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Combination of capillary micellar liquid chromatography with on-chip microfluidic chemiluminescence detection for direct analysis of buspirone in human plasma

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ABSTRACT

Microfluidic based chemiluminescence (CL) detector having novel channel design for enhanced mixing has been developed and investigated in terms of its applicability with micellar mode of liquid chromatography (MLC). The newly developed detector was found to be highly sensitive and an alternative detection technique to combine with capillary MLC. This combination was successfully employed for direct detection of a model analyte using Ru(III)-peroxydisulphate CL system. The selected analyte, buspirone hydrochloride (BUS), was detected selectively at therapeutic concentration levels in human plasma without any sample pretreatment. By incorporating eight flow split units within the spiral channel of microfluidic chip, an enhancement of 140% in CL emission was observed. We also evaluated the effect of non- ionic surfactant, Brij-35, which used as mobile phase modifier in MLC, on CL emission. The CL signal was improved by 52% compared to aqueous-organic mobile phase combinations. Various parameters influencing the micellar chromatographic performance and the CL emission were optimized. This allowed highly sensitive analysis of BUS with limit of detection (LOD) of 0.27 ng mL^{-1} ($3\sigma/s$) and limit of quantification (LOQ) of 0.89 ng mL^{-1} ($10\sigma/s$). The analyte recovery from human plasma at three different concentration level ranges from 88% to 96% (RSD 1.9–5.3%). The direct analysis of BUS in human plasma was achieved within 6 min. Therefore, combining microfluidic CL detection with micellar mode of separation is an efficient, cost-effective and highly sensitive technique that can utilize MLC in its full capacity for various bioanalytical procedures.

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1. Introduction

Developing *green* separation methods with highly sensitive and selective detection techniques is a challenging task. Micellar liquid chromatography (MLC) is among the most promising separation approaches that can provide high selectivity and throughput. In this approach, a mobile phase containing a surfactant at concentrations above its critical micellar concentration (CMC) is used. MLC has received a lot of attention as an attractive alternative to conventional reverse phase chromatography due to its environmental friendly nature, applicability in physicochemical studies, enhanced retention, selectivity and cost effectiveness [1–3]. Another key advantage of MLC is the ability of direct on-column injection of biological fluids and the elimination of laborious and extensive sample pre-treatment steps [4,5]. However, most of the reported MLC methods utilize uv–visible spectrophotometric detection methods. Although, uv–visible spectroscopy is simple and almost universal, the detection limits are usually high, and in many cases, the method is not suitable for the determination of an analyte in biological samples. The major limitation of MLC is its incompatibility with mass spectrometry, a highly sensitive detector commonly used for the determination of analytes in biological fluids. On the other hand, MLC is completely compatible with chemiluminescence (CL) detection techniques. Moreover, the combination of CL and MLC offers an added advantage by enhancing the CL emission of several CL reactions [6–8].

The combination of CL detection with HPLC has been extensively studied. By coupling the CL detection with the selectivity of a powerful separation technique, provides highly sensitive detection that can be easily manipulated for developing many robust applications for biological, pharmaceutical, food and environmental samples [9–11]. CL detection is usually carried out using a post column reaction of column eluent with CL reagents. Moreover, the reactions adopted in HPLC-CL systems are very rapid, and the emission intensity depends on many environmental factors, such as the pH, reagent concentration, temperature, solvent and ionic strength. Therefore, several factors must be considered while interfacing HPLC with CL detection, including compatibility of

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mobile phase conditions and the CL reaction, rate of CL reaction, the mixing efficiency and the volume of flow cell. In most of the cases, and especially in the tris(2,2′-bipyridyl)ruthenium CL system, the use of organic solvents affect the CL emission negatively. This has been observed in the case of methanol, which remarkably increases the background CL signal of the system [8]. It is worth mentioning here that the use of these organic solvents in HPLC-CL is essential for better chromatographic performance, but at the expense of the CL sensitivity. In this work, we propose a micellar mode of separation as an alternative to conventional reverse phase chromatography to utilize the added benefit of enhanced CL intensity due to surfactants in mobile phase.

