Journal of Chromatography B, 878 (2010) 2852–2856

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous production of immunoaffinity membranes

Youji Shimazaki*, Masayuki Miyamoto

Graduate school of Science and Engineering (Science Section) and Venture Business Laboratory, Ehime University, Matsuyama, Japan

ARTICLE INFO

Article history:
Received 29 June 2010
Accepted 27 August 2010
Available online 6 September 2010

Keywords:
Non-denaturing 2-DE
Transferrin
C3
Haptoglobin
Transthyretin
Immobilization

ABSTRACT

We simultaneously separated antibodies for transferrin, the third component of complement (C3), haptoglobin and transthyretin by multi-sample non-denaturing two-dimensional electrophoresis (2-DE), transferred them to a polyvinylidene difluoride (PVDF) membrane and then stained them using direct blue 71 to obtain membrane-immobilized antibodies. The antigens, transferrin, C3, haptoglobin and transthyretin were specifically bound to the membrane-immobilized antibodies and were eluted only after rinsing the membrane with acid solution. The antigens specifically bound to the membrane-immobilized antibodies were separated by SDS-PAGE and identified by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Furthermore, transferrin and transthyretin were trapped and eluted by each membrane-immobilized antibody and detected by MALDI-TOF MS directly without separations. Using membrane-immobilized anti-transferrin antibody, transferrin in flowing blood was directly trapped and analyzed. The results indicated that membrane-immobilized antibodies are simultaneously produced, and that the immunoaffinity membranes can capture specific substances in flowing fluids.

1. Introduction

Microfiltration devices such as hollow fiber microporous or dialysis membrane are believed to be useful for the capture of fluid substances. In fact, it has been reported that xenoreactive antibodies from blood are selectively removed using immunoaffinity adsorption on hollow fiber microporous or dialysis membrane [1,2], thus demonstrating that fluid substances can be selectivity captured by membrane-immobilized (or fiber-immobilized) antibodies. We have reported that the antigen, transferrin, can be selectively captured using the membrane-immobilized anti-transferrin antibody, which is produced after purification of antiserum using non-denaturing two-dimensional electrophoresis (2-DE), and is then transferred to a membrane and stained [3]. The membrane-immobilized antibody can be used to capture the antigens in fluids. Since these membranes can be deposited on the inner wall of tubes, fluids such as blood can flow without restrictions. Thus, it is believed that the antigens in flowing fluids can be directly captured by the membrane-immobilized antibodies.

To capture various substances, specific antibodies are separated and simultaneously immobilized on membranes. It has been reported that several kinds of samples have been simultaneously separated by multi-sample non-denaturing 2-DE, and transferred to membranes [4]. Many type of membrane-immobilized antibodies can, therefore, be produced.

In the present study, we found that after separation, transferring and staining, immunoaffinity membranes of anti-transferrin, C3, haptoglobin and transthyretin antibodies were simultaneously produced. The antigens of transferrin and transthyretin were captured by their corresponding immunoaffinity membrane and directly analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Furthermore, the immunoaffinity membrane could be utilized to capture antigens in fluids such as blood. These results indicate that membrane-immobilized antibodies were simultaneously produced, and the immunoaffinity membranes might capture specific substances in flowing fluids.

2. Materials and methods

2.1. Reagents and chemicals

Acrylamide, carrier ampholyte (Pharmalyte, pH 3–10), and polyclonal rabbit anti-human transferrin, C3, haptoglobin and transthyretin antibodies were purchased from Kishida Chemicals

Abbreviations: 2-DE, two-dimensional electrophoresis; PVDF, polyvinylidene difluoride; IEF, isoelectric focusing; TEMED, N,N,N′, N′-tetramethylenediamine; TFA, trifluoroacetic acid; CBB, Coomassie Brilliant Blue; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; C3, the third component of complement.

