Fieldable flow injection analysis system with 1,1′-oxalyldiimidazole chemiluminescence detection capable of quantifying acetylcholine†‡

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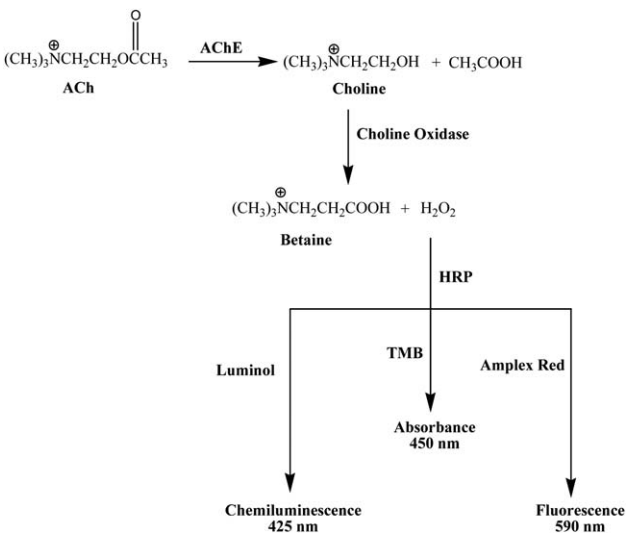
We developed a highly sensitive biosensor for quantifying acetylcholine (ACh) using flow injection analysis system with 1,1′-oxalyldiimidazole chemiluminescence (ODI-CL) detection designed based on the principle of liquid core waveguide. ACh in Tris-HCl (pH 8.5) was incubated with the mixture of 1.0 U/ml acetylcholinesterase, 0.5 U/ml choline oxidase, 0.04 U/ml horseradish peroxidase, and 1.0 μM Amplex Red in PBS (pH 7.4) for 15 min at room temperature. The concentration of resorufin formed from the consecutive enzyme reactions was proportional to the concentration of ACh in analytical sample. The dynamic range of linear calibration curve ($y = 12444x + 11617$, $R^2 = 0.998$) for the quantification of ACh using the biosensor with ODI CL detection was 0.7∼11.3 μM. The limit of detection (LOD = background noise + 3σ) of the biosensor was as low as 0.14 μM. Based on the results of recovery test and linearity study, finally, we confirmed that FIA system with ODI CL detection is accurate, precise, and reproducible.

Introduction

Acetylcholine (ACh) is an important neurotransmitter of central nervous system (CNS) and peripheral nervous system (PNS).[1,2] The roles of ACh in the CNS are related to various activities such as arousal, attention, learning and memory. ACh in the PNS activates muscles. The change of ACh concentration in organisms causes various nerve disorders. For example, neurological disorders such as Parkinson's disease and Alzheimer's disease are often associated with the decrease of ACh.[1] The chemical warfare nerve agents and organophosphate pesticides are potent and irreversible inhibitors resulting in excessive accumulation of ACh.[2] The increase of ACh due to the exposure to chemical warfare nerve agents or organophosphate pesticides causes muscle contraction such as neuromuscular paralysis.[2] Therefore, there is a need for a highly sensitive biosensors capable of monitoring imbalance of ACh with a wide dynamic range.

Capillary electrophoresis,[3,4] liquid chromatography,[5–7] and a couple of enzyme-based procedures[8–10] have previously been applied to separate ACh from serum or plasma containing various biological materials. Enzyme-based procedures performed in a small container such as strip-well and detection cell is simpler and more cost-effective than large-scale capillary electrophoresis and liquid chromatography. The concentration of ACh in serum or plasma has been determined with various detections such as chemiluminescence,[5,8,9] electrochemical,[3] fluorescence.[7,10] and mass spectrometry.[4,6]

As shown in Scheme 1, ACh is converted to choline in the presence of acetylcholinesterase (AChE). Then, choline converts to betaine with H_2O_2 in the presence of choline oxides (ChOX). The H_2O_2 formed is used as an oxidation agent of substrate added for measuring absorbance,[11] chemiluminescence,[12] or fluorescence.[13] Horseradish peroxidase (HRP) is an enzyme catalyst in these reactions. All the procedures shown in Scheme 1 occur consecutively in a small container. Thus, the strength of signal observed with one of the three detections is proportional to the concentration of Ach in the absence of choline. As shown in Scheme 1, choline existing in human samples such as serum, plasma, and urine can be quantified in the absence of AChE. Thus, Scheme 1 indicates that the concentration of ACh existing in human sample can be quantified with the difference between



Scheme 1 Enzyme-based analyses for the quantification of ACh

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the concentration of choline determined in the presence of AChE and that of choline obtained in the absence of AChE.