In most commercial HPLC-CL systems, coiled-tubing flow cells with T- or Y-shaped connectors are used as CL flow cells [12,13]. Many research groups fabricated flow cells for CL detection using Perspex, Teflon, Polycarbonate or White Acetal disks or thick sheets with spiral or serpentine channels machined or engraved into them and sealed with transparent plates or films [14]. These flow cells had many merits over the coiled-tubing approach in terms of mixing and fabrication capabilities; however, they have not received widespread acceptance. One of the major reasons is due to the usage of relatively high quantities of CL reagents for efficient mixing. The total internal volume of these cells are moderately high (133–275 μL), and therefore, it is necessary to infuse the CL reagents at high flow rates [15]. Conventional HPLC systems can be operated at 0.8–1.2 mL min^{-1}, but the CL reagents need to be infused at a rate of minimum 2–3 mL min^{-1} for efficient mixing. Even flow rate as high as 15 mL min^{-1} was also reported [16]. The high CL reagent flow rates in HPLC-CL systems result in additional dilution of the analytes, post column band broadening and poor resolution. Microbore HPLC columns have been utilized as an alternative to reduce the consumption of CL reagents, resulting in improved resolution and sensitivity; however, the flow rates of CL reagents remain high compared with typical flow rates of capillary systems [17–20]. In capillary HPLC systems, the minimum CL reagent flow rate reported is 400 μL min^{-1} while a typical mobile phase flow rate of 100 μL min^{-1} is used and even a CL-flow rate as high as 2 mL min^{-1} was used with 0.2 μL min^{-1} flow rate of the mobile phase [20]. Generally, high flow rates of CL reagents are used in HPLC-CL to achieve good sensitivity which confines developing applications for routine analysis due to the expensive and hazardous nature of CL reagents. Additionally, long and wide tubes used in conventional CL detector can have an impact on the separation and band broadening. These limitations make HPLC-CL systems relatively unpopular even though it possesses many analytical merits. Microfluidics is an ideal alternative to overcome the limitations associated with high flow rates as it is a proven efficient mixing device at low flow rates. The operating flow rate of capillary HPLC instruments with microbore columns (20–200 μL) is very well suitable and the tolerable flow rates suitable for reagent mixing in microfluidic chips generally falls within these limits. In addition, it can be easily fabricated for enhanced mixing according to the rate of CL reaction.

Here, we introduce a novel approach to overcome the problems associated with high flow rates generally used in CL detectors of microbore HPLC systems by interfacing it with on-chip detection system. Microfluidic chips can be easily fabricated to be used simultaneously as a *microflow cell* and *micromixer* for CL reaction. By incorporating suitable channel designs, it is possible to enhance the reagent mixing with analyte and resultant chemiluminescence efficiency. We have investigated the appropriateness and advantages of such setup in combination with capillary HPLC and their impacts on CL emission. Tris(2,2′-bipyridyl)ruthenium(III)-ammonium peroxydisulphate (Ru-Oxidant) CL system with buspirone hydrochloride (BUS) was selected as a model system. BUS belongs to the azapirone chemical class of drug molecules, which are generally used as anxiolytics and antipsychotics drugs. Generally, these molecules are suitable for CL detection using Ru- Oxidant due to the presence of an aliphatic tertiary nitrogen atom in the molecule. The $Ru(bpy)_3^{3+}$, generated via photoinduced chemical oxidization of Ru(II) using peroxydisulphate in presence of light, can be reduced by tertiary amine to an electronically excited $[Ru(bpy)_3^{2+}]^*$ species from which light emission is observed. A reducing intermediate of amine with sufficient energy to produce an excited species was supposed to be formed by one-electron oxidation followed by deprotonation and the resultant neutral amine radical subsequently reduces the $[Ru(bpy)_3^{3+}]$ to $[Ru(bpy)_3^{2+}]^*$ (Scheme 1) [21–23]. The effect of pH on the CL intensity substantiates the deportation step as slightly basic pH was always observed as the most suitable condition for maximum CL emission.

$$Ru(bpy)_3^{2+} + h\upsilon \rightarrow Ru(bpy)_3^{2+*}$$

$$Ru(bpy)_3^{2+*} + S_2O_8^{2-} \rightarrow Ru(bpy)_3^{3+} + SO_4^{2-} + SO_4^{\cdot-}$$

$$Ru(bpy)_3^{2+*} + SO_4^{\cdot-} \rightarrow Ru(bpy)_3^{3+}$$

$$Ru(bpy)_3^{3+} + R_3N \rightarrow Ru(bpy)_3^{3+} + R_3\dot{N}^+$$

$$R_3\dot{N}^+ \rightarrow R_3\dot{N} + H^+$$

$$R_3\dot{N} + Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\upsilon$$

Scheme 1

In this paper, we demonstrated for the first time the development, performance and validation of an instrumental combination of a capillary HPLC in micellar mode of separation and on-chip chemiluminescence detection with novel microfluidic channel design for enhanced luminescence emission of Ru(III)-Peroxydisulphate CL system.