* Corresponding author at: Graduate school of Science and Engineering (Science Section), Ehime University, Matsuyama City 790-8577, Japan. Tel.: +81 89 927 9617; fax: +81 89 927 9590.
E-mail address: yoji@dpc.ehime-u.ac.jp (Y. Shimazaki).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.08.042

(Osaka), GE healthcare (Uppsala) and Dako (Glostrup), respectively. Polyvinylidene difluoride (PVDF) membrane and C18 Zip-Tip were purchased from Millipore (Bedford). Bovine serum albumin, angiotensin II, adrenocorticotropic hormone (ACTH), α-cyano-4-hydroxycinnamic acid and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) were purchased from Sigma–Aldrich (St. Louis, MO). Bovine trypsin (sequence grade) was purchased from Roche (Mannheim). All other reagents such as ammonium persulfate, N,N,N′, N′-tetramethylenediamine (TEMED), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), glycine, trifluoroacetic acid (TFA), sodium dodecylsulfate (SDS), Coomassie Brilliant Blue (CBB), direct blue 71, dithiothreitol (DTT), ammonium hydrogen carbonate, iodeacetamide and acetonitrile were purchased from Wako Pure Chemicals (Osaka) or Nacalai Tesque (Osaka).

2.2. Separation of antibodies by non-denaturing 2-DE and elecroblotting

Sucrose was added to the purchased antibody to a concentration of 40% (w/v). Five to ten microliter of antibodies (10–20 μg) were subjected to microscale non-denaturing 2-DE using a previously reported method [3,5]. Isoelectric focusing (IEF) was done on rod gels (35 mm long × 1.3 mm i.d.). A mixed solution of 4% (w/v) acrylamide (0.2%, w/v bisacrylamide) containing 2% (v/v) pharmalyte (pH 3–10), 0.05% ammonium persulfate and 0.029% (v/v) TEMED was used. The electrode solutions were 0.04 M NaOH (cathode), and 0.01 M H_3PO_4 (anode). The resultant IEF gel was placed on top of the second-dimension slab gel, which was run on a 4–17% (w/v) acrylamide linear gradient (0.2–0.85%, w/v Bis gradient). The IEF gel was equilibrated in a buffer containing 0.01 M Tris and 0.076 M glycine, pH 8.3. The electrode buffer contained 0.05 M Tris and 0.38 M glycine (pH 8.3).

After the proteins were separated using non-denaturing 2-DE, they were transferred to a polyvinylidene difluoride (PVDF) membrane, using a semi-dry-type transblotting apparatus, to immobilize them [3]. To detect the proteins, the membrane was soaked in 0.01% (w/v) direct blue 71 in 10 mL of 0.1 M acetate buffer (pH 5.5) overnight at 4 °C, and destained with H_2O.

2.3. Antigen–antibody interaction on the membrane and antigen analysis by SDS-PAGE

After the antibody was separated, blotted onto membranes and stained with dye. These spots were then transferred to a 0.5 mL polypropylene microcentrifuge tube. 10–20 μL of human plasma was added, and the tube was incubated for 1 h. To capture antigens from blood directly, the spot on the membrane was placed in the tube of a flow system. Untreated collected blood was then applied to the system as shown in Fig. 1. For the purpose of control, the procedure was also performed utilizing a membrane of the same size that was soaked in 10 μL of 1.0 mg/mL IgG which had not been immunoreacted to specific antigens. When a substantial amount of protein in human plasma bound non-specifically to the membrane, the spots were incubated with 0.5% (w/v) bovine serum albumin in 0.1 M Tris–HCl (pH 7.0) for 2 h before addition of human plasma to minimize non-specific absorption on the membrane.

