Discrimination of glycoproteins via two-color laser-induced fluorescence detection coupled with postcolumn derivatization in capillary electrophoresis

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Here, we report a novel method consisting of capillary electrophoretic separation followed by two-color laser-induced fluorescence detection with postcolumn derivatization. The method can be used to discriminate glycoproteins in a protein mixture containing both glycosylated and unglycosylated proteins. The detector-permitted simultaneous measurements of two electropherograms obtained by 450-nm (diode laser) and 532-nm (Nd:YAG laser) lasers excited native proteins following postcolumn derivatization with naphthalene-2,3-dicarboxaldehyde and concanavalin A labeled with tetramethylrhodamine (Rh-Con A), respectively. So, a protein can be assigned as glycosylated if it shows a peak at the same migration time in both electropherograms. According to the proposed principle, in a single run we discriminated a glycosylated protein (thyroglobulin) from an unglycosylated protein (albumin) in the presence of Rh-Con A. Because the methodology permits the simultaneous detection of native proteins and their complexes with a fluorescently labeled probe, it should have broad applicability to binding assays.

1 Introduction

In biological systems, several chemical modifications of molecules play important roles for regulating life activity. Proteins are subject to post-translational modifications such as glycosylation and phosphorylation, which lead to the functionalization of proteins in biological systems. In particular, glycosylated proteins participate in some of the key roles in cellular recognition, protein folding, and protein trafficking [1]. In addition, several clinical biomarkers and therapeutic targets in cancer are glycoproteins, since different changes in O- and N-linked glycoproteins are observed in cancer cells [2,3].

A variety of analytical methods have been developed for glycoproteins, glycans, and glycoforms, e.g., current technologies include a microarray that is based on the selective binding of lectins [4,5], lectin affinity chromatography [6,7], high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) [8], western blotting [9], and capillary electrophoresis (CE) [10]. In general, HPLC-MS is an excellent technique for the analyses of glycans and glycopeptides produced by enzymatic digestion of a glycoprotein [8], since the molecular masses of glycans and glycopeptides are determined precisely. However, intact glycoproteins are too large to be measured directly by MS. Conversely, in these techniques, CE has several advantages because of its high resolution, rapid separation, and low consumption of samples. In fact, CE showed excellent performance in the analyses of glycans [11], glycoforms [12], and glycoproteins [13].

In the studies of glycans, MS [14-16] and laser-induced fluorescence (LIF) [17,18] were frequently coupled with CE. In terms of sensitivity, LIF is superior to the other detection methods, while MS has the advantage of providing information on molecular masses. Such an advantage for LIF makes it suitable for biological samples that are frequently difficult to obtain on a large scale. Therefore, LIF is attractive for analyses not only of glycans, but also for other biomolecules such as proteins and DNA.

In protein analyses, LIF has demonstrated a high sensitivity that realizes the detection of single molecules [19] and the analysis of single cells [20]. One of the problems with LIF is the need to label proteins with a fluorescent dye before separation. To solve that problem, postcolumn [21] and oncolumn [22] derivatizations are useful since native proteins can be injected directly into a capillary with no labeling. We also reported the postcolumn derivatization of proteins separated by capillary sieving electrophoresis, which permitted direct injection of denatured proteins in LIF detection [23,24].

To enhance the information in LIF, a two-color excitation has been developed for CE separations in the analyses of DNA [27-29], cytokines [30], immune complexes [31], metabolites in a single cell [32], and conjugates of green fluorescent protein and protein kinase C [33]. Light-emitting diodes are also employed for two-color excitation where some fluorescent dyes are detected by ultraviolet and visible lights [34]. Although there is no report so far, coupling of two-color excitation with postcolumn derivatization is

quite promising as a method used to extend the applicability of a two-color excitation detection scheme. When using postcolumn derivatization, at least one of the target chemical species can be detected without prelabeling and be injected in its native form. Herein we report a novel LIF method with two visible lasers coupled with postcolumn derivatization. We validated this method when identifying glycoproteins bound with a probe lectin, concanavalin A, labeled with tetramethylrhodamine.

2 Materials and methods

2.1 Materials

Sodium tetraborate decahydrate, albumin (from human serum), sodium fluorescein, hydrochloric acid, sodium chloride, sodium dodecyl sulfate, methanol, sodium hydroxide, rhodamine B, and 2-mercaptoethanol were obtained from Wako Pure Chemicals (Osaka, Japan). Manganese chloride tetrahydrate was from Ishizu pharmaceutical (Osaka, Japan). Thyroglobulin, naphthalene-2,3-dicarboxaldehyde (NDA), and calcium chloride dehydrate was purchased from Sigma-Aldrich (MO, USA). A solution of rhodamine labeled concanavalin A (Rh-Con A), which is labeled with tetramethylrhodamine isothiocyanate, was from Vector Laboratories (CA, USA) (5.0 mg/ml active conjugate, molar ratio, Rh/Con A = 3.5). The solution contained 10 mM HEPES (pH 7.5), 0.15 M NaCl, 0.1 mM Ca^{2+}, 0.01 mM Mn^{2+}, and 0.08% sodium azide. Water used in all experiments was

purified by means of an ultrapure MILLI-Q system (Millipore, Molsheim, France).