It is well-known that cost-effective and simple chemiluminescence detection is more sensitive than colorimeter and fluorescence operated with light source such as halogen, tungsten, xenon, and laser.[14] In particular, peroxyoxalate chemiluminescence (PO-CL) is more sensitive and selective than other chemiluminescence using luminol or 1,2-dioxetane.[14] Unfortunately, conventional PO-CL using bis(2,4-dinitrophenyl) oxalate (DNPO) or bis(2,4,6-trichlorophenyl) oxalate (TCPO) in the presence of an appropriate catalyst (*e.g.*, imidazole, sodium salicylate) is difficult to apply in the quantification of analytes formed from the enzyme reaction due to the rapid decomposition of DNPO and TCPO in aqueous solution.[14,15]

1,1′-oxalyldiimidazole (ODI) derivatives are formed from the reaction between oxalate ester (*e.g.*, DNPO, TCPO) and imidazole derivatives such as imidazole (ImH), 2-metylimidazole (2MImH), and 4-methylimidazole (4MImH) in organic solvent such as ethyl acetate and acetonitrile.[16] High-energy intermediate (**X**) formed from the reaction of ODI and H_2O_2 transfers energy to fluorescent molecules based on chemically initiated electron-exchange luminescence (CIEEL) mechanism.[15–17] Then, fluorescent molecules in the excited state emit bright light in the process of coming down to the ground state. CL measured in the presence of excess fluorescent molecules is self-quenched like that observed in fluorescence measurement. The sensitivity of ODI CL is much better than that of conventional PO-CL. This is because ODI CL reaction is so fast that it is possible to emit bright CL before ODI derivatives are decomposed in aqueous solution[15] even though they are also unstable in aqueous solution like DNPO and TCPO. The time (τ_{max}) necessary to attain maximum intensity (I_{max}) in ODI-CL in aqueous solution is less than 0.3 s.[18]

In this paper, we described in detail the development of an enzyme-based biosensor with ODI CL detection capable of rapidly quantifying and monitoring ACh in human serum.

Experimental

Chemical and material

Bis(2,4,6-trichlorophenyl)oxalate (TCPO) was purchased from TCI-America. 4-Methylimidazole (4MImH, 98%), Acetylcholine bromide (AChBr) was purchased from Alfa Aesar. Type 1 horseradish peroxidase (HRP, 5KU), phosphate buffer (1M, pH 6.6), acetylcholine esterase (AChE, type V–S, from *electric eel*, 658 U/mg), choline chloride (>99.0%), and choline oxidase (ChOx, from *Alcaligenes* species, 14.16 U/mg) were purchased from Sigma. 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was purchased from Cayman Chemical Company. Ethyl acetate (EA) and deionized water (H_2O) were purchased from J. T. Baker (HPLC grade). Hydrogen peroxide (H_2O_2, 3%) and isopropyl alcohol (ACS grade) were purchased from VWR. Dimethyl sulfoxide (DMSO) was purchased from Calbiochem. Sodium phosphate buffer (1M, pH 6.0, 7.0), and tris-HCl buffer (1M, pH 7.0, 7.5, 8.0, 8.5) were purchased from Teknova. Phospate buffer saline (PBS, 0.1M, pH7.4) was purchased from EMD. Sodium bicarbonate-sodium carbonate buffer (1M, pH 9.6 ± 0.2) and borate buffer (0.5M, pH 8.5 ± 0.2) were purchased from Polysciences, Inc. The "0" calibrator of human serum was purchased from Monobind, Inc. Washington County Hospital (Hagerstown, MD, USA) kindly provided coded human serums.

Preparations of standards and analytical samples

Various concentrations (0.1∼100.0 μM) of ACh standard solutions containing 0.5% "0" calibrator of human serum and analytical samples containing 0.5% coded human serums were prepared in Tris-HCl buffer solution (pH 8.5).

