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Rapid labelling of amino neurotransmitters with a fluorescent thiol in the presence of o-phthalaldehyde

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Abstract

Laser-induced fluorescence (LIF) detection often requires labelling of analytes with fluorophores; and fast fluorescent derivatization is valuable for high-throughput analysis with flow-gated capillary electrophoresis (CE). Here, we report a fast fluorescein-labelling scheme for amino neurotransmitters, which were then rapidly separated and detected in flow-gated CE. This scheme was based on the reaction between primary amines and o-phthalaldehyde (OPA) in the presence of a fluorescent thiol, 2-[(5-fluoresceinyl)aminocarbonyl]ethyl mercaptan (FACE-SH). The short reaction time (<30 s) was suited for on-line mixing and derivatization that was directly coupled with flow-gated CE for rapid electrophoretic separation and sensitive LIF detection. To maintain the effective concentration of reactive FACE-SH, Tris(2-carboxyethyl)phosphine (TCEP) was added to the derivatization reagents to prevent thiol loss due to oxidation. This labelling scheme was applied to the detection of neurotransmitters by coupling in vitro microdialysis with on-line derivatization and flow-gated CE. It is also anticipated that this fluorophore tagging scheme would be valuable for on-chip labelling of proteins retained on support in solid phase extraction.

Keywords

Laser-induced fluorescence; Flow-gate capillary electrophoresis; fluorescent labelling; neurotransmitter; microdialysis

1 Introduction

Flow-gated capillary electrophoresis (CE) or microchip CE coupled with microdialysis has become an emerging tool for monitoring the dynamics of biological processes, such as variations of neurotransmitters in brain and chemical release of cells upon stimulation [1-7]. The advantages of flow-gated CE include rapid separation, reduced consumption of samples, and ease of integration. However, CE suffers in poor detection sensitivity compared with HPLC. To improve the sensitivity, laser-induced fluorescence (LIF) is commonly used in CE and microchip CE. Nonetheless, many analytes of interest lack fluorophores; therefore, fluorescent derivatization, i.e. tagging external fluorophores to analytes, is a common strategy to take advantage of the sensitivity in LIF detection.

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Fluorescent derivatization involves the chemical reaction between analytes and derivatization reagents. It is essential for the derivation to have fast reaction kinetics so that rapid overall analysis can be performed on the advantageous CE platform. For compounds carrying primary amine groups, numerous labelling routes are able to produce high sensitive fluorescence [4,8-12], but most of these schemes take a long time to complete derivatization. Alternatively, o-phthalaldehyde (OPA) is able to rapidly derivatize compounds (<1 minute) bearing primary amine groups in the presence of a thiol [13-17], typically β -mercaptoethanol (BME) [17-19]. The derivatives, isoindoles, have optimum emission at ~455 nm under the optimum excitation at ~340 nm. OPA has been employed in the detection of peptides and amino acids with the LODs of ~100 nM after electrophoretic separations [20-22]. The disadvantages of this scheme include instability of derivatives, low quantum yield, and UV laser for excitation. To take advantage of its fast reaction kinetics, a fluorescent thiol has been used to replace the non-fluorescent thiol in the derivatization mixture [23]. Hapuarachchi and Aspinwall have used (5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino)fluorescein) (SAMSA-F) to label amino compounds in the presence of OPA, in which the LODs of involved analytes were lowered to less than 2 nM while preserving the fast reaction kinetics (< 10 s). However, SAMSA-F has two isomers which correspondingly generate two derivative isomers for each analyte and thus complicating electropherograms. Therefore, a different fluorescent thiol with a defined structure may improve the detectability of analytes by cleaning extra peaks in an electropherogram.

In this paper, we report a novel fluorescent thiol, 2-[(5-fluoresceinyl)aminocarbonyl]ethyl mercaptan (FACE-SH), which was tagged to amino neurotransmitters under the assistance of OPA. As usual, the reaction kinetics was fast so that on-line derivatization was feasible; high quantum yield of fluorescein was able to enhance the detection sensitivity; and simplified electropherograms were observed for resolving multiple amino compounds. Additionally, TCEP (Tris(2-carboxyethyl)phosphine) was proposed to mix with the derivatization mixture to prevent dimerization of FACE-SH, which would otherwise decrease the effective amount of FACE-SH for the derivatization reaction. Finally, this scheme was applied to the detection of amino acids neurotransmitters in cerebrospinal fluid (CSF) via in vitro microdialysis coupled with on-line derivatization and rapid electrophoretic separations.