The spots were then transferred to a 0.5 mL polypropylene microcentrifuge tube, and washed three times with 100 μL of 50 mM Tris–HCl (pH 7.0) for 10 min. To extract the bound proteins, the spot was rinsed with 20 μL of 0.1% (v/v) TFA without shaking. The extraction was concentrated under vacuum and centrifuge. For analysis, the antigens were solubilized by 5 μL of sample buffer containing 0.3 M Tris–HCl (pH 6.8), 0.1% (w/v) SDS and 0.2% (w/v) sucrose, then separated by SDS-PAGE (4–17%, w/v acrylamide

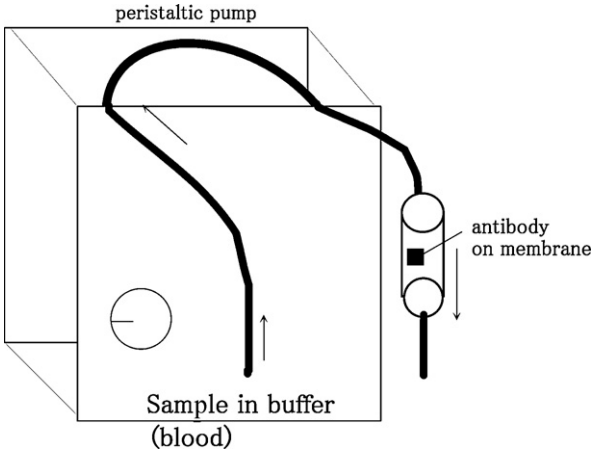

Fig. 1. Schematic drawing of a flow system when the antibody on the membrane was placed in the tube of a flow system, and collected blood was applied to the system.

linear gradient). After electrophoresis, the gel was stained in 0.1% (w/v) CBB solution and then destained.

2.4. Direct analysis of antigens using MALDI-TOF MS

For analysis of antigens by MALDI-TOF MS, the following preparation was used: 1 μL of 0.1% (v/v) TFA was added to the extraction, and then a 1 μL portion was spotted onto a stainless steel target plate and dried. In addition, 1 μL of a matrix solution containing 20 mg/mL of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 0.1% (v/v) TFA in 60% (v/v) acetonitrile was spotted at the same position of the sample on the plate. The target plate was then dried. MALDI-TOF MS analysis was performed using a Voyager DE PRO (Applied Biosystems, Framingham). The instrument was operated in positive-ion linear mode at 25 kV accelerating voltage, with the nitrogen laser at 337 nm and 3 Hz. Internal or external calibration was performed using the average mass of bovine serum albumin (m/z 66430.9).

2.5. Peptide mass fingerprinting for protein identification

The peptide mass fingerprinting (PMF) essentially followed a previously published protocol [6–9]. The protein spots were excised and transferred to a 0.5 mL polypropylene microcentrifuge tube. The excised gel was washed with 200 μL water and was shrunk with acetonitrile. After removing all liquid, the gel was dried in a vacuum evaporator centrifuge (VEC-50, Asahi Techno Glass). This gel was incubated in the presence of 0.1% (w/v) SDS and 10 mM DTT in 0.1 M ammonium hydrogen carbonate at 56 °C for 2 h, and it was then washed and shrunk with acetonitrile. After removing all liquid, the gel was incubated with 55 mM iodeacetamide in 0.1 M ammonium hydrogen carbonate for 20 min at room temperature in the dark. After the incubation, it was washed three times in 50 mM ammonium hydrogen carbonate. The gel was dehydrated with acetonitrile and dried in a vacuum evaporator centrifuge for 30 min, and was incubated in 15 μL of bovine trypsin (12.5 ng/μL) in 50 mM ammonium hydrogen carbonate at 4 °C for 30 min. The remaining supernatant was removed and 5–25 μL of 50 mM ammonium hydrogen carbonate solution without trypsin was added to the gel, which was incubated at 37 °C for 8 h. The digested polypeptides were recovered with 10 μL solution containing 0.1% (v/v) TFA and 50% (v/v) acetonitrile. The extracts were concentrated in the vacuum evaporator centrifuge, and were suspended in 20 μL of 0.1% (v/v) TFA. The polypeptides obtained were concentrated and desalted using C18 Zip-Tip from Millipore (Bedford),

