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Chemiluminescence Resonance Energy Transfer-based Detection for Microchip Electrophoresis

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Abstract

Since the channels in micro- and nanofluidic devices are extremely small, a sensitive detection is required following microchip electrophoresis (MCE). This work describes a highly sensitive and yet universal detection scheme based on chemiluminescence resonance energy transfer (CRET) for MCE. It was found that an efficient CRET occurred between a luminol donor and a CdTe quantum dot (QD) acceptor in the luminol-NaBrO-QD system, and that it was sensitively suppressed by the presence of certain organic compounds of biological interest including biogenic amines and thiols, amino acids, organic acids, and steroids. These findings allowed developing sensitive MCE-CL assays for the tested compounds. The proposed MCE-CL methods showed desired analytical figures of merit such as a wide concentration range of linear response. Detection limits obtained were $\sim 10^{-9}$ M for biogenic amines including dopamine and epinephrine, and $\sim 10^{-8}$ M for biogenic thiols (e.g. glutathione and acetylcysteine), organic acids (i.e. ascorbic acid and uric acid), estrogens, and native amino acids. These were 10 to 1000 times more sensitive than those of previously reported MCE-based methods with chemiluminescence, electrochemical, or laser induced fluorescence detection for quantifying corresponding compounds. To evaluate the applicability of the present MCE-CL method for analyzing real biological samples, it was used to determine amino acids in individual human red blood cells. Nine amino acids including Lys, Ser, Ala, Glu, Trp, etc. were detected. The contents ranged from 3 to 31 amol /cell. The assay proved to be simple, quick, reproducible, and very sensitive.

Micro- and nanofluidic devices are great platforms where to perform chemical analyses.^{1–3} Electrophoresis carried out in these devices is known as microchip electrophoresis (MCE).^{4–7} Compared with conventional capillary electrophoresis (CE), MCE offers advantages such as the possibility of integrating sample injection, pre-concentration, digestion /cell lysing, separation, and detection onto one single chip. MCE is becoming an attractive alternative to CE. However, effectively detecting the separated analytes after MCE remains a challenge due to the small dimensions (10–100 μm) of microfluidic channels.^{3, 6} One of the most significant applications of MCE in analytical chemistry is analyzing single cells. Ramsey and co-workers used a glass microfluidic device to achieve high throughput analysis of Jurkat cells.⁸ Klepárník and Horký reported a microfluidic device integrated on a plastic disk for the detection of DNA fragmentation in single apoptotic cardiomyocytes.⁹ Fang group developed a microfluidic system for the analysis of glutathione (GSH) and several reactive oxygen species (ROS) in individual human erythrocytes.¹⁰ Zare and co-workers reported the determination of amino

acids in single Jurkat cells using an integrated microfluidic device.¹¹ In all of these works, laser induced fluorescence (LIF) detection was deployed to achieve the assay sensitivity needed.

Chemiluminescence (CL) detection is another sensitive detection scheme used in micro-scale separations such as CE. Compared with other detection schemes, CL detection offers many advantages such as high detection sensitivity, a wide linear range for quantification, and no need for a light source that allows the use of a simple instrumental set-up.¹² Since a CL detector can be easily miniaturized, this detection technique is unequally well suited for in-line detection in microchip electrophoresis (MCE). Several MCE-CL systems were reported. Determination of glutathione was carried out on microchips coupled with CL detection.^{13–15} Also, CL detection was integrated with capillary electrophoresis microchip for quantification of dopamine and catechol.¹⁶ However, since the microfluidic channels were extremely small, the sensitivity of these MCE-CL methods was in the range from 10^{-5} ~ 10^{-7} M (limit of detection, LOD), which in many cases was hardly sufficient for analysis of real biological samples (e.g. single cell analysis). Recently, we reported our efforts in improving the sensitivity of MCE-CL assays. After pre-assay CL labeling with N-(4-aminobutyl)-N-ethylisoluminol, small peptides present in physiological fluid samples at the 10^{-8} M level were quantified.¹⁷ Intracellular glutathione in individual human red blood cells was effectively labeled by diazo-luminol, a CL tagging reagent, and subsequently quantified by MCE-CL.¹⁸ The improved MCE-CL assays had the sensitivity in the range from 10^{-7} to 10^{-9} M of analytes (LOD). However, although assay sensitivity was significantly improved in these assays, an additional experimental step had to be performed for labeling the analytes.