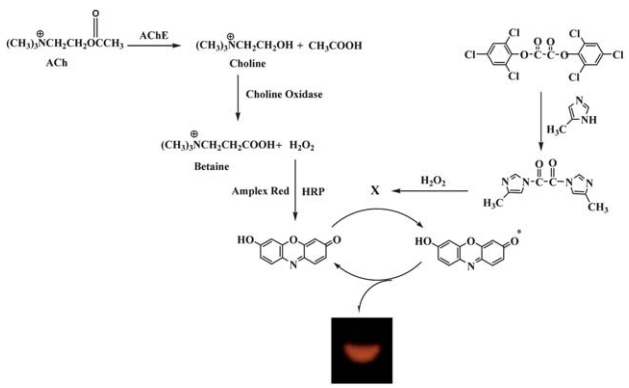
Preparation of stock solution

AChE (500 U/ml), ChOx (50 U/ml), and HRP (1000 U/ml) were prepared in deionized water as stock solutions. 15 mM Amplex Red used as a chemiluminescent substrate was prepared in DMSO. They were stored in a freezer (−20 °C). 2.0 mM TCPO was prepared daily in ethyl acetate due to the instability of TCPO solution. 12.0 mM 4MImH dissolved in ethyl acetate was stored under ambient condition. 0.1 M H_2O_2 was prepared in isopropyl alcohol.

Measurement of ODI-CL

In order to measure relative CL intensity of resorufin emitted based on the reaction mechanism shown in Scheme 2, flow injection analysis (FIA) system with three syringe pumps (975 Harvard Apparatus Compact Infusion Pump) was developed (See Fig. 1). Pump 1 was used for delivering 0.1 M H_2O_2 solution in a 50 ml-syringe into the flow cell (10 mm path, 80 μl, Agilent Technology) through the cross mixer (OD: 1/16 inch, ID: 0.25 mm, Valco VICI Cheminert) as shown in Fig. 1. Pump 2 was applied to deliver PBS (pH 7.4) and resorufin to the flow cell. Pump 3 was operated to deliver ODI, formed from the reaction between 10 μM TCPO and 50 μM 4MImH in a beaker for 30 min, to the flow cell through the mixer. The flow rate of all three pumps was 1.1 ml min^{-1}.

Based on the reaction mechanism shown in Scheme 2, resorufin was formed in a vial when 1.0 ml of standard or sample solution containing ACh was mixed and incubated for 15 min with 1.0 ml of PBS (pH 7.4) containing AChE, ChOx, HRP, and



Scheme 2 Quantification of Ach using ODI CL detection. 1. Resorufin, 2. TCPO, 3. 4-methylimidazole, X. high-energy intermediate (4-methylimidazolehydroperoxydioxatanone[16,17]) capable of transferring energy to resorufin, Ach. Acetylcholine, AChE. Acetylcholinesterase.

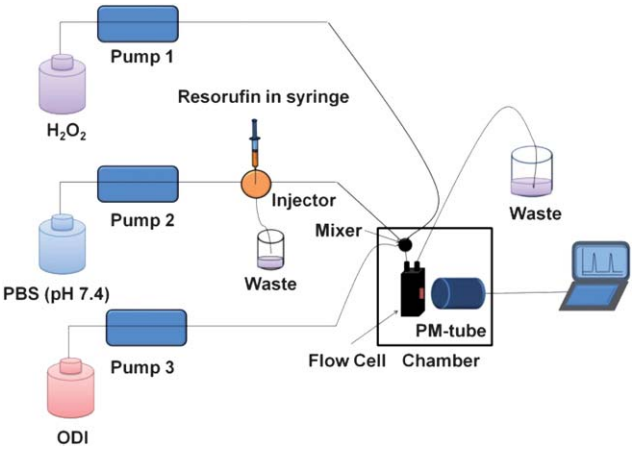


Fig. 1 FIA system with ODI CL detection.