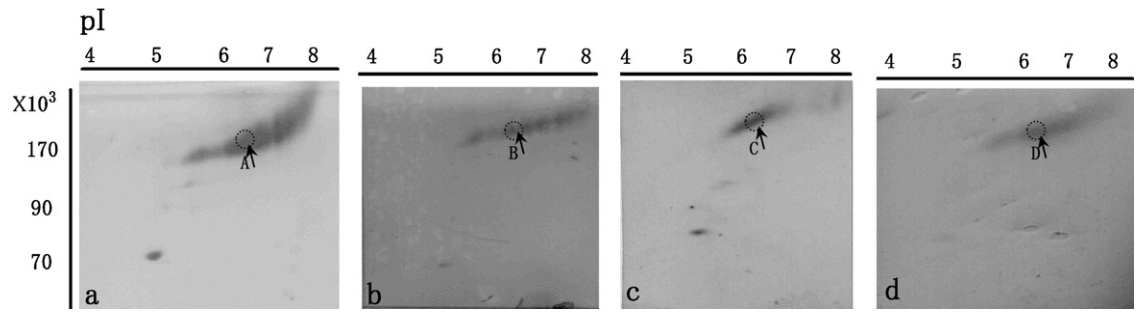

Fig. 2. Non-denaturing 2-DE patterns of polyclonal rabbit anti-human C3 (a), haptoglobin (b), transferrin (c), and transthyretin (d) antiserum proteins. Spots (A–D) on the membrane were excised and incubated with human proteins.

and were mixed with α-cyano-4-hydroxycinnamic acid. Mass analysis was done using MALDI-TOF MS, operating in a positive-ion reflector mode. The spectra were analyzed using the mass values for monoisotopic peaks that were used for searches (Mascot, http://www.matrixscience.com/) against the Swiss-Prot database. Monoisotopic peaks of angiotensin II (*m*/*z* 1046.5423) and ACTH 18-39 (*m*/*z* 2465.1989) were used for internal calibration. The database was searched using the following terms: taxonomy (*Homo sapiens*), trypsin digest (one missed cleavage allowed), cysteine modified by carbamidomethylation, and mass tolerance of 50 ppm, using internal calibration and oxidation of methionines. The criteria used to accept identification included the extent of sequence coverage, the number of peptides matched, and the probabilistic score (the required probability for a random match was <0.05).

3. Results and discussion

Fig. 2 shows direct blue 71 staining of polyclonal rabbit anti-human C3 (a), haptoglobin (b), transferrin (c), and transthyretin (d) antibodies after separation by non-denaturing 2-DE and electroblotting onto a membrane. IgG in each sample was separated and identified by PMF using MALDI-TOF MS (arrows in Fig. 2). It has been reported that four samples were simultaneously separated and transferred to membranes by multi-sample non-denaturing 2-DE [4]. Hence, the same method was applied for the simultaneous separation and immobilization of antibodies in the present study. Since we have already reported that the antigen–antibody interaction is retained even after staining by direct blue 71 in the acetate buffer (pH 5.5) [3], all antibodies in the present study are thought to bind to antigens. Proteins bound to each antibody on the membrane (A-D in Fig. 2) were separated by SDS-PAGE as shown in Fig. 3. The protein bands in the SDS-PAGE were identified by PMF using MALDI-TOF MS (Table 1). The results indicate that antigens C3, haptoglobin, transferrin and transthyretin were bound to spots A–D, respectively, on the membrane, as shown in Fig. 2. Therefore, it was proved that the specific antigen–antibody interaction could be retained even after separation, electroblotting and staining by direct blue 71. It is well known that the antigen–antibody interactions on the membrane take place after the antigens are separated by SDS-PAGE and electroblotted to membranes [10]. Further, antibodies are purified by antigens on the membranes after the targeted antigens are separated by SDS-PAGE, and are electroblotted to membranes [11]. In these cases, it is considered that the antibodies recognize the linear epitope of denatured antigens because antigens are treated with SDS. On the other hand, because the immunoaffinity membranes in the present study are thought to recognize not only amino acid sequences but also antigen conformation, it can be examined whether the antibodies interact with denatured antigens and/or native antigens.