Resonance energy transfer (RET) involves non-radiative (dipole-dipole) energy transfer between a donor and an acceptor that are in close proximity (normally <10 nm). RET that occurs between two fluorophores is known as fluorescence RET (FRET). Numerous publications have been seen on FRET being used in various areas such as signal transduction in living cells.^{19–21} Chemiluminescence RET (CRET) involves non-radiative energy transfer from a chemiluminescent (CL) donor to a fluorophore acceptor.²² CRET occurs by the oxidation of a CL compound that then excites the fluorescent acceptor. Very little study has been reported on CRET so far.^{23–24} These works described the observation of CRET between luminol oxidized by H_2O_2 and horse radish peroxidase (HRP)-conjugated quantum dots. Luminescent semiconductor nanocrystals (also called quantum dots, QDs) have optical properties of broad excitation spectrum, high quantum yield, and size-dependent emission-wavelength tunability. They are very suitable materials for developing RET approaches. QDs have been used as both donors and acceptors in FRET.^{25–27}

The aim of this work was to improve detection sensitivity in MCE by developing a facile, sensitive, and universal detection scheme. In our study on MCE-CL assays, we observed an efficient CRET between a luminol donor and a CdTe QD from the luminol-NaBrO-QD system. Furthermore, we noticed that the CRET was very sensitively inhibited by the presence of certain organic compounds. These findings inspired us to carry out the development of a CRET-based detection scheme for MCE. Several categories of organic compounds of biological interest including biogenic amines and thiols, amino acids, organic acids, and steroids were tested as model analytes. The applicability of the proposed MCE-CL method for analyzing real biological samples was demonstrated by quantifying amino acids in individual human red blood cells.

EXPERIMENTAL SECTION

Chemicals and solutions

Luminol, amino acids, epinephrine (E), isoprenaline (IP), dopamine (DA), glutathione (GSH), cysteine (Cys), N-acetylcysteine (NAC), glycylcysteine (GlyCys), estrone (E1), estradiol (E2),

estriol (E3), ascorbic acid (AA), uric acid(UA), sodium borate, Te powder, CdCl₂, NaHB₄ and mercaptopropyl acid (MPA) were obtained from Sigma Chemicals (St. Louis, MO, USA). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was obtained from Shanghai Qcbio Science & Technologies Co. Ltd. (Shanghai, China). Sylgard 184 (PDMS) silicone elastomer and curing agent was obtained from Dow Corning (Midland, MI, USA). All other chemicals used in this work were of analytical grade. Water was purified with a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. All solutions were filtered through a 0.22 μm membrane filter. The PBS solution for storage of red blood cells contained 135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM NaH₂PO₄, 10 mM HEPES and 2 mM CaCl₂ at pH 7.4 (adjusted with a 1 M NaOH solution). The electrophoresis buffer was 30 mM borate solution containing 1.0 mM luminol and 0.1 mM CdTe QDs at pH 9.4 (adjusted with 1 M NaOH solution). The CL reaction buffer was 50 mM borate solution containing 1.0 mM NaBrO at pH 10.5. Human blood samples were taken from healthy adult volunteers.

Apparatus

CL spectra were measured with a LS-55 luminescence spectrometer (Perkin-Elmer, USA); UV-visible spectra were measured with a TU-1901 UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd. China). CE experiments and Analysis of single cells were carried out using the laboratory-built MCE-CL system described previously.¹⁸ A multi-terminal high voltage power supply, variable in the range of 0–5000 V (Shandong Normal University, Jinan, China), was used for cell loading, lysing, and MCE separation.