We also presented, a highly selective and sensitive analytical method for the determination of BUS hydrochloride. The feasibility of direct on column injection of biological fluids using *micellar-capillary HPLC with microfluidic CL* detection has been explored. Several validated methods are available for the determination of BUS and other drugs in azapirone class, including radioimmunoassay [24], GC–MS [25], liquid chromatography [26–30] with different detection techniques, such as electrochemical detection [31], mass spectrometry and tandem mass spectrometry [32]. Detection limits as low as 0.2 ng mL^{-1} have been reported for BUS using MS and MS/MS. However, these methods require extensive sample cleaning, protein separation and pre-concentration of analyte. To the best of our knowledge, no such method has been reported for the analysis of any such drugs from this class, including BUS, using capillary micellar liquid chromatography and on-chip CL detection. Additionally, the developed HPLC method is a cost-effective "*green*" analytical solution.

2. Experimental

2.1. Chemicals and reagents

All reagents were of analytical grade unless otherwise specified and consumed without further purification. The solutions were prepared with ultrapure water obtained from a MilliQ water system (Millipore S.A.S. France). Acetonitrile (HPLC Grade), Brij 35, ammonium peroxydisulphate, sodium hydroxide and potassium dihydrogen phosphate (Fluka Biochemika, Ultra for Molecular biology) were purchased from Sigma Aldrich, Germany. Tris(2,2′-bipyridyl)ruthenium(II) chloride ($Ru(bpy)_3^{2+}$) was purchased from Aldrich (USA). The buspirone hydrochloride standard was a gift from a quality control laboratory, Ministry of Health (Muscat, Sultanate Oman).

2.2. Preparation of reagent and standard solutions

$Ru(bpy)_3^{2+}$ (2.0×10^{-3} mol L^{-1}) was prepared by dissolving 14.9 mg of tris(2,2′-bipyridyl)ruthenium(II)chloride hexahydrate in 10 mL of deionized water, and the oxidant, ammonium peroxydisulphate, was prepared by dissolving 5.7 mg in deionized water and diluting to 25.0 mL to give a 1.0×10^{-3} mol L^{-1} stock solution. Phosphate buffer (0.02 M, pH 6.0) was prepared using potassium hydrogen phosphate, and the pH was adjusted using a 1 M NaOH solution. A stock standard solution of BUS (50 μg mL^{-1}) was prepared separately by dissolving 5.0 mg of pure drug in 100 mL of deionized water. The stock solution was kept in a cold, dark place and working solutions were prepared daily by appropriate dilutions of the stock solution with phosphate buffer (0.02 M, pH 6).

2.3. Mobile phase preparation

An aqueous-organic mobile phase was prepared by mixing acetonitrile and phosphate buffer (0.02 M, pH 6) in a 30:70 ratio followed by filtration and sonication. The micellar mobile phase was prepared by dissolving appropriate amounts of Brij-35 in phosphate buffer (0.02 M, pH 6). The effect of surfactant concentration was evaluated by preparing Brij-35 at different concentrations ranging from 0.15 mM to 1.0 mM, otherwise all of the analysis were performed at 1.0 mM of Brij-35 in buffer.

2.4. Instrumentation

2.4.1. Microfluidic channel design

The chip fabrications were performed at Micronit (Netherlands) as per the designs provided for the two models: spiral (SP) and spiral with eight flow split units (FS). A tear drop (TD) chip design (Micronit, Netherlands) was used as third model without any change. For the first two models, Borofloat glass (same as TD) was used as the top and bottom layer with thicknesses of 1.1 mm and 0.7 mm, respectively, and dimensions of $45 \times 15 \times 1.8$ mm. The channel length, depth, width and volume of these two models were adjusted in such a way that both have same internal dimensions of 80 mm, 150 μm, 200 μm and 3 μL, respectively. The chip itself served as the detection window as it is made of transparent material.

2.4.2. Microfluidic CL detector setup

The microfluidics chips, fluidics connect 4515 and fused silica capillary were from Micronit (Netherlands). A dual syringe infusion pump (KD Scientific, USA) was used for CL reagent delivery, and a photomultiplier tube (PMT, H7155-2, Hamamatsu, Japan) was used to detect the signal and documented using C-8855 counting software program (Hamamatsu, Japan).