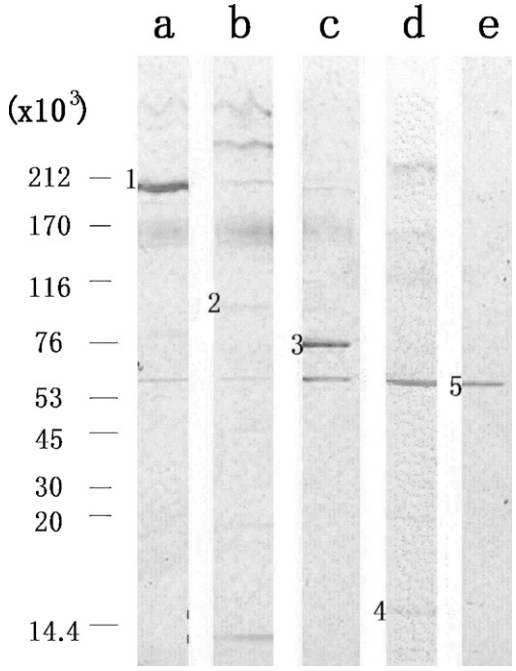

Fig. 3. SDS-PAGE of the binding proteins after the spot of anti-human C3 antibody (line a), haptoglobin antibody (line b), transferrin antibody (line c), transthyretin antibody (line d) or non-immunoreacted antibody (line e) was incubated with human plasma.

Fig. 4 shows the MALDI-TOF MS spectra of the extraction by 0.1% TFA solution, after spot C in Fig. 2c (a), spot D in Fig. 2d

Table 1
Protein spots analysis of SDS-PAGE and PMF using MALDI-TOF MS.

Spot number	Entry name	Protein name	Accession number	Protein score[a]	Sequence coverage (%)
1	CO3_HUMAN	Complement C3	P01024	78	19
2	HPT_HUMAN	Haptoglobin	P00738	70	13
3	TRFE_HUMAN	Serotransferrin	P02787	76	8
4	TTHY_HUMAN	Transthyretin	P02766	70	55
5	ALBU_HUMAN	Serum albumin	Q9P157	67	13

The proteins are as numbered in Fig. 3.

[a] Proteins score is $-10\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 60 are significant ($P<0.05$).