Microchip

A schematic layout of the glass/PDMS hybrid microchip (98 mm × 25 mm) is shown in Figure 1. The microchip consisted of 60 μm wide × 20 μm deep sample load and separation channels, 300 μm wide × 20 μm deep oxidizer introduction channel, as well as holes of 4.0 mm in diameter and 1.5 mm in depth drilled into the chip to facilitate access to the channels and to serve as reservoirs (labeled as S, SW, B, BW and R in Figure 1). The channel between reservoir S and SW was used for sampling, the one between B and the join-point of the oxidizer introduction channel with the separation channel was used for the separation, and the one between R and BW was used for oxidizer introduction. The join-point of the oxidizer introduction channel with the separation channel was the viewing point for CL emission.

Preparation of CdTe QDs

The procedure used for preparing mercapto-propyl acid (MPA) capped CdTe QDs was similar to that described by Li et al.²⁸ with minor modifications. An Ar-saturated cadmium chloride solution was added to a NaHTe solution which was prepared by the reaction between NaHB₄ and tellurium powder in the presence of MPA. The concentration of Cd²⁺ was 2 mM, and the molar ratio of Cd²⁺: Te²⁻: MPA was maintained at 1: 0.5: 2.5. After mixing, the solution was heated with microwave at different reaction time to prepare different-sized QDs. The prepared CdTe QDs were purified by selective precipitation with isopropanol and re-dispersed in Milli-Q water. The CdTe QDs were further purified by dialysis using a dialysis membrane with molecular weight cut off 7000 in 10 mM NaOH. The concentration of MPA-capped CdTe QDs was defined by the number of cadmium atoms contained in the sample. Particle size distribution was obtained by a NaNo-ZS90 particle size and Zeta potentiometer analyzer.

Cell sample pretreatment

About 25 μL of a human blood sample was transferred into a 1.0-mL vial containing 975 μL PBS solution. After gently mixing, the cell suspension was centrifuged at 2000 rpm for 5 min and the supernatant was removed. PBS solution (975 μL) was added to the vial. The washing

was repeated for seven times until the supernatant was clear and transparent. Cells were suspended in 1.0 mL PBS solution and stored in portions at 4 °C. Before analysis, the cell suspension was diluted to 1.0×10^5 cell /mL with PBS solution.

MCE-CL assay

The microchannels were rinsed sequentially with 0.1 M NaOH, water and electrophoretic buffer for 5 min each. Prior to the MCE separation, the reservoirs B, S, SW and BW were filled with the electrophoretic buffer, and reservoir R was filled with oxidizer solution. Vacuum was applied to the reservoir BW in order to fill the separation channel with the electrophoresis buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample, a set of electrical potentials were applied to the reservoirs: reservoir S at 700 V, reservoir B at 250 V, reservoir BW at 350 V, reservoir SW at grounded, and reservoir R floating. The sample was transported from reservoir S to SW in pinched mode. After 20 s, potentials applied were changed as following: reservoir B at 2300, S at 1400, SW at 1400, and R at 350 V, while reservoir BW was grounded for separation and detection.

Analysis of Single Cells

The microchannels were preconditions as described above. The electrophoretic buffer solution in reservoir S was replaced by cell suspension, and reservoir SW was emptied. Owing to the differences in liquid levels in the reservoirs S and SW, the cell suspension flowed from reservoir S through the double “T” intersection to reservoir SW. When a single cell moved within the double “T” intersection of the channels as being observed under the microscope, reservoir SW was filled quickly with the electrophoretic buffer to stop the cell suspension flow. The sampled cell settled within the channel of double “T” intersection adhering to its wall. The docked cell was lysed by a set of electrical shocks (1 s each) with reservoir B at 900 V / reservoir BW at ground or reservoir BW at ground /reservoir B at 950 V as described previously.¹⁰ The MCE separation was performed by applying a 2300 V voltage to reservoir B, 1400 V and 1400 V voltage to reservoirs S and SW with the reservoir BW held at ground. Reservoir R was set at 350 V to generate a stream of the NaBrO oxidizer solution.