2 Materials and methods

2.1 Chemicals

Amino acids, dopamine hydrochloride (DA), D/L-norepinephrine hydrochloride (NE), sodium tetraborate, and TCEP were purchased from Sigma (St. Louis, MO, USA). OPA was purchased from MP Biomedicals (OH, USA). Dimethylformamide (DMF), sodium hydroxide, tetrasodium salt of ethylenediaminetetraacetic acid (EDTA), and chemicals (NaCl, KCl, MgSO₄, CaCl₂, and Na₂HPO₄) for preparing artificial cerebrospinal fluid (aCSF) were purchased from Fisher Scientific (Chicago, IL, USA).

FACE-SH was ordered from Toronto Research Chemicals (Toronto, ON, Canada). Cerebrospinal fluid (CSF) collected from the striatum of Sprague Dawley rats was

purchased from BioreclamationIVT (Long Island, NY, USA). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

The FACE-SH stock solution at 10.0 mM was prepared in anhydrous DMF and stored at -20°C , and the 1.0 mM of FACE-SH solution was prepared by diluting the stock solution with deionized (DI) water and methanol in the ratio of 1:1 (v/v) on a daily basis. The stock solution of OPA at 50.0 mM was prepared in 10% methanol/water by volume. The stock solution of TCEP at 50.0 mM was prepared in DI water. Borate buffer was prepared by dissolving tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 100.0 mM) in DI water, and its pH was adjusted to desired values using 1.0-M NaOH. The stock solution of EDTA at 100.0 mM was prepared in DI water without pH adjustment. DA and NE stock solutions at 10.0 mM each were respectively prepared in 20 mM HCl solutions with pH 2.5 on a weekly basis, and they were stored in a refrigerator. Amine solutions (arginine, glutamine, gamma-aminobutyric acid (GABA), serine, alanine, taurine, glycine, glutamine, glutamate, and aspartate) at 10 mM were prepared in DI water, and were diluted to appropriate concentrations with aCSF. The aCSF consisted of 145.0 mM NaCl, 2.7 mM KCl, 1.0 mM MgSO_4 , 1.2 mM CaCl_2 , and 2.0 mM Na_2HPO_4 , and its pH was adjusted to 7.4 with NaOH [24].

2.2 Instrumentation

A similar custom-built detection system has been described elsewhere [25,26]. Briefly, a 491-nm laser (Cobolt Inc., San Jose, CA, USA) beam was spectroscopically filtered through an interference bandpass filter at 492 ± 5 nm and then focused on the separation capillary through a 100x oil-immersion objective with the numerical aperture of 1.3 (Carl Zeiss Microscopy, Thornwood, NY, USA). The fluorescence was collected by using the same objective and then passed to a photomultiplier tube (PMT) with the part #R11540 (Hamamatsu Photonics, Japan) after emission filtration with a bandpass filter (520 ± 18 nm). The current signal generated by the PMT was pre-amplified and then converted to voltages by an SR570 current preamplifier (Stanford Research Systems, Sunnyvale, CA, USA). Voltage signals were finally recorded by a LabVIEW program.

For on-line mixing, a 4-syringe pump (Chemyx Inc., Stafford, TX, USA) was used to supply sample and derivatization reagents through two gastight Hamilton syringes (Reno, Nevada, USA). The flow gate and other interfaces were fabricated with poly(dimethylsiloxane) (PDMS) as described earlier [25,27]. Sample injection and separation was performed by applying a negative voltage on the capillary outlet reservoir, while the inlet side was grounded, with a high-voltage power supply (Model CZE1000R) purchased from Spellman High Voltage Electronics Corporation (Hauppauge, NY, USA). This process was controlled by a LabVIEW program.

2.3 General experimental conditions

The separation capillary was 10 μm in ID and 360 μm in OD. It was 17.0 cm in total length and 10.0 cm in effective length. Fresh separation buffer was supplied via a 50-mL plastic syringe powered by a syringe pump purchased from New Era Pump Systems, Inc. (Model # NE-1010, Farmingdale, NY, USA). The gating flow of the separation buffer was controlled with a 3-way solenoid pinch valve purchased from Cole-Parmer (Vernon Hills, IL, USA).