Standard Solutions of NDA and 2-mercaptoethanol were prepared by dissolving the reagents in methanol so as to form concentrations of 5 and 80 mM, respectively. Borate buffer solutions were prepared by dissolving the appropriate amounts of sodium tetraborate dehydrate in water. The running buffer solution was prepared by dissolving 1.25 mmol of sodium tetraborate in 100 mL of water (50 mM borate buffer, pH 9.2). A derivatizing solution was prepared each day by mixing the standard solutions of 5 mM NDA, 80 mM 2-mercaptoethanol, and 0.1 M borate buffer (pH 9.2) at the ratio of 2:1:7, resulting in a buffer solution containing 1 mM NDA, 8 mM 2-mercaptethanol, and 30% methanol. The derivatizing solution was filtered using 0.2 μm syringe filters (13CP020AS, ADVANTEC, Tokyo, Japan) to remove particulates before use.

Sample solutions of proteins were dissolved in the running buffer. The eggs of hens were purchased from a local supermarket. After separating the egg white from the yolk, 500 μL of the egg white was diluted with 1 mL of 0.5 M NaCl solution. In the reaction mixtures of the standard proteins and egg samples with Rh-Con A, 0.5 mM concentrations of Ca^{2+} and Mn^{2+} were added. Sodium fluorescein was also added to the reaction mixture as an internal standard to correct the migration times and peak areas of the analytes. All samples were measured three times to evaluate reproducibility of migration times.

2.2 Apparatus

A schematic illustration of the instrumentation is shown in Fig. 1. The apparatus for CE was a homemade system equipped with a postcolumn reactor using sheath flow, which is the same alignment as that reported by Rose and Jorgenson [35]. The CE system was housed in a cage equipped with a safety interlock to prevent electric shock from the high voltage that was applied for electrophoresis. In the postcolumn reactor, a tapered capillary with 50 μm i.d., 360 μm o.d., and 40 cm length (PicoTip EMITTER, TT360-50-50-CE-5, coating; P200P, NEW OBJECTIVE, MA, USA) and a large bore capillary with 530 μm i.d., 660 μm o.d., and 13 cm length (GL Science, Tokyo, Japan) were used for the separation and reaction capillaries, respectively. The separation capillary was connected into the reaction capillary by a tee connector (P-727 PEEK Tee, GL Science, Tokyo, Japan). The derivatizing solution was introduced from the side of the tee connector where the separation capillary was inserted into the reaction capillary, so the derivatizing solution flowed along outside the separation capillary. The derivatizing solution was pumped by a micro syringe pump (MSPE-1, AS ONE, Osaka, Japan).

In LIF detection, a 40 mW Nd:YAG laser emitting at 532 nm (Z40M18B-F-532-pz, Z-LASER, Germany) and a 20 mW diode laser emitting at 450 nm (Z20M18H-F-450-pe, Z-LASER, Germany) were used for excitation light sources. Two solenoid beam stoppers (F116-1, SURUGA SEIKI, Shizuoka, Japan) controlled by a shutter controller

(F77-7, SURUGA SEIKI, Shizuoka, Japan) were located on the pathways of the lasers. The laser beams were overlapped by a dichroic filter (XF2077, Omega Optical, Inc., VT, USA), and then were focused at the point 750 μm away from the tapered end of the separation capillary. One of two lasers irradiated the capillary by opening and closing the beam stopper alternately at 2 Hz and data acquisition of fluorescence was synchronized with the opening and closing of the beam stoppers. Therefore, each electropherogram obtained by at excitations of either 450 or 532 nm was recorded at 1 Hz of sampling frequency. Fluorescence was collected by a photomultiplier tube (Model R3896, Hamamatsu, Shizuoka, Japan) biased at 800 V with a power supply (Model C3830, Hamamatsu, Shizuoka, Japan) after passing through a long-pass filter (HQ470LP, Chroma Technology Corporation, VT, USA), notch filters (NF01-532U-25, Semrock, NY, USA and 532nm Rugate Notch Filter, Edmund Optics Inc., NJ, USA), and a special filter (0.5 mm pinhole). The collection of the fluorescence signals and the switching of the beam stoppers was regulated by a homemade LabView program (National Instruments, CA, USA) using a personal computer (Panasonic, Japan) equipped with an A/D converter (NI 9215, National Instruments, CA, USA).

The separation capillary and the reaction capillary were filled with the running buffer and the derivatizing solution, respectively. Sample solutions were hydrodynamically injected for 10 s into the capillary from the sample vial raised 10 cm above the outlet vial.

The separation capillary was initially conditioned by rinsing with 0.1 M NaOH, deionized water, and the running buffer. After the experiments, the separation capillary was filled with 0.1 M NaOH to keep the surface of the capillary activated. The reaction capillary was conditioned by rinsing with water and the running buffer sequentially, and after the experiment, the reaction capillary was flushed with 0.1 M NaOH and was filled with water. A constant potential of 10 kV was applied to the separation and reaction capillaries by a high-voltage power supply (HCZE-30PN0.25, Matsusada Precision, Shiga, Japan), and the derivatizing solution was flowed into the reaction capillary at a flow rate of 0.2 μl min^{-1} during the separation.