The two-chip microfluidic setup was used as the chemiluminescence detector. The first chip (chip-1) was a micro-reactor having a serpentine design and 13-μL internal chip volume. A light source was placed on top of it to catalyze the oxidation reaction of the chemiluminescence reagents, $Ru(bpy)_3^{2+}$ and peroxydisulphate, injected from the syringe pump via chip inlets. The second chip (chip-2) functioned as a micro-mixer and flow cell with an internal channel volume of 3 μL and a spiral shape, which had eight flow splitting units. It was connected to the outlet of chip-1 via a fused silica capillary (100 μm I.D.). The PMT was placed on top of the second chip and designed to collect the maximum output of the CL signal. The entire detector setup was encased in a light-tight housing and connected the UV detector outlet of a microbore HPLC using a fused silica capillary (50 μm, I.D.)

2.4.3. Interfacing capillary liquid chromatography with on-chip detection device

A capillary LC (Agilent 1260 Infinity) equipped with a uv detector, binary capillary LC pump, low flow autosampler and thermostated column compartment with six port micro column switching valve was used for interfacing with on-chip CL detection system. A column of Pursuit 3 diphenyl (100×1.00 mm, 5 μm, Agilent Technologies, USA) was utilized and the column temperature was maintained at room temperature. Fig. 1 shows the schematic diagram of interfacing capillary LC with the proposed microfluidic chip setup. The internal diameter (i.d) of fused silica capillary tubing used to connect the capillary LC pump to autosampler, microbore column via a micro column switching valve and uv detector was 50 μm. A larger i.d fused silica tubing (100 μm) was used while connecting the outlet of the uv detector to the inlet of chip-2 in microfluidic setup. This will reduce the back pressure generated by microfluidics chips. The total back pressure observed by coupling the microfluidic detection device to capillary LC was less than 10 bar and this level was maintained throughout the experiments. The mobile phase flow rate and sample injection volume was optimized at 100 μL min^{-1} and 20 μL, respectively. The chromatographic system was controlled using Open Lab ChemStation (version C.01.04).

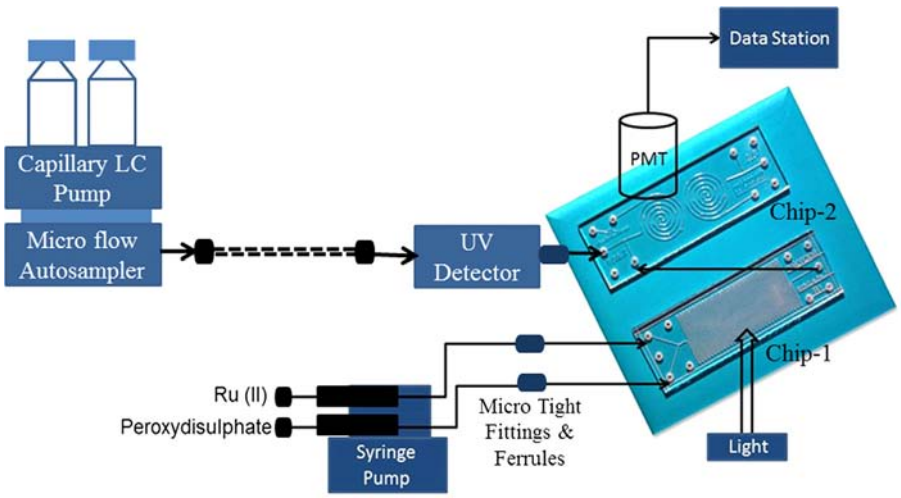

Fig. 1. Schematic representation of capillary HPLC combined with microfluidic *on-chip* CL detection device.

2.5. Biological sample preparation

The blank human plasma samples were collected (University hospital blood bank) and stored at 4 °C. Approximately 100 μL of stock solutions of respective high concentrations of BUS were spiked into the blank plasma (400 μL). The sample solutions were further diluted with micellar mobile phase, and the final content was in a 1:1 ratio. Plasma samples were prepared at 5 ng mL^{-1}, 10 ng mL^{-1} and 50 ng mL^{-1} to study the recovery.

3. Results and discussions

The preliminary experiments for investigating the parameters affecting the CL signal intensity were carried out independently without using the column by direct injection mode via the six port column switching valve. The effect of the chip design and experimental parameters, such as buffer pH, concentration of reagents and flow rate, were investigated systematically and optimized for maximum CL signal intensity. Similarly, the suitable chromatographic conditions for MLC were established without interfacing the capillary HPLC system with the MF-CL detector. After the successful completion of preliminary evaluations and optimisations, the MF-CL setup was interfaced with the capillary LC, which was then utilized for further studies and performance evaluation.