RESULT AND DISCUSSION

CRET in luminol-NaBrO-CdTe QD system

Little study has been so far reported on CRET or its application in chemical analysis. Luminol- H_2O_2 CL reaction is one of the most sensitive CL reactions and widely used in CL bioassays. Luminol CL spectrum has a maximum at 430 nm that overlaps well with the absorption spectrum of CdTe QDs. This satisfies the essential condition for CRET. However, no CRET was observed from luminol- H_2O_2 -QDs system in our studies. Further, we investigated various oxidizers including H_2O_2 , $\text{K}_3\text{Fe}(\text{CN})_6$, KMnO_4 , and NaBrO. These compounds are effective to oxidize luminol, producing CL emission. Fortunately, an efficient CRET was observed from the luminol-NaBrO-QDs system as shown in Figure 2A. As can be seen, in addition to the luminol CL emission with a maximum at ~ 430 nm another CL emission maximum was observed. The emission maximum wavelength matched the photoluminescent emission maximum of QDs (i.e. the acceptor). This is one of the most important characteristics of CRET. When QDs of different sizes were used, the CRET emission maximum changed with the size of the QDs (e.g. 660 nm for 4.1 nm diameter QDs). We recorded the photoluminescent emission maxima at 550, 572, 596, 614 and 660 nm for QDs with diameters of 2.31 nm, 2.93 nm, 3.82 nm, 4.08 nm and 4.41 nm, respectively. It's worth noting that no other oxidizers tested induced CRET in the luminol-oxidizer-QD systems. Figure 2B shows the CL spectra from luminol- H_2O_2 -QDs solutions. As can be seen, only CL emission from oxidized luminol was observed at ~430 nm.

Inhibition of the CRET

To our knowledge, no study on CRET inhibition has been reported so far. In this work, we studied the effects of various organic compounds on the CRET by adding these compounds into a luminol-NaBrO-QD solution and recorded the CL spectrum from the solution. It was found that some of the compounds tested inhibited effectively the CRET even at a very low concentration (e.g. 10^{-7} M). These compounds included many of biological interest such as biogenic amines and thiols, amino acids, organic acids (i.e. uric acid and ascorbic acid), and steroids. Figure 3 shows the CL spectra obtained from luminol-NaBrO-QD solutions with or without test compounds. As can be seen, biogenic amines and thiols exhibit stronger inhibitory effects on the CRET than amino acids and steroids. Coupling this very sensitive detection scheme with MCE, we carried out MCE separations with CRET-based detection in a microfluidic chip illustrated in Fig. 1. Luminol and CdTe QDs were added to the MCE running buffer. The NaBrO oxidizer solution was introduced and mixed with the MCE running buffer post-column. Fig. 4 shows an electropherogram obtained from separating a mixture of 20 protein amino acids (at a concentration of 5.0×10^{-7} M each). Under the MCE conditions, 12 amino acids were well separated and could be identified by their migration times. The other 8 amino acids were co-eluted into four peaks. Reproducibility of the migration times was $< 5.0\%$ (RSD, $n = 3$).