Amplex Red. Resorufin formed from the reaction was injected into the FIA system through a 6 port injection valve (Valco VICI Cheminert 04Y-0016H) having 5 μl loop. The volume (5 μl) injected into the FIA system was the same as HPLC system[5] and larger than electrophoresis system.[3] Resorfin was rapidly mixed with ODI CL reagents in the cross mixer shown in Fig. 1. Then, the mixture was flowed into the flow cell. The relative CL intensity of resorufin emitted in the flow cell was measured using a photomultiplier tube module (H7818-01 and M9003, Hamamatsu).

Results and discussion

1. Effect of pH

1.1 Standard and sample. As shown in Scheme 1 and 2, H_2O_2 is formed when ACh is incubated with AChE and ChOx for a certain amount of time. Fig. 2 indicates that the yield of H_2O_2 obtained from the reaction is dependent on pH as well as the properties of buffer solution used to prepare standards and samples. Based on the result shown in Fig. 2, standards and samples containing ACh were prepared in Tris-HCl buffer solution (pH 8.5).

1.2. Working solution containing AChE, ChOx, HRP and Amplex Red. As shown in Scheme 2, resorufin is formed when

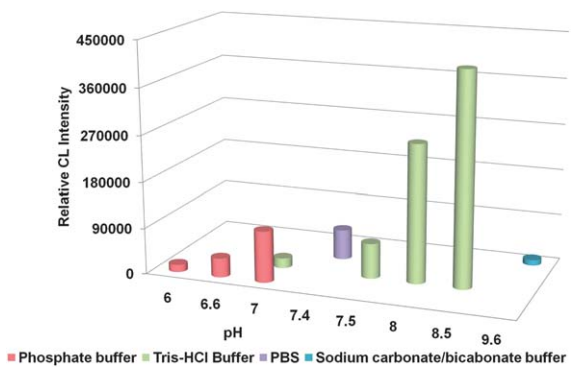


Fig. 2 Effect of pH in standards and samples.

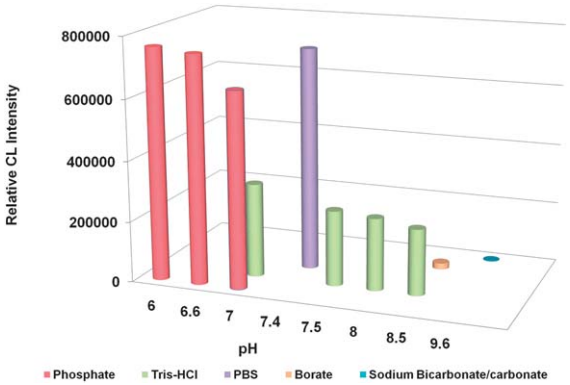


Fig. 3 Effect of pH in working solution.

Amplex Red reacts with H_2O_2 in the presence of HRP. The yield of resorufin obtained from the reaction depends on pH and the properties of buffer solution. We selected PBS as a buffer solution to prepare working solutions containing Amplex Red and HRP even though relative CL intensity measured in PBS (pH 7.4) is similar to those obtained in three phosphate buffer solutions (pH 6.0 and 6.5) as shown in Fig. 3. This is because H_2O_2 and resorufin are consecutively formed when the standard prepared in Tris-HCl buffer solution (pH 8.5) is incubated with the working solution containing AChE, ChOx, HRP and Amplex Red. Based on the results shown in Fig. 2, we expect that the yield of H_2O_2 formed in the mixture of two buffer solutions of pH 8.5 Tris-HCl and pH 7.4 PBS is higher than that in the mixture of two buffer solutions of pH 8.5 Tris-HCl and pH 6.0 phosphate.

2. Effect of enzyme and amplex red

2.1. Acetylcholinesterase (AChE). As shown in Fig. 4, relative CL intensity was enhanced with the increase of AChE activity. However, relative CL intensity of resorufin formed from the consecutive enzyme reaction shown in Scheme 2 began to be self-quenched in the presence of higher AChE activity than 1.0 U/ml. This is because the concentration of resorufin formed from the reaction between Amplex Red and H_2O_2 in the presence of HRP is dependent on the concentration of H_2O_2 formed from the enzyme reaction occurring with the addition of different activities of AChE. Based on the results shown in Fig. 4, 1.0 U/ml AChE was used for the quantification of ACh in human samples using ODI CL detection.