3 Results and discussion

3.1 Two-color laser excitation

To characterize the two-color excitation LIF, solutions of fluorescein, rhodamine B, and their mixture were separated without postcolumn derivatization. When two fluorophores, fluorescein and rhodamine B, were injected into a capillary independently, each fluorophore provided an intense peak in the electropherograms at excitations of 450 and 532 nm, respectively. The electropherograms of their mixture showed a clear separation of the two dyes and each dye represented an intense peak in the electropherograms obtained at 450- and 532-nm excitations with no interference.

3.2 Migration behavior of tetramethylrhodamine-labeled concanavalin A

Concanavaline A (Con A), a well-known lectin, is a complex molecule that binds with metal ions such as Ca^{2+} and Mn^{2+} and exists as a dimer or tetramer depending on pH. A structural dependence of Con A on pH has been reported, as Con A exhibits a reversible dimer-tetramer transition at pH that ranges from 6.0 to 7.2 [36]. This molecular transition was nearly complete at pH 7.2 so that Con A was expected to exist as a tetramer under the present experimental conditions at pH 9.2.

Conversely, metal ions such as Ca^{2+} and Mn^{2+} play important roles in the binding of Con A with sugars. The conformation of Con A that binds metals tightly exhibits a high affinity for sugars and is referred to as a "locked" form while an "unlocked" form containing no metal ions cannot interact with sugars [37]. Therefore, we attempted to clarify the conformational change in Rh-Con A at different concentrations of metal ions, Ca^{2+} and Mn^{2+}. The electropherograms of Rh-Con A at the different concentrations of metal ions are shown in Fig. 2. Freshly prepared Rh-Con A before the electrophoretic run, which contained 0.01 mM Ca^{2+} and 0.001 mM Mn^{2+}, appeared as a broadened peak in the electropherogram at an excitation of 532 nm (Fig. 2(a)). However, after standing for 24 h at room temperature, the peak was split into two peaks (Fig. 2(b)). The peak splitting was attributed to the separation of free Rh-Con A and its metal complex.

Conversely, the peak of Rh-Con A became narrower than that of the fresh solution with no splitting of the peak in the presence of 0.5 mM of Ca^{2+} and Mn^{2+} ((Fig. 2(c)). Furthermore, an independent addition of Mn^{2+} prevented the peak splitting of Rh-Con A, whereas the addition of Ca^{2+} showed no influence on the electropherogram. Therefore, we confirmed that the two peaks generated from Rh-Con A corresponded to the complex with Ca^{2+} and Mn^{2+}, and to the complex without Mn^{2+}. Consequently, we added 0.5 mM of Ca^{2+} and Mn^{2+} to sample solutions to prevent a conformational change in Rh-Con A.

3.3 Discrimination of glycosylated and unglycosylated proteins

Thyroglobulin is a glycoprotein with sugar moieties that bind with Con A [38]. Thus, thyroglobulin was reacted with Rh-Con A to detect the complex of thyroglobulin with Rh-Con A using two-color excitation. We added different amounts of Rh-Con A to solutions of thyroglobulin and obtained the electropherograms. The results are shown in Fig. 3. A peak of free thyroglobulin appeared only in the electropherogram by 450-nm excitation as seen in Fig. 3(a) since thyroglobulin was reacted with NDA in the postcolumn reactor, resulting in a fluorescent derivative which can be excited by the 450-nm laser. It should be noted that the migration time of free thyroglobulin is 431.7± 4.9 s, which is much different from that of Rh-Con A (326.8±0.6 s in Fig. 2(c)). So, if thyroglobulin has no interaction with Rh-Con A, they would be separated completely.

When we analyzed a mixture of thyroglobulin and Rh-Con A at a concentration ratio of thyroglobulin : Rh-Con A = 1 : 1, the mixture showed a single peak at the same migration time in both electropherograms obtained by excitations at 450 and 532 nm (Fig. 3(b)). The migration time of the peak was 412.8±4.0 s (n=3), which was slightly earlier than that of free thyroglobulin (431.7±4.9 s). Obviously, we can assign the peak to the complex between thyroglobulin and Rh-Con A since the peaks corresponding to free thyroglobulin and Rh-Con A disappeared completely, as shown in Fig. 3(b).

With an increasing concentration ratio of Rh-Con A, the peak shape of thyroglobulin varied with a shift in the migration time. At a concentration ratio of thyroglobulin : Rh-Con A = 1 : 0.25, the peak top was slightly different between the electropherograms at excitations of 450 and 532 nm. This suggested that free thyroglobulin coexisted with the complex at that ratio. Further increases in the Rh-Con A concentration (thyroglobulin : Rh-Con A = 1 : 0.5) led to a peak narrowing of the complex with no deviation in the peak top observed in the electropherograms at excitations of 450 and 532 nm, although the large peak in the electropherograms at excitation of 450 had a small shouldered peak corresponding to free thyroglobulin. However, when the equivalent concentration was exceeded (thyroglobulin:Rh-Con A = 1 : 1.5), shot noises appeared and there was a distortion of the peak for the complex (Fig. 3(c)). The peak intensity then decreased at a concentration ratio of 1 : 2, which suggested the formation of precipitation (Fig. 3(d)).