MCE assays with CRET-based CL detection

Certain organic compounds that are of biological interest were selected as model analytes to evaluate the analytical figures of merit for the proposed MCE assays with CRET-based detection. These compounds included biogenic amines and thiols, steroids, amino acids, and organic acids. Seven standard mixture solutions at various concentrations were prepared for each of these groups. They were then injected into the MCE-CL system and analyzed under the conditions described above in the "Experimental Section". All compounds in each group were well separated from each other (the separation of amino acids is shown in Fig. 4). For each compound, a calibration curve was established by performing a linear regression on the peak height – concentration data. From this calibration curve, the limit of detection was estimated (signal / noise = 3). Analytical figures of merit for quantifying the tested compounds are summarized in Table 1. In all cases, a linear calibration curve was obtained ($r^2 > 0.99$) with a linear range of > 2 orders of magnitude. Three biogenic amines were tested, i.e. epinephrine, isoprenaline, and dopamine. The assay sensitivity (LOD) was in the range of 6.4 ~10.3 nM. For analysis of biogenic thiols, the present method has detection limits of 6.4, 8.7, 10.3, and 10.7 nM for glutathione (GSH), cysteine (Cys), N-acetylcysteine (NAC), and glycylcysteine (GlyCys), respectively. The present method is also very sensitive for quantifying amino acids, steroids, and organic acids with LODs at the level of 10^{-8} M. Based on the results from a literature survey (Table 2), the proposed CRET-based detection coupled with MCE is 10 to 1000 times more sensitive than the other detection schemes used in MCE including electrochemical, chemiluminescence (CL), laser induced fluorescence (LIF), and fluorescence (Flu) for quantifying the tested compounds except dopamine. It's worth noting that LOD data of the MCE-CL assays developed by our lab are also included in the table and it's more reasonable to compare them with those of the present method because the MCE-CL systems used in all these studies were either identical or very similar. To evaluate the reproducibility of the proposed assay, a standard mixture solution of Arg, Ser, and Gly (1.0×10^{-7} M each) was analyzed six times. The relative standard deviation (RSD) in peak height was found to be $< 3.8\%$ for all the three components.

Quantification of amino acids in individual cells

To access the applicability of the proposed MCE assay with CRET-based detection for analyzing real biological samples, the method was used to quantify amino acids in individual

human red blood cells. Two typical electropherograms from such analyses are shown in Figure 5. Peaks corresponding to 9 amino acids, i.e. Arg, Lys, Ser, Ala, Asn, Glu, Asp, Gly, and Trp were identified based on the migration times. The peak identification was confirmed by separating a cell homogenate sample spiked with the 9 amino acids. The analytical results of intracellular amino acids in individual human red blood cells are summarized in Table 3. The amino acid contents differed from cell to cell. As shown in Figure 5, Arg and Trp were detected in cell #A (Fig. 5A), but not in cell #B (Fig. 5B). The average intracellular contents of Arg, Lys, Ser, Ala, Asn, Glu, Asp, Gly, and Trp were 4.5 ± 3.3 , 12.1 ± 5.8 , 13.8 ± 4.9 , 22.0 ± 6.0 , 9.5 ± 5.4 , 14.4 ± 4.1 , 6.0 ± 4.4 , 20.5 ± 5.3 , and 2.6 ± 2.8 amol /cell, respectively ($n = 20$). In a previously reported study, contents of amino acids in individual human erythrocytes were found in the range from 3.8–32 amol /single cell by using a CE-LIF method.³⁹ From the analytical results, Ser, Ala, Glu, and Gly were the predominant amino acids in human red blood cells.

CONCLUSION

An efficient CRET between a luminol donor and a CdTe QD acceptor was observed from the luminol-NaBrO-QD system. Moreover, it was found that the CRET was very sensitively suppressed by the presence of certain organic compounds of biological interest. These allowed developing sensitive MCE assays with CRET-based detection. Five categories of organic compounds were selected as model analytes including biogenic amines and thiols, amino acids, organic acids, and steroids. Studies on the analytical figures of merit showed that linear calibration curves were obtained for all the compounds tested with a wide linear range (> 2 orders of magnitude). Limits of detection were in the range from 10^{-8} to 10^{-9} M. The proposed MCE assays were 10 – 1000 times more sensitive than the previously reported MCE methods with CL, LIF, Flu, or electrochemical detection for quantifying the corresponding compounds. Amino acids in individual red blood cells were determined by using the present method. Nine amino acids including Ser, Ala, Glu, and Gly were detected at the level of amol / cell. The assay proved to be simple, quick, reproducible, and sensitive. To the best of our knowledge, the proposed CRET-based detection is the most sensitive detection scheme for MCE that has been reported so far.