2.2 Choline oxidase (ChOx). Relative CL intensity was enhanced with the increase of ChOx activity added in the consecutive enzyme reaction (see Fig. S-1 of the supplemental information). Relative CL intensity obtained from the consecutive enzyme reaction in the presence of higher ChOX than 0.75 U/ml was self-quenched. Based on the results, we select 0.5 U/ml ChOx for the research.

2.3 Horseradish peroxidase (HRP). As shown in Fig. S-2 of the supplemental information, the activity of HRP for the quantification of ACh from the consecutive enzyme reaction is much lower than those of AChE and ChOx. Relative CL

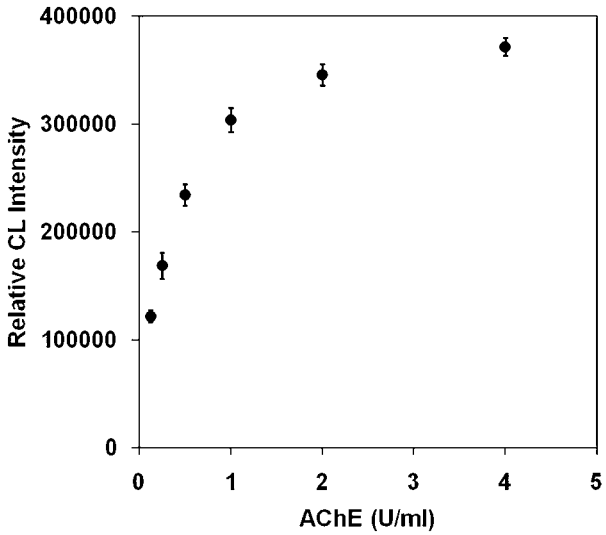


Fig. 4 Effect of AChE. Condition: [ACh] = 3.0 μM, [ChOx] = 1.0 U/ml, [HRP] = 1.0 U/ml, [Amplex Red] = 1.25 μM, [TCPO] = 15.0 μM, [4MImH] = 75.0 μM, [H_2O_2] = 0.1 M, Incubation time: 20 min.

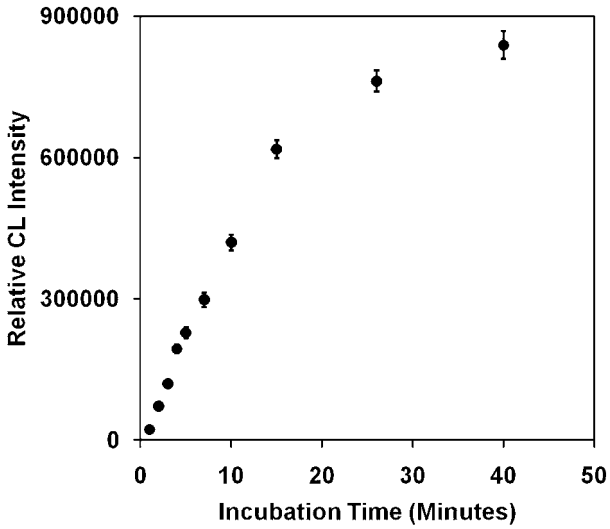


Fig. 5 Effect of incubation time. Condition: [ACh] = 3.0 μM, [AChE] = 1.0 U/ml, [ChOx] = 0.5 U/ml, [HRP] = 0.04 U/ml, [Amplex Red] = 1.0 μM, [TCPO] = 15.0 μM, [4MImH] = 75.0 μM, [H_2O_2] = 0.1 M.

intensity was sharply enhanced with the increase of HRP activity up to 0.04 U/ml. Then, it dropped down due to the self-quenching of excess resorufin formed from the consecutive enzyme reaction in the presence of higher HRP than 0.04 U/ml. Based on the results shown in Fig. S-2 of the supplemental information, 0.04 U/ml was selected to monitor ACh in human serum and plasma samples using FIA system with ODI CL detection.

2.4 Amplex red. Relative CL intensity of resorufin formed from the consecutive enzyme reaction was dependent on the concentration of Amplex Red (0.5∼10.0 μM) used as a substrate for measuring ODI CL. We obtained the highest CL intensity of resorufin when 1.0 μM Amplex Red was mixed with three enzymes (1.0 U/ml of AChE, 0.5 U/ml of ChOx, and 0.04 U/ml of HRP) for the consecutive enzyme reaction for 15 min. Relative CL intensities measured with the addition of higher Amplex Red (2.0∼10.0 μM) than 1.0 μM was similar to or lower than that obtained in the presence of 1.0 μM Amplex Red due to the self-quenching of excess resorufin converted from Amplex Red. In addition, relative CL intensity measured in the presence of 0.5 μM Amplex Red was lower than that obtained with 1.0 μM Amplex Red. Based on the results, 1.0 μM Amplex Red was used for sensing trace levels of ACh in human samples.