Similar to an earlier study [39], precipitation at a high concentration of Rh-Con A under the present experimental conditions also supports the complex formation between thyroglobulin and Rh-Con A. These results suggest that a 1:1 complex is a soluble form in an aqueous solution at pH 9.2. Therefore, the composition of the complex also can be determined by titrating a glycoprotein with a suitable lectin using the proposed method.

To confirm the selective binding of Rh-Con A with thyroglobulin, we employed albumin (unglycosylated protein) as a negative control. Both in the absence and the presence of Rh-Con A, we observed a single peak corresponding to albumin in the electropherograms at an excitation of 450 nm, and there was no overlap with the peak of Rh-Con A (Fig. 4(a) and 4(b)). Thus, we can verify that albumin is not glycosylated since no peak appeared at the same migration time as albumin in the electropherogram at an excitation of 532 nm. In Fig. 4(b), the peak of Rh-Con A is split in the presence of albumin, that is, Mn^{2+} will be dissociated from Rh-Con A. It is known that albumin binds with several metal ions including Mn^{2+} [40], so the result of Fig. 4(b) indicates that albumin induces the dissociation of Mn^{2+} in Rh-Con A.

The limits of detection (LOD, S/N=3) for free thyroglobulin, albumin, Rh-Con A, and the complex between thyroglobulin and Rh-Con A were estimated to be 320 nM (450 nm), 340 nM (450 nm), 57 nM (532 nm), and 5.9 nM (532 nm), respectively. Here we assumed that thyroglobulin forms the complex with Rh-Con A completely at the

concentration ratio of 1:1. No difference in the LODs was observed between thyroglobulin and albumin, whereas the complex showed the lowest LOD. The LODs of proteins obtained by 450-nm excitation may be improved by increasing the laser power since the power of 532-nm laser is two-fold higher than that of 450-nm laser in this study.

We also attempted the separation of thyroglobulin and albumin in the absence and in the presence of Rh-Con A. Thyroglobulin co-migrated with albumin without Rh-Con A (Fig. 4(c)), whereas the complex between thyroglobulin and Rh-Con A was slightly separated from albumin in the presence of Rh-Con A (black line in Fig. 4(d)). In addition, only the peak corresponding to the complex was observed in the electropherogram at an excitation of 532 nm (gray line in Fig. 4(d)). The separation of thyroglobulin from albumin was achieved due to a narrowing of the peak and the mobility shift by the complex formation between thyroglobulin and Rh-Con A. However, with only a 450-nm excitation, the mobility shift was too small to completely separate thyroglobulin from albumin. These results indicated that the present technique could be used to discriminate a glycosylated protein from an unglycosylated protein even when they were co-eluted as a single peak.

4 Conclusions

Two-color excitation LIF coupled with postcolumn derivatization represents a

powerful means for identifying the complex of a glycoprotein and a probe lectin that has been labeled with a fluorescent tag. A blue laser emitting at 450 nm excited the proteins derivatized with NDA in the postcolumn reactor while a green laser emitting at 532 nm was used for the excitation of Rh-ConA as a probe for the sugar chains of glycoproteins.

Thyroglobulin interacted with Rh-ConA, resulting in a single peak in both electropherograms obtained at excitations of 450 and 532 nm, whereas albumin, which is an unglycosylated protein, showed a single peak only in the electropherogram with an excitation of 450 nm because there was no interaction with Rh-ConA due to a lack of sugar chains. Therefore, the detection method facilitated the identification of a protein as being either glycosylated or unglycosylated.

In a mixture of thyroglobulin and albumin, thyroglobulin was apparently discriminated from albumin in the presence of Rh-ConA, although they co-migrated in the absence of Rh-ConA. Therefore, the present detection scheme is significantly promising for the discrimination of glycosylated and unglycosylated proteins in a protein mixture. Furthermore, the two-color excitation method would be applicable to several fields since we can detect native proteins and labeled molecules simultaneously. So, the results of the present study are expected to promote further research on applications of the proposed method.

The proposed method will determine whether a protein is glycosylated or not, using an

appropriate lectin. However, in this study, the method is only applicable to the detection of glycoproteins which bind with Con A. So, further investigation using different lectins is necessary for extending the applicability of the detection scheme. Nevertheless, the two-color detection coupled with postcolumn derivatization will be promising since no prelabeling of proteins are necessary, native proteins react with a fluorescent probe molecule directly, and two electropherograms are compared directly without no correction of the migration times.

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5 References

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Figure Legends

Figure 1. Schematic illustration of experimental setup.

Figure 2. Electropherograms of Rh-Con A.

(a) Rh-Con A prepared freshly, (b) a solution of (a) stored at room temperature for 24 h, (c) Rh-Con A solution containing 0.5 mM $CaCl_2$ and $MnCl_2$ stored at room temperature for 24 h. Black line, 450-nm excitation; gray line, 532-nm excitation. The concentration of Rh-Con A was 4.9 μM. The separation capillary, 50 μm i.d. and 40 cm length; the reaction capillary, 530 μm i.d. and 13 cm length; applied potential, 10 kV. Derivatizing solution, 1 mM NDA, 8 mM 2-mercaptethanol, and 30% methanol in 70 mM borate buffer (pH 9.2); flow rate of the derivatizing solution, 0.2 μl min^{-1}. The migration buffer contained 50 mM borate. The sample solutions were injected for 10 s by raising the sample vial 10 cm above the outlet vial.