3.1. The effect of the microfluidic channel design

We have previously reported 250% signal enhancement using TD design in a multichip setup for the analysis of chlorpheniramine maleate compared with a serpentine geometry. This is due to the enhanced mixing in TD chips using three parallel mixing mechanisms: flow folding, chaotic advection and flow splitting with recombination [33,34]. However, relatively low precision in the signal intensity and high back pressure was observed while interfacing with capillary LC systems. Moreover, frequent chip clogging was a bottleneck when using TD chip continuously, which ends up in gradual increase in the background signal. It is worth mentioning here that repetitive additional cleaning procedures were required for the TD chip to obtain consistent performance. The TD chip consists of three layers and the middle layer has holes to connect the two layers, which could be the reason for such difficulties. These drawbacks were not observed in the systems reported earlier, as the total flow rate was lower than the current system. Moreover, surfactant was not used in the previous systems, and therefore, the viscosity of the solvents used in the current system is higher, which further increases the pressure. Therefore, it was necessary to find an alternative two-layer chip designs (instead of the three layer TD design), which is simple but at the same time can efficiently mix the CL reagents and analyte. Flow cells having spiral designs were generally used to improve mixing and CL emission [15]. Therefore, a microfluidic chip with spiral channel was initially designed (Fig. 2a) and used for the analysis of BUS. Relatively good CL signal intensity was obtained. We then used another chip (FS) design, where eight flow split units have been introduced within the spiral channel as shown in Fig. 2b. The CL emission was enhanced by 140% as can be clearly observed in Fig. 2c and d. Although the difference in channel design between the two chips is not too large, the enhancement of the CL signal intensity in the FS compared with the SP design was considerable. This difference is attributed to a multi-mixing mechanism in the FS rather than the single mixing mechanism