Two solutions were prepared separately for off-line or on-line mixing at 50/50 by volume: (a) derivatization reagents, consisting of 600 μM of FACE-SH, 5.0 mM TCEP, 10.0 mM OPA, 10.0 mM EDTA, 20.0 mM tetraborate buffer at pH 10.6, and 10% organic solvent (9% methanol and 1% DMF), unless otherwise stated; (b) sample, aCSF with amino compounds dissolved, or aCSF for microdialysis sampling. For condition optimization, off-line mixing was performed by pipetting samples and derivatization reagents to be mixed in a centrifuge tube. A silica capillary with 100 μm in ID and 15.0 cm in length was used for on-line mixing and derivatization. Other capillaries were 40 μm in ID for fluid transport. Unless otherwise stated, the separation buffer consisted of 20.0 mM tetraborate at pH 10.6.

3 Results and discussion

3.1 Characterization of FACE-SH with CE

FACE-SH used here was a commercial product with the purity ~94% determined by HPLC (provided by the manufacturer). The stock solution of FACE-SH was prepared in anhydrous DMF to prevent oxidation in long-term storage. To look at its fluorescent purity, a FACE-SH standard sample at 50 μM in tetraborate buffer (20 mM) at pH 10.6 was run on the flow-gated CE system. As shown in Fig. 1b-i, two major peaks were observed, and the first peak was assigned to disulfides (FACE-S-S-FACE), and the second was for sulfides (FACE-S⁻). The disulfide peak was increasing while the sulfide peak was decreasing with time when sequential injections and separations were performed (see Fig. 1a), which indicates that sulfides were being oxidized to disulfides continuously. Moreover, this oxidation reaction was kinetically fast, which was demonstrated by the rapid decrease of the sulfide peak as seen in Fig. 1a. However, the fluorescence intensity of disulfides was not proportional to their concentration in terms of fluorescein associated with disulfides, which indicates there was fluorescence quenching effect when disulfides were produced. In addition, several small unknown peaks were also observed as seen in Fig. 1b-i, which were attributed to impurities in the FACE-SH dye. Among these peaks, one unknown peak (Unknown 1 in Fig. 1b-i) was increasing with time, which was presumably regarded as another disulfide compound produced by FACE-SH and a thiol impurity. FACE-SH, like other thiol compounds, tends to be oxidized to form disulfides by molecular oxygen dissolved in aqueous solutions [28]. This oxidation can be favorable under the catalysis of some metal ions such as Cu²⁺, Fe³⁺, and Ni²⁺ in a basic solution [28]. The pH used here was 10.6 because the derivatization scheme involving a thiol, OPA, and primary amines required a basic pH [20]. The conversion of FACE-SH to disulfides would reduce the effective amount of FACE-SH for the derivatization of amines in the presence of OPA and thus should be prevented.

To maintain FACE-SH in the form of sulfide, a reducing reagent such as TCEP or dithiothreitol (DTT) could be added to the thiol solution and/or the derivatization mixture. Considering DTT contains thiol groups which may compete with FACE-SH for amines during the derivatization process, TCEP was selected as the reducing reagent. As shown in Fig. 1b-ii, both disulfide peaks (including Unknown 1) disappeared while the sulfide peak increased when TCEP at 1.0 mM was added to a solution with 50 μM FACE-SH, which indicates that TCEP was able to efficiently convert disulfides to sulfides. The reducing effectiveness by TCEP was dependent on the relative amount of TCEP to disulfides because

of the 1/1 stoichiometry [29], and an over amount of TCEP was used to ensure complete reduction of disulfides in a timely manner. It should be addressed that the existence of TCEP in the derivatization mixture did not affect the reaction involving FACE-SH, OPA and amines.

3.2 Buffer pH effect on migration of FACE-SH

For complete derivatization of amines at low concentrations such as in a sub-nanomolar range, high concentrations of OPA and FACE-SH kinetically and dynamically favor the tagging reaction for amines. However, FACE-SH is highly fluorescent and appears as a huge peak in the electropherogram if a high concentration is used. Therefore, its location in an electropherogram should be away from the analyte peaks of interest. A series of experiments with the separation buffer at different pH values was performed to adjust the location of FACE-SH along the time axis. As shown in Fig. 2, FACE-SH peak moved backward along the time axis relative to other amino acid peaks, and pH higher than 10 shifted FACE-SH peak between glycine and glutamate peaks. A further increase in pH only slightly changed the FACE-SH position relative to other peaks. The shift of FACE-SH peak was attributed to the ionization of the sulfhydryl group with the increase in pH, and a higher pH favored sulfide anions. The total charge on single FACE-S⁻ molecules was -3 including -2 on the fluorescein group at pH 10.6 [30]. Most derivatives of amino acids also held -3 charges but their sizes were larger than FACE-S⁻; and arginine derivative held -2, while glutamate and aspartate -4, which were reflected on the order of their migration times as demonstrated in Fig. 2. Therefore, pH 10.6 with 20.0 mM of tetraborate buffer was chosen for further experiments.