Figure 3. Electropherograms of thyroglobulin.

(a) 4.9 μM thyroglobulin, (b) a mixture of 4.9 μM thyroglobulin and 4.9 μM Rh-Con A (1 : 1), (c) a mixture of 4.9 μM thyroglobulin and 7.35 μM Rh-Con A (1 : 1.5), (d) a mixture of 4.9 μM thyroglobulin and 9.8 μM Rh-Con A (1 : 2). Black line, 450-nm excitation; gray line, 532-nm excitation. Inset in (c) is magnification at a range from 390 to

430 s. All sample solutions contained 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$. The conditions for electrophoresis are the same as those shown in Figure 2.

Figure 4. Electropherograms of albumin and a mixture of albumin and thyroglobulin. (a) 49 μM albumin, (b) 49 μM albumin with 4.9 μM Rh-Con A, (c) 4.9 μM thyroglobulin and 49 μM albumin, (d) 4.9 μM thyroglobulin and 49 μM albumin with 4.9 μM Rh-Con A. Black line, 450-nm excitation; gray line, 532-nm excitation. Peak assignment: (a) 1= albumin (migration time, 444 s), (b) 1= albumin (migration time, 448 s), 2=Rh-Con A (migration time, 327.5 s), (c) 3=albumin and thyroglobulin (migration time, 492 s), (d) 1= albumin (migration time, 469 s), 4=complex of thyroglobulin with Rh-Con A (migration time, 444.5 s). The conditions for electrophoresis are the same as those shown in Figure 2.

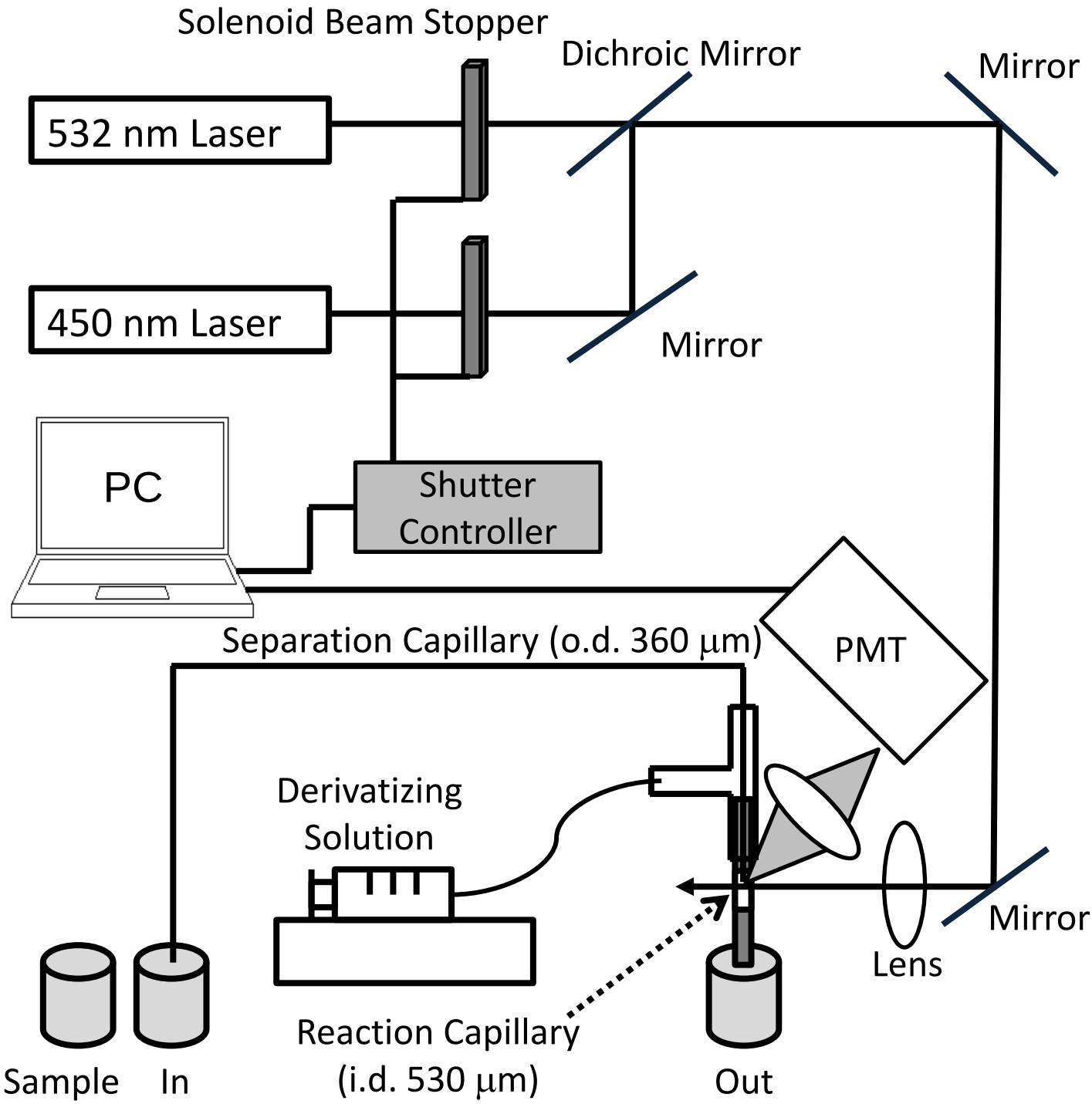
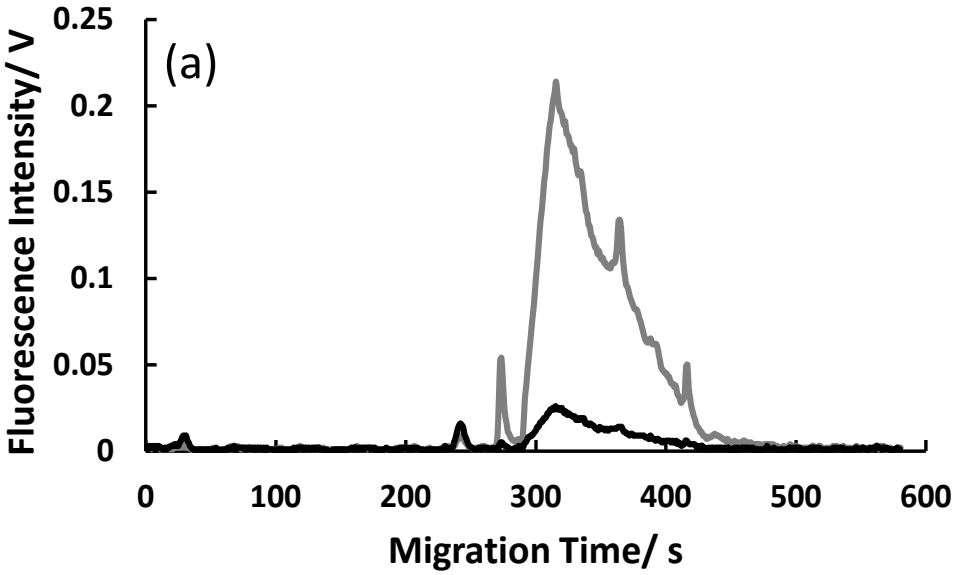