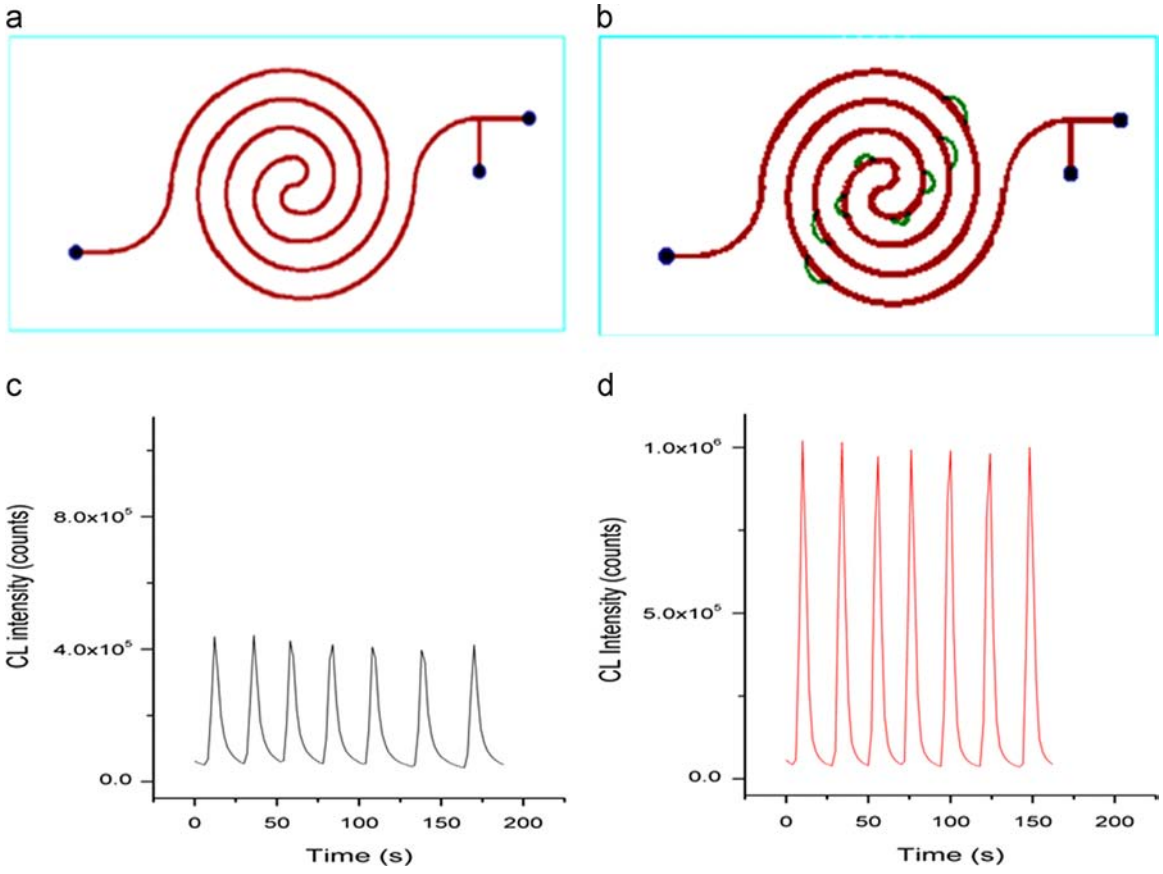

Fig. 2. A model of the microfluidic chip designs evaluated for use as CL reaction detection *microflow cells* and the effect of channel design on the CL signal intensity for 500 ng mL^{-1} of BUS solution in Ru-Oxidant CL system, Spiral ((a) and (c)) and Spiral with eight flow splitting units ((b) and (d)).

in the SP. In FS, sudden changes due to the flow division in the direction of liquid flow create disturbances and result in increased radial mixing. The background signal was found to be very stable, and the FS design was considered for further use with capillary LC as a micro-mixer CL flow cell. The FS design is very simple compared with the TD and cheaper as it consists of only two layers of chip rather than three layers.

3.2. Method development and optimization of the chromatographic conditions—Reverse phase and micellar mode

The isocratic chromatographic conditions for BUS was developed using a Pursuit-3-diphenyl column at 215 nm as the detection wavelength. Combinations of acetonitrile and 0.02 M phosphate buffer were used as the mobile phases. The phenyl column was selected to obtain reduced retention for BUS as most of the C18 based columns show relatively long retention times. Initially, the pH of the buffer was kept at 6.5, and mobile phase combinations over the range of 50–20% acetonitrile were evaluated. A ratio of 70:30 buffer: acetonitrile resulted in good retention and peak shape. The effect of acetonitrile on the Ru-Oxidant chemiluminescence emission was studied by comparing the CL intensity of 30% acetonitrile in buffer with that of aqueous buffer, a standard carrier solvent used in microfluidic setups via direct injection. The mobile phase using acetonitrile yielded only 56% of the observed intensity compared with the aqueous buffer. Similarly, the influence of surfactants on CL emission has been studied and preliminary investigations using Brij-35 were found to have a strong enhancement effect on CL emission compared with the mobile phase that had acetonitrile. Although the addition of surfactant to the mobile phase reduced the CL intensity by approximately 15% compared with the intensity in aqueous buffer, as seen in Fig. 3, an enhancement of 52% was observed by replacing acetonitrile with Brij-35 in the mobile phase. Therefore, Brij-35 above its CMC in phosphate buffer was used as the micellar mobile phase. The effect of the concentration of Brij-35 on the chromatographic parameters of BUS was investigated over the range of 0.2–1.0 mM using capillary HPLC with UV detection at 215 nm. At lower concentrations of surfactants, as shown in Fig. 4a, relatively long retention of analyte with peak broadening and tailing was observed. This result may be due to the partially modified stationary phase at a lower concentration of surfactant in the mobile phase, and it is expected that the difference in the surfactant adsorption on the stationary phase can significantly affect the retention behavior. Furthermore, the relationship between the surfactant concentration and its adsorption on the stationary phase was seen from the rapid column pre-conditioning observed at higher surfactant concentrations. The retention and peak shape were improved up to 0.8 mM and no further improvements above 1.0 mM of Brij-35 in the mobile phase were observed. The retention time of BUS at optimized surfactant concentrations was 3.34 min (Fig. 4b), and the standard deviation in retention time of fourteen replicate injections was ± 0.01 min. The retention factor (k') was calculated at different concentrations and $k' \geq 10$ was obtained when 1.0 mM surfactant was used in the mobile phase, as shown in Fig. 4c. Therefore, further studies were performed using 1.0 mM Brij-35 in phosphate buffer. Additionally, the effect of the concentration of the phosphate buffer (0.01–0.05 M) and pH (5.5–6.5) on the retention time in micellar mode was investigated. There were no significant changes found in the peak shape or retention time, which may be due to non-ionic nature of the surfactant used.