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Figure 1. Dimensions and layout of the glass /PDMS microchip used in this work. S: sample reservoir; B: buffer reservoir; SW: sample waste reservoir; BW: buffer waste reservoir; and R: the oxidizer reagent reservoir.

Figure 2.

CL spectra of various luminol-oxidizer solutions containing different sized QDs (a-f): A) luminol-NaBrO solutions; and B) luminol-H₂O₂ solutions. Peaks (QD size): a) no QDs; b) 2.31 nm; c) 2.93 nm; d) 3.82 nm; e) 4.08 nm; and f) 4.41 nm.

Figure 3. CL spectra of the luminol-NaBrO-QD system in the presence of analytes. Peaks: a. GSH; b. Epinephrine; c. Ala; d. Estradiol; e. no analyte; CL reaction buffer (pH10.5) was 30 mM borate solution containing 1.5×10^{-4} M luminol, 3.0×10^{-7} M analyte and 5.0×10^{-5} M CdTe QDs (diameter, 4.43 nm). The concentration of NaBrO in borate buffer (pH10.5) was 1.5×10^{-4} M.

Figure 4.

Electropherogram obtained from the separation of 20 protein amino acids. MCE conditions: electrophoretic buffer was 30 mM borate solution (pH 9.4) containing 1.0 mM luminol and 0.1 mM CdTe QDs; oxidizer solution (pH 10.5) was 1.0 mM NaBrO in 50 mM borate buffer. Concentrations of amino acids were 5.0×10^{-7} M. Peaks: 1. Arg; 2. Lys; 3. Ala; 4. Pro+Val; 5. Leu+ Ile; 6. Phe+His; 7. Tyr; 8. Gln+Met; 9. Ser; 10. Asn; 11. Thr; 12. Cys; 13. Gly; 14. Trp; 15. Glu; and 16. Asp.

Figure 5. Two typical electropherograms obtained from analysis of individual human red blood cells. Nine amino acids were detected at the level of amol /cell. MCE conditions were as in Figure 4.

Table 1

Analytical figures of merit

Analyte	Linear regression Equation ^a	Linear rang (10 ⁻⁸ M)	Correlation coefficient(r ²)	LOD (10 ⁻⁹ M)
Biogenic amines				
IP	$\Delta H = 16.037C + 1.767$	2.0–200	0.9952	6.4
E	$\Delta H = 10.356C + 3.036$	3.0–300	0.9985	8.7
DA	$\Delta H = 7.554C + 4.188$	3.5–350	0.9906	10.3
Biogenic thiols				
GlyCys	$\Delta H = 9.777C + 1.320$	3.0–300	0.9909	10.9
NAC	$\Delta H = 9.987C + 1.704$	3.0–300	0.9955	10.3
Cys	$\Delta H = 15.497C - 1.582$	2.0–200	0.9927	8.8
GSH	$\Delta H = 16.747C - 0.608$	2.0–200	0.9961	7.5
Steroids				
E ₁	$\Delta H = 4.191C - 1.327$	8.0–800	0.9970	32
E ₂	$\Delta H = 4.454C - 0.934$	7.0–700	0.9946	29
E ₃	$\Delta H = 4.708C + 0.253$	7.0–700	0.9973	27
Organic acids				
UA	$\Delta H = 9.280C + 3.282$	3.0–300	0.9929	9.4
AA	$\Delta H = 8.964C + 3.165$	3.0–300	0.9936	9.8
Amino acids				
Arg	$\Delta H = 6.007C + 0.675$	5.0–500	0.9970	19
Lys	$\Delta H = 5.774C - 1.368$	5.0–500	0.9946	23
Ala	$\Delta H = 5.578C + 0.406$	5.0–500	0.9973	21
Glu	$\Delta H = 4.937C - 2.450$	6.0–500	0.9915	29
Asp	$\Delta H = 4.840C - 3.046$	6.0–500	0.9923	31
Ser	$\Delta H = 5.994C + 0.265$	5.0–500	0.9960	20
Asn	$\Delta H = 5.852C - 0.311$	5.0–500	0.9951	21
Gly	$\Delta H = 5.755C - 0.308$	5.0–500	0.9951	21
Trp	$\Delta H = 5.646C - 0.424$	5.0–500	0.9951	22