3. Determination of incubation time

As shown in Fig. 5, the consecutive enzyme reaction is dependent on the incubation time. The relative CL intensity was bi-phasic with a proportional increase in intensity when the reaction was allowed to incubate for15 min, then incubation times greater than 15 min were self-quenched. Based on the experimental results, the consecutive enzyme reaction was performed for 15 min for the quantification of ACh using FIA system with ODI CL detection. The incubation time for the quantification of ACh using FIA system with ODI CL detection was two times shorter than that (30 min) with commercially available fluorescence detection (Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit, Invitrogen) because ODI CL is more sensitive than fluorescence.

4. Effect of ODI CL reagent

4.1 ODI. As shown in Scheme 2, ODI is formed from the reaction of TCPO and 4MImH. Based on the previous research results,[15,18–20] the concentration ratio between TCPO and 4MImH was 1 : 5 for maintaining the stability of ODI for at least 4 h. The best concentrations of TCPO and 4MImH to measure the highest CL intensity of resorufin using the photomultiplier (PM)-tube of FIA system were 10 μM and 50 μM, respectively. The relative CL intensity was enhanced with the proportional increase of TCPO (1∼10 μM) and 4MImH concentrations (5∼50 μM). However, the light emitted in the presence of ODI formed from the reaction of higher TCPO and 4MImH than 10.0 μM and 50.0 μM is so strong that the relative CL intensity measured using the highly sensitive PM-tube of the FIA system was overloaded.

4.2 H_2O_2. The most appropriate concentration of H_2O_2 dissolved in isopropyl alcohol was 0.08 M. The CL intensity observed with the increase of H_2O_2 concentration up to 0.08 M (0.01∼0.08 M) was enhanced proportionally and then slightly increased in the presence of higher H_2O_2 concentrations (*e.g.*, 0.1 and 0.2 M) than 0.08 M. The trend observed in this research was consistent with the previous research results.[17–20]

5. Effect of flow rate

As shown in Scheme 2, H_2O_2 formed from the enzyme reaction reacts with Amplex Red to produce resorufin. The CL intensity of resorufin measured using ODI CL detection is enhanced with the increase of H_2O_2 concentration formed from the enzyme reaction. Also, Towne and his collaborators reported that

resorufin is converted to non-fluorescent compounds such as resazurin in the presence of excess H_2O_2 when HRP acts as a catalyst.[21] This result indicates that CL of resorufin measured using ODI CL detection will be reduced due to the enzymatic oxidation of resorufin in the presence of excess H_2O_2. As shown in Scheme 2, high concentration of H_2O_2 in isopropyl alcohol acts an ODI CL reagent. If resorufin molecules formed from the enzyme reaction are mixed with excess H_2O_2 used as an ODI CL reagent in a vial for a certain amount of time, part of resorufin molecules may be converted to resazurin. In order to prevent this unnecessary reaction, resorufin formed from the enzyme reaction and H_2O_2 in isopropyl alcohol were inserted into the FIA system using two different syringe pumps, as shown in Fig. 1.

As shown in Fig. S-3 of the supplemental information, the time (τ_{max}) necessary for attaining maximum CL intensity (I_{max}) of resorufin is as short as 0.4 s. Also, the time (τ_{half}) necessary for attaining the half of I_{max} from I_{max} is only 0.8 s. This result indicates that resorufin and ODI CL reagents should be mixed in the flow cell to measure flash CL emission. Unfortunately, no commercial flow cell that has three inlets and a outlet is available. Thus, we connected a cross mixer with a commercially available flow cell that has a inlet and a outlet as shown in Fig. 1. The distance between the cross mixer and the flow cell was 5.0 cm. Thus, the 1/16-inch OD PTFE tubing used for the connection of the cross mixer and the flow cell was applied as a liquid core waveguide for the detection of flash CL emitted from rapid ODI CL reaction.