Solenoid Beam Stopper
Dichroic Mirror
Mirror
532 nm Laser
450 nm Laser
Mirror
PC
Shutter Controller
Separation Capillary (o.d. 360 μm)
PMT
Derivatizing Solution
Lens
Mirror
Reaction Capillary (i.d. 530 μm)
Sample
In
Out

Figure 1


(a)
Fluorescence Intensity/ V
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0.15
0.1
0.05
0
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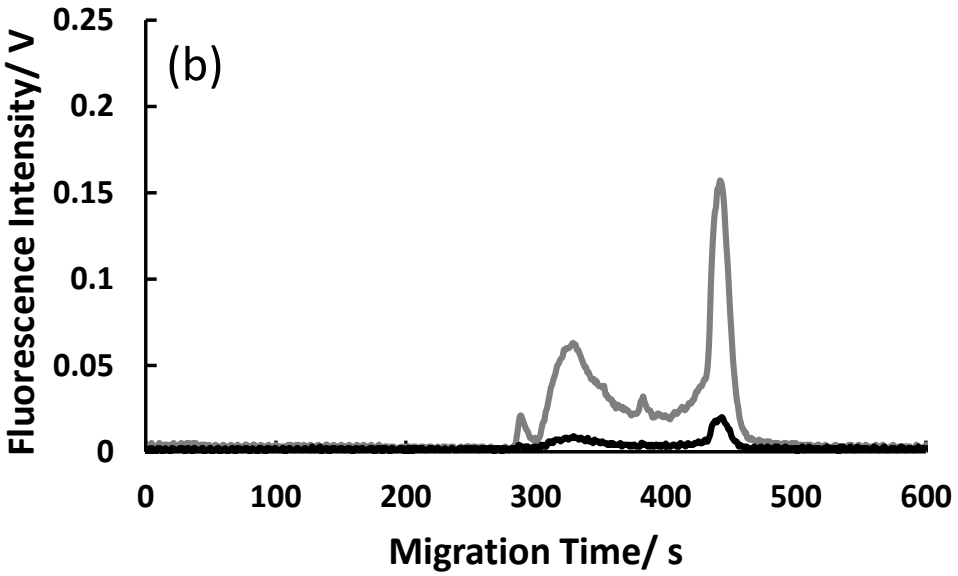
200
300
400
500
600
Migration Time/ s


(b)
Fluorescence Intensity/ V
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
500
600
Migration Time/ s