3.3 Cysteine effect on amino acid derivatization

Biological fluids (plasma, urine, and CSF) contain thiol-bearing compounds such as cysteine, homocysteine, and glutathione [31,32]. These thiols may compete with FACE-SH for the amino acids in the derivatization mixture. The typical level of free cysteine in human blood plasma is in the range of 40-80 μ M while only a trace level (<1.0 μ M) exists in CSF [32-34]. To test the effect of cysteine on the derivatization of FACE-SH, a series of experiments was performed using various concentrations of cysteine added to the derivatization mixture. As shown in Fig. 3a, the presence of cysteine up to 100 μ M imposed negligible influence on the sizes of Glu and Asp for unknown reasons. On the other hand, cysteine was also derivatized by FACE-SH and OPA via the amine groups thus showing multiple peaks and the decreased peak of FACE-SH as shown in Fig. 3a-i. These peaks were presumably attributed to the chain-reaction derivatives involving cysteine which had two functional groups: -SH and -NH₂. However, these peaks were relatively small even at the cysteine concentration of 100 μ M (Fig. 3b), and thus the trace amount of cysteine in CSF would fairly affect the detection of amino acid neurotransmitters based on the present derivatization strategy. The adverse effect when the sample contains a large concentration of cysteine would be the interfering peaks associated with cysteine.

3.4 On-line mixing and derivatization

The derivatization reagents included OPA, FACE-SH, TCEP, and EDTA; and the sample was dissolved in aCSF. Two micro-syringes were used to respectively deliver the reagents and

the sample to mix at a PDMS-T interface and the derivatization was occurring in a capillary at 100 μm in ID and 15 cm in length. It allowed >1.5 min for the reaction to complete at a total flow rate of 600 nL/min. A typical electropherogram is shown in Fig. 3b. As can be seen, the selected amines were labelled with fluorophores and separated. It is important to point out that a total 22 μM of amines were mixed with 50 μM of FACE-SH and 5.0 mM of OPA (before mixing), and the remaining FACE-SH generated a large peak (over-range) as seen in Fig. 4b. On the other hand, Fig. 4a demonstrates a typical electropherogram that was obtained with the same conditions as Fig. 4b except no amino compounds added. Comparing Fig. 4b to Fig. 4a, additional peaks, although small, were observed in Fig. 4b, which were attributed to byproducts in the derivatization process.

The derivatization scheme was intended to improve the detection sensitivity associated with OPA/BME while preserving its favorable reaction kinetics. Limits of detection (LODs) were determined for DA, NE, Glu and Asp to be below 0.5 nM at 3-fold S/N with standards dissolved in aCSF, which was favored by a low concentration of FACE-SH (such as 10 μM). As can be seen in Fig. 4, byproducts and impurities in the FACE-SH dye produced numerous interfering peaks, and the broad range of these interferences also elevated the background signal. For CSF sample, a high total concentration of amino compounds required a large concentration of FACE-SH to ensure complete derivatization of specific analytes, and consequently, the background interferences increased proportionally with the increase of the FACE-SH concentration. Therefore, 600 μM of FACE-SH generated LODs of below 5 nM for DA, NE, Glu, and Asp, which are still much lower than typical 100 nM obtained with the OPA/BME scheme [21].

3.5 Analysis of CSF sample via in vitro microdialysis

To demonstrate the capability of the derivatization scheme, in vitro microdialysis was coupled with on-line derivatization for analyzing CSF and its spiking. A microdialysis probe at 3.5 mm in active length and 200 μm in OD was prepared according to the reference [24] and was immersed in CSF first and then its spiking contained in centrifuge tubes. The perfusate of aCSF was pumped at 300 nL/min and the dialysate was mixed with derivatization reagents. Typical electropherograms are shown in Fig. 5a which presented the increases of Glu and Asp peaks after spiking. In this series of experiments, original 600 μM of FACE-SH was used and the remaining FACE-SH after derivatization left a large peak, which indicates the 600 μM was more than enough to derivatize amines sampled through the microdialysis probe. In addition, numerous other amines were also observed in the electropherogram (not identified), which demonstrates the capability of this derivatization scheme for simultaneously measuring multiple analytes. Fig. 5b shows the peak size variations of Glu and Asp in the CSF sample and its spiking. The probe was maintained in the spiked sample for 10 minutes, and then manually moved back to the CSF sample in a short time (~ 10 s). As can be seen, the increase in peak size exactly reflected the concentration difference, and the temporal resolution was less than 50 s. This in vitro demonstration mimicked the in vivo application of other derivatization methods in neurotransmitter monitoring in rat brain [4,22].