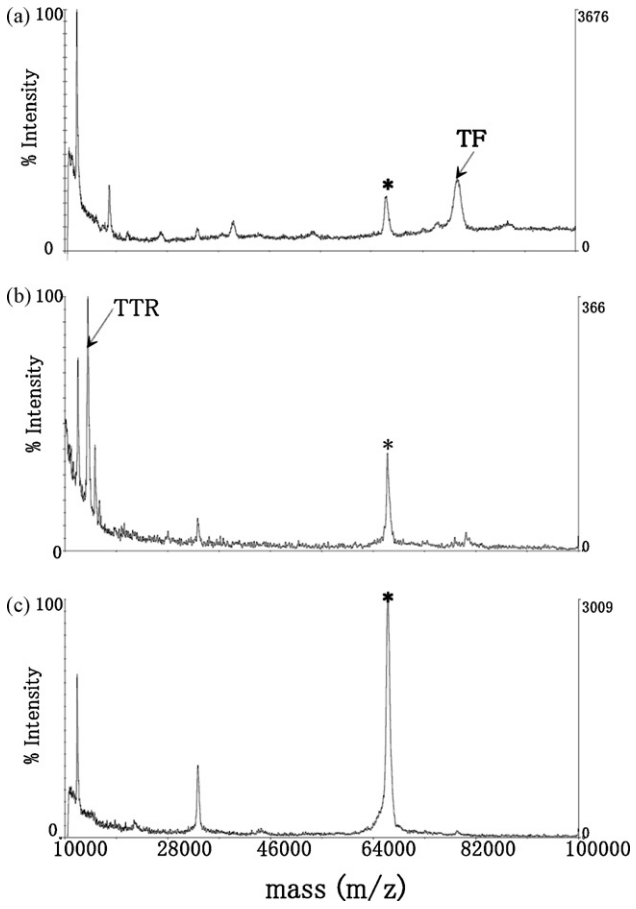

Fig. 4. MALDI-TOF MS spectra of the proteins bound to the membrane-immobilized anti-human transferrin antibody (a), anti-human transthyretin antibody (b) or non-immunoreacted antibody (c). The peaks at TF and TTY corresponded to human transferrin and transthyretin, respectively. The peak at *m*/*z* 66,590 (* in each spectrum) was non-specifically bound to the membrane-immobilized antibodies.

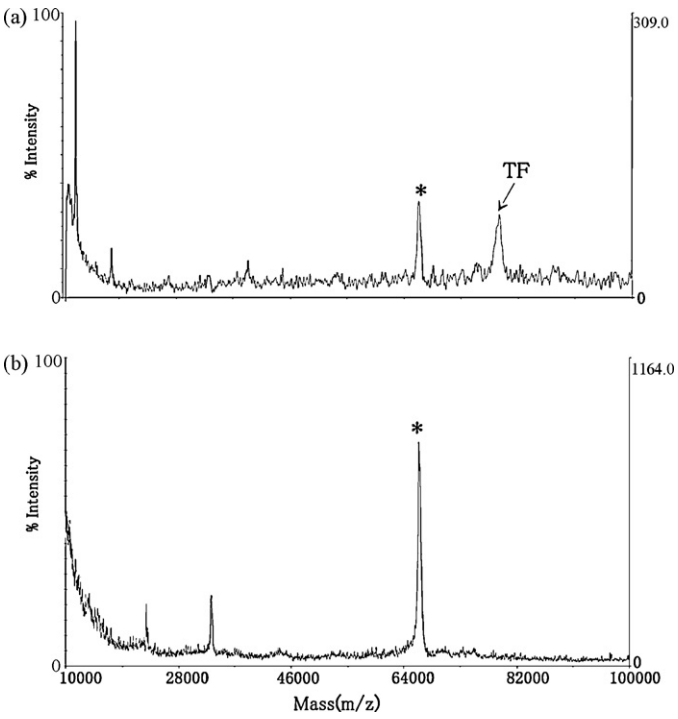

Fig. 5. MALDI-TOF MS spectra of the membrane-immobilized anti-human transferrin antibody (a) or non-immunoreacted antibody (b) binding to the membrane in the flow system; where fluid antigens in blood were directly captured by the membranes. TF, transferrin; *, non-specific binding protein.