^a) H, peak height (relative CL intensity, μV); and C, concentration of the analyte in 10⁻⁸ M.

Table 2

Sensitivity comparison of MCE-based methods with different detection techniques

Detection tech.	Analyte	Limit of detection	Reference
Electrochemical	dopamine	5.9×10^{-8} M	30
Electrochemical	dopamine	1.0×10^{-6} M	38
CL	dopamine	10^{-8} M	31*
LIF	GSH	2.5×10^{-7} M	10
CL	GSH	$>1.0 \times 10^{-7}$ M	13 – 15
CL	Luminol-labeled GSH	3.6×10^{-9} M	18*
Electro-CL	ascorbic acid	1.0×10^{-8} M	35
CL	ascorbic acid	1.3×10^{-6} M	36*
Electrochemical	uric acid	1.0×10^{-6} M	38
Flu	OPA-amino acids	$> 8.0 \times 10^{-7}$ M	34
Flu	NBD-Phe	2.8×10^{-5} M	29
CL	dansyl-Gly	3.9×10^{-7} M	32
CL	Trp	2.1×10^{-6} M	36*
UV-LIF	Trp	1.0×10^{-7} M	33

* Our work previously reported. A similar MCE-CL system was used.

Table 3

Contents of amino acids in single human red blood cells (amol)

Cell	Arg	Lys	Ser	Ala	Asn	Glu	Asp	Gly	Trp
1	9.8	18.0	15.5	27.5	16.4	18.5	8.9	19.3	7.1
2	5.6	12.4	11.4	18.3	10.6	15.7	11.9	23.4	ND
3	3.5	9.2	8.7	17.6	8.6	12.4	7.4	24.2	5.4
4	6.4	17.1	16.8	15.4	9.5	19.3	12.4	15.7	4.5
5	8.1	15.0	21.2	23.4	15.7	16.1	8.7	27.8	6.4
6	ND	9.8	7.9	31.2	ND	9.5	ND	21.7	ND
7	4.1	8.4	9.9	12.9	9.9	10.4	5.1	22.6	ND
8	6.8	19.4	17.6	29.8	14.8	18.0	8.3	30.5	3.9
9	ND	11.6	8.9	13.7	9.2	13.5	7.0	11.6	ND
10	7.8	19.4	20.5	27.4	17.1	21.0	10.5	18.3	ND
11	9.2	22.8	24.3	30.7	19.0	19.2	9.4	20.1	6.7
12	7.6	16.0	14.1	14.2	8.3	12.9	5.6	21.2	4.8
13	5.9	14.8	12.4	20.9	9.4	14.3	ND	27.8	ND
14	ND	7.5	10.0	16.8	ND	10.2	ND	14.6	ND
15	5.4	10.6	7.6	25.7	11.3	8.8	7.1	19.5	5.5
16	4.6	12.6	16.7	19.1	6.4	16.7	8.4	16.0	4.0
17	ND	5.4	9.8	22.8	7.1	8.9	ND	10.7	ND
18	3.0	ND	10.4	18.6	ND	9.2	ND	25.4	4.3
19	2.87	7.4	18.6	24.5	9.6	20.1	9.9	22.8	ND
20	ND	4.8	13.2	28.7	6.9	13.5	ND	16.9	ND

ND: not detected.