After setting up the FIA system shown in Fig. 1, we studied the effect of flow rate in order to measure the brightest CL emitted in the flow cell. For this research, the range of flow rate for each pump was from 0.59 to 2.2 ml min^{-1} (0.59, 0.82, 1.1, 1.6, and 2.2 ml min^{-1}). In order to mix the three reagents smoothly in the cross mixer, the flow rate of the pump were the same. With the increase of flow rate, the width of the peak became narrow. For example, the width of peak obtained when the flow rate was 0.59 ml min^{-1} was about 2.0 times wider than that when the flow rate was 2.2 ml min^{-1}. I_{max} of peak measured when the flow rate was 1.1 ml min^{-1} was higher than those when the flow rate was slower or faster than 1.1 ml min^{-1}. Based on the experimental result, the flow rate of FIA system for the quantification of ACh in human serum and plasma was 1.1 ml min^{-1}.

6. Validation of FIA system with ODI CL detection

Seven different concentrations (0.70, 1.4, 2.8, 5.6. 11.3, 22.5, 45.0 μM) of ACh standards were prepared in the "0" calibrator of human serum 200-time diluted in Tris-HCl buffer solution (pH 8.5). As shown in Fig. S-4 of the supplement information, a linear calibration curve ($y = 12444x + 11617$, $R^2 = 0.998$) for the quantification of ACh in human serum was obtained under the optimum conditions of various variables (*e.g.*, pH, enzymes, ODI CL reagents, flow rate) described above. The dynamic range of the linear calibration curve was 0.70∼11.3 μM. The relative CL intensity measured in the presence of higher ACh concentration of 11.3 μM was lower than those predicted using the linear calibration curve due to the self-quenching of excess resorufin formed from the enzyme reaction. The limit of detection (LOD = background noise + 3σ) of the FIA system with ODI CL detection for the quantification of ACh was 0.14 μM. σ is the standard deviation of average background noise (n = 20). LOD determined using our FIA system with ODI CL detection was lower than those recently reported using various detection methods such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS),[22] liquid chromatography with electrochemiluminescence detection,[5] and capillary electrophoresis with electrocatalytic microelectrode detection.[3] Also, the limit of quantification (LOQ = background noise + 10σ) of the FIA system with ODI CL detection was 0.47 μM.

Table 1 Recovery test (n = 7) of FIA system with ODI CL detection

Sample number	ACh/ μM	ACh added/ μM	Expected value/μM	Result/ μM	Recovery (%)
1[a]	1.00	5.00	3.00	3.15	105
2[b]	1.00	5.00	3.00	2.82	94
3[c]	1.00	5.00	3.00	3.09	103
4[a]	2.00	6.00	4.00	4.31	108
5[a]	5.00	8.00	6.50	6.35	106

[a] "0" calibrator of human serum 200-time diluted in Tris-HCl buffer (pH 8.5). [b] human serum (male) 200-time diluted in Tris-HCl buffer (pH 8.5). [c] human serum (female) 200-time diluted in Tris-HCl buffer (pH 8.5).

Table 2 Linearity (n = 7) of FIA system with ODI CL detection

Dilution Ratio (R)	Expected value/μM	Result/ μM	Result × 1/R/μM
1	10.00	9.67	9.67
1/2	5.00	5.23	10.46
1/4	2.50	2.77	10.44
1/8	1.25	1.32	10.56

Watanabe and his collaborators reported that the blood ACh levels of healthy subjects varied over a wide range with a geometric mean of 0.49 μM.[23] Thus, patient serums were diluted 200 times with pH 8.5 Tris-HCl buffer to perform the recovery test (Table 1) and linearity study (Table 2) to validate the new analytical method with the removal of errors occurring from undiluted human serums containing different concentations of ACh and choline. The concentrations of ACh as well as choline existing in patient serum 200 times diluted with pH 8.5 Tris-HCl buffer are too low to be detected using FIA system with ODI CL detection before spiking ACh (1∼10 μM) in diluted human serums. The results of recovery test shown in Table 1 indicate that FIA system with ODI CL detection can quantify ACh with good accuracy and precision. After various dilutions of a sample containing high concentration of ACh (10.0 μM), each diluted sample was quantified with FIA system with ODI CL detection in order to study the linearity of the new analytical method. As shown in Table 2, the concentration of each of the diluted sample was similar to what we expected within the acceptable error range (≤5.6%).