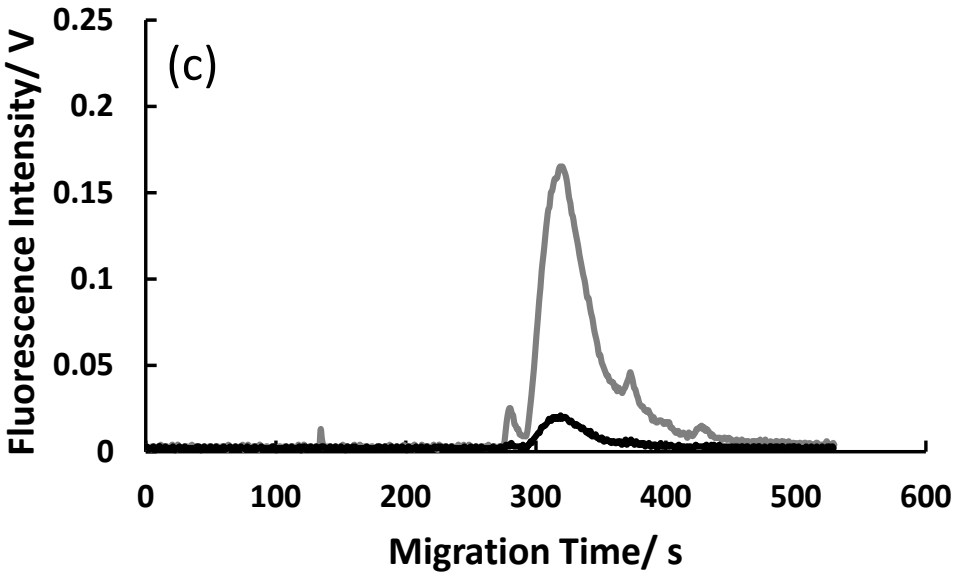

(c)
Fluorescence Intensity/ V
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
500
600
Migration Time/ s