(b) and the membrane-immobilized IgG (c) were incubated with human plasma. Peaks at *m*/*z* 66,590 were obtained in both spectra and are shown in Fig. 4a–c. Because the peak at *m*/*z* 66,590 corresponds to human serum albumin, the protein can bind non-specifically to the PVDF membrane. The protein was also extracted from the membrane when IgG (non-immunoreacted to human transferrin and transthyretine) was immobilized onto the membrane as shown in Fig. 4c. On the other hand, a peak at *m*/*z* 79,167 was observed when spot C was incubated with human plasma (arrow in Fig. 4a). From the molecular weight in SDS-PAGE (Fig. 3), the peak at *m*/*z* 79,167 corresponds to human transferrin. Further, the peak at *m*/*z* 13,860 was observed when spot D was incubated with human plasma (arrow in Fig. 4b). From the molecular weight in SDS-PAGE (Fig. 3), the peak at *m*/*z* 13,860 corresponds to human transthyretin. In both spectra, the peak at *m*/*z* 66,590 was observed (Fig. 4a and b), and the peak was also observed in the control as shown in Fig. 4c. The peak at *m*/*z* 66,590 was non-specifically bound to the membrane-immobilized antibodies. The bound protein is thought to be human serum albumin. On the other hand, specific antigens were not obtained by MALDI-TOF MS when anti-human C3 and haptoglobin antibodies were immobilized onto membranes in the present study. This may be because it is difficult to detect a peak of more than *m*/*z* 90,000 by MALDI-TOF MS spectrum as the amount of captured antigens are below the detectable limit of MALDI-TOF MS, or because it is difficult to crystallize antigens of such high molecular mass. In fact, it has been reported that antigens of less than *m*/*z* 30,000 such as serum amyloid P (*m*/*z* 25,458), serum amyloid A (*m*/*z* 11,678) and C-reactive protein (*m*/*z* 23,038) are quantitatively analyzed by MALDI-TOF MS after being isolated by antibody-immobilized membranes [12]. Fig. 5 shows the MALDI-TOF MS spectra of the binding protein to the membrane-immobilized anti-human transferrin antibody (a) and non-immunoreacted antibody (b), when human blood was applied to a flow system equipped with immunoaffinity membranes, as shown in Fig. 1. The spectra indicate that transferrin was specifically captured by the membrane-immobilized anti-human transferrin antibody (arrow in Fig. 5a), and the protein was not captured by the membrane-immobilized non-immunoreacted antibody. From these results, fluid antigens are thought to be captured by the immunoaffinity membrane in the flow system. It has been reported that cells can be captured by an antibody-coated microdevice by binding to antigen on the cell membrane [13]. Thus, the immunoaffinity membranes in the present study are thought to be used to capture flowing cells in the same manner.

4. Conclusions

The present study indicated that after separation, transferring and staining, immunoaffinity membranes of anti-transferrin, C3, haptoglobin and transthyretin antibodies were simultaneously produced. The antigens of transferrin and transthyretin were directly analyzed by MALDI-TOF MS after each antigen was captured by each immunoaffinity membrane. Furthermore, it was demonstrated that the immunoaffinity membrane can be utilized to capture antigens in flowing fluids such as blood. These results indicated that membrane-immobilized antibodies were simultaneously produced, and that the immunoaffinity membrane can capture specific substances in flowing fluids.

References

[1] S. Karoor, J. Molina, C.R. Buchmann, C. Colton, J.S. Logan, L.W. Henderson, Biotechnol. Bioeng. 81 (2003) 134.
[2] C. Charcosset, Z. Su, S. Karnoor, G. Daun, C.K. Colton, Biotechnol. Bioeng. 48 (1995) 415.
[3] Y. Shimazaki, A. Kodama, Anal. Chim. Acta 643 (2009) 61.
[4] T. Manabe, Y. Takahashi, T. Okuyama, Anal. Biochem. 143 (1984) 39.
[5] Y. Shimazaki, Y. Sugawara, Y. Ohtsuka, T. Manabe, Proteomics 3 (2003) 2002.
[6] W.J. Henzel, T.M. Billeci, J.T. Stults, S.C. Wong, C. Grimley, C. Watanabe, Proc. Natl. Acad. Sci. 90 (1993) 5011.
[7] P. James, M. Quadroni, E. Carafoli, G. Gonnet, Biochem. Biophys. Res. Commun. 195 (1993) 58.
[8] M. Mann, P. Hojrup, P. Roepstorff, Biol. Mass Spectrom. 22 (1993) 338.
[9] C.J.C. Pappin, P. Hojrup, A.J. Bleaby, Curr. Biol. 3 (1993) 327.
[10] B.T. Kurien, R.H. Scofield, Methods 38 (2006) 283.
[11] J.B. Olmsted, J. Biol. Chem. 256 (1981) 11955.
[12] S.H. Chen, H.K. Liao, C.Y. Chang, C.G. Juo, J.H. Chen, S.I. Chan, Y.J. Chen, Proteomics 7 (2007) 3038.
[13] R.D. Reif, M.M. Martinez, K. Wang, D. Pappas, Anal. Bioanal. Chem. 395 (2009) 787.