Conclusions

Using FIA system with ODI CL detection capable of sensing resorufin, we developed a new analytical method for quantifying

trace levels of ACh in human samples. This is because the concentration of resorufin formed in the consecutive enzyme reaction is proportional to the concentration of ACh in patient sample. Thus, we expect that the FIA system with ODI CL detection can be applied as a cost-effective, simple, and rapid analytical method to diagnose or monitor Alzheimer Disease or neuromuscular paralysis occurring with the exposure of chemical warfare nerve agents or organophosphate pesticides. Also, we expect that the results observed with the FIA system designed based on the principle of liquid core waveguide can be applied to fabricate a microfluidic device as a fieldable biosensor for the quantification and monitoring of ACh.

Notes and references

1 L. Frolich, A. Dirr, M. E. Gotz, W. Gsell, H. Reichmann, P. Riederer and K. Maurer, *J. Neural Transm.*, 1998, **105**, 961.
2 A. Raghunath, F. Perez-Branguli, L. Smith and J. O. Dolly, *J. Neurosci.*, 2008, **28**, 3683.
3 J. Mukherjee and J. R. Kirchhoff, *Anal. Chem.*, 2009, **81**, 6996.
4 T. Lapainis, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2009, **81**, 5858.
5 H. Yoshida, A. Yamada, K. Todoroki, O. Imakyure, H. Nohta and M. Yamaguchi, *Luminescence*, 2009, **24**, 306.
6 L. Prokai, P. Frycak, S. M. Stevens and V. Nguyen, *Chromatographia*, 2008, **68**, S101.
7 J. G. Bruno, M. P. Carrillo, T. Phillips and B. King, *In Vitro Cell. Dev. Biol.: Anim.*, 2008, **44**, 63.
8 X. F. Wang, Y. Zhou, J. J. Xu and H. Y. Chen, *Adv. Funct. Mater.*, 2009, **19**, 1444.
9 D. Yao, A. G. Vlessidis and N. P. Evmiridis, *Anal. Chim. Acta*, 2002, **462**, 199.
10 T. Jin, *Sensors*, 2010, **10**, 2438.
11 F. C. Gong, D. Z. Li, R. Yang, J. K. Wei, Z. Cao, S. Z. Tan and Y. F. Tan, *Sci. China, Ser. B: Chem.*, 2009, **52**, 1142.
12 S. Bi, H. Zhou and S. S. Zhang, *Biosens. Bioelectron.*, 2009, **24**, 2961.
13 S. H. Kim, B. Kim, V. K. Yadavalli and M. V. Pishko, *Anal. Chem.*, 2005, **77**, 6828.
14 M. Tsunoda and K. Imai, *Anal. Chim. Acta*, 2005, **541**, 13.
15 J. H. Lee, J. Je, J. Hur, M. A. Schlautman and E. R. Carraway, *Analyst*, 2003, **128**, 1257.
16 J. H. Lee, J. Je, M. A. Schlautman and E. R. Carraway, *Chem. Commun.*, 2003, 270.
17 J. H. Lee, J. C. Rock, S. B. Park, M. A. Schlautman and E. R. Carraway, *J. Chem. Soc., Perkin Trans.*, 2002, **2**, 802.
18 J. H. Choi, Y. T. Kim and J. H. Lee, *Analyst*, 2010, **135**, 2445.
19 Y. T. Kim, S. O. Ko and J. H. Lee, *Talanta*, 2009, **78**, 998.
20 J. H. Lee, J. R. Rho, T. D. Rho and J. G. Newby, *Biosens. Bioelectron.*, 2010, **26**, 377.
21 V. Towne, M. Will, B. Oswald and O. J. Zhao, *Anal. Biochem.*, 2004, **334**, 290.
22 M. Persike, M. Zimmermann, J. Klein and M. Karas, *Anal. Chem.*, 2010, **82**, 922.
23 M. Watanabe, A. Kimura, K. Akasaka and S. Hayashi, *Biochem. Med. Metab. Biol.*, 1986, **36**, 355.