Figure 2

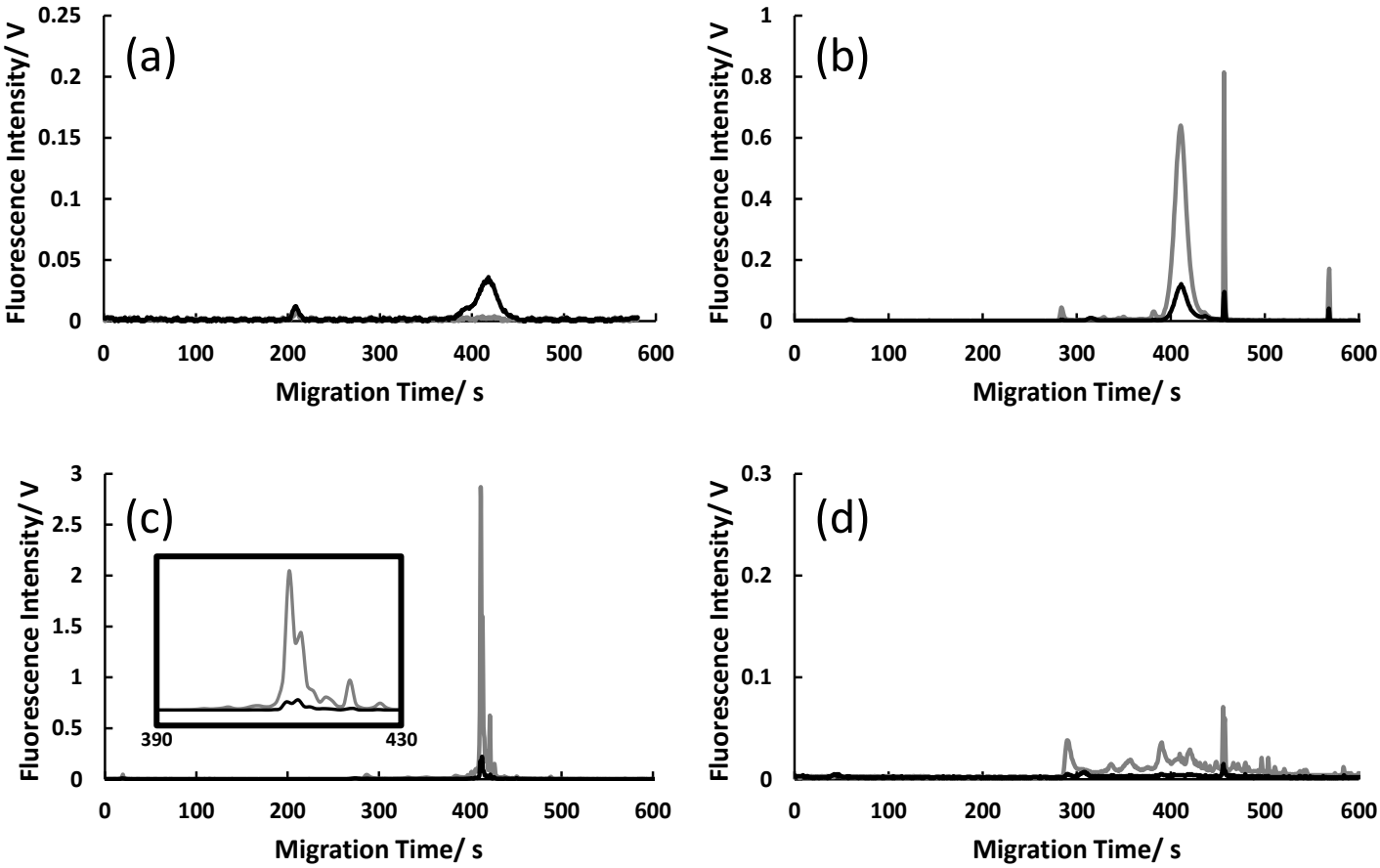

(a)
Fluorescence Intensity/ V
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
500
600
Migration Time/ s
(b)
Fluorescence Intensity/ V
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
Migration Time/ s
(c)
Fluorescence Intensity/ V
3
2.5
2
1.5
1
0.5
0
390
430
0
100
200
300
400
500
600
Migration Time/ s
(d)
Fluorescence Intensity/ V
0.3
0.2
0.1
0
0
100
200
300
400
500
600
Migration Time/ s

Figure 3

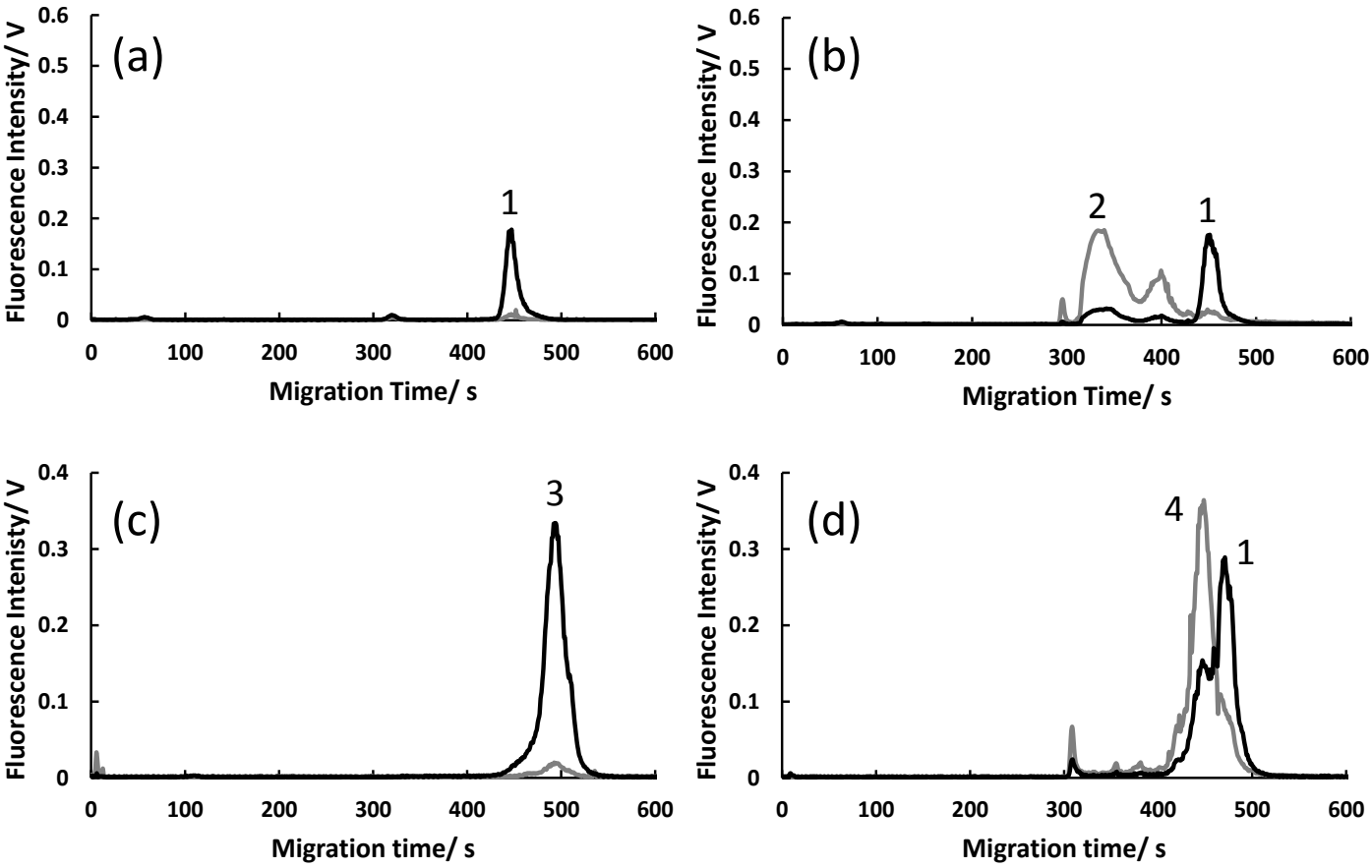

(a)
Fluorescence Intensity/ V
0.6
0.5
0.4
0.3
0.2
0.1
0
1
0
100
200
300
400
500
600
Migration Time/ s
(b)
Fluorescence Intensity/ V
2
1
Migration Time/ s
(c)
Fluorescence Intenisty/ V
0.4
3
Migration time/ s
(d)
Fluorescence Intensity/ V
4
1
Migration time/ s

Figure 4