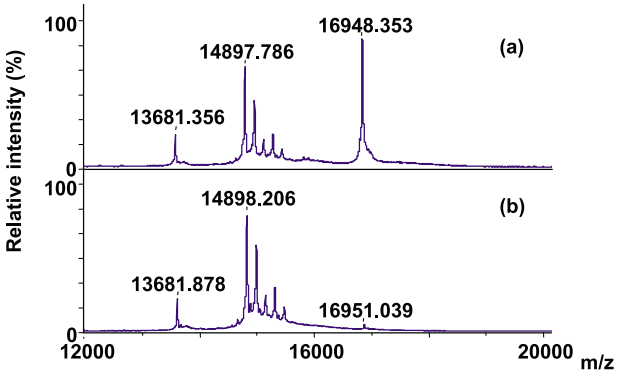


Figure 3. MALDI spectrum of mixture of RNase B and myoglobin (**a**) in solution and (**b**) from APBA beads reacted with the mixture of RNase B and myoglobin. The concentrations of RNase B and myoglobin used in each experiment are same.

on magnetic beads by trypsin. Fluorescence image of FITC-tagged protein acquired with confocal laser microscopy showed the difference of immobilization efficiency between glycol- and nonglycoproteins. The methods developed within this work allow for the simple treatment and enrichment of glycoproteins as well as direct determination of protein on beads.

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