4 Concluding remarks

A similar derivatization approach for amino compounds using a fluorescent thiol (SAMS-A-F) and OPA has been developed [23], but SAMS-A-F has two isomers which complicate the electropherogram. Here, FACE-SH is a novel fluorescent thiol commercially available. Although its oxidation by oxygen would cause the loss of effective FACE-SH, TCEP could efficiently reduce disulfides to FACE-SH thus preserving these reactive thiol molecules. The derivatization scheme has been coupled with in vitro microdialysis and on-line derivatization so that it could be potentially used for in vivo neurotransmitter measurements. Similar to the reported method [23], the reaction kinetics was fast, and the detection sensitivity was greatly improved relative to the OPA/BME scheme. However, some impurities in the thiol dye and by-products generated during derivatization may interfere with the detection of some amino compounds of interest. Despite these limitations, the fluorophore-tagging with the present approach is fast and sensitive, which would be valuable in specific analytical situations. For example, the OPA/FACE-SH scheme could be potentially applied to fast on-chip labelling of proteins retained on a solid phase [35].

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Abbreviations

OPA	o-phthalaldehyde
FACE-SH	2-[(5-fluoresceinyl)aminocarbonyl]ethyl mercaptan
TCEP	Tris(2-carboxyethyl)phosphine
BME	β -mercaptoethanol
aCSF	artificial cerebrospinal fluid
DA	dopamine
NE	norepinephrine

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Figure 1.

(a) Oxidation of FACE-SH and formation of FACE-S-S-FACE with time. The first injection occurred in 2 minutes since the sample got ready. 50 μ M of FACE-SH was dissolved in 20 mM of tetraborate buffer at pH 10.6. (b-i) The electropherogram of No. 15 injection without TCEP, and (b-ii) 50 μ M of FACE-SH with 1.0 mM of TCEP. Injection at $-5 \text{ kV} \times 0.5 \text{ s}$ and separation at -25 kV .

Figure 2.

FACE-SH peak shift with pH along the time axis. Derivatization reagents contained 50 μ M of FACE-SH, 2 mM of TCEP, 2 mM of OPA, 10 mM of EDTA, and 20 mM of tetraborate, pH 10.6; Sample consisted of arginine, glutamine, glycine, glutamate and aspartate at 2 μ M each. Off-line derivatization was performed by mixing the derivatization reagents and the sample at 1:1 ratio by volume. Separation buffer contained 20 mM of tetraborate with pH adjusted using NaOH. Injection at -5 kV $\times$ 0.5 s and separation at -25 kV.

Figure 3.

Electropherograms showing Cysteine effect on amino acid derivatization with FACE-SH and OPA. (b) is the zoom-in graph of (a). U=unknown. Concentrations of FACE-SH, Glu, and Asp were 50 μM , 10 μM , and 10 μM , respectively. Separation buffer was 20-mM tetraborate at pH 10.6. Other conditions were the same as in Fig. 2.

Figure 4.

Comparison of electropherograms with and without amino compounds. (a) A typical electropherogram of FACE-SH (50 μ M) plus OPA (5 mM); (b) A typical electropherogram of FACE-SH (50 μ M) and OPA (5 mM) plus 11 amino acids at 2 μ M each. Derivatization reagents: 50 mM of FACE-SH, 5 mM OPA, 2 mM TCEP and 10 mM EDTA in 20 mM tetraborate at pH 9.6; Sample: amino compounds dissolved in aCSF. On-line mixing at 1/1. Injection at $-5 \text{ kV} \times 0.5 \text{ s}$ and separation at -25 kV . 1-norepinephrine, 2-dopamine, 3-glutamine, 4-GABA, 5-serine, 6-alanine, and 7-taurine & glycine.

Figure 5.

(a) Electropherograms obtained via in vitro microdialysis of CSF and its spiking. The sample spiking was prepared using 100 μM of Glu and 50 μM of Asp in aCSF (sample/aCSF = 90/10 by volume). The CSF sample was diluted with the same volume as the spiking. (b) Peak height variations after spiking for Glu and Asp, respectively. Initial microdialysis probe was immersed in CSF, and then moved to the spiking sample for 10 min, and finally moved the probe back to the CSF. On-line mixing of FACE-SH 600 μM , 5 mM TCEP, and 10 mM OPA, and 10 mM of EDTA. Injection at $-5\text{ kV} \times 0.5\text{ s}$, and separation at -27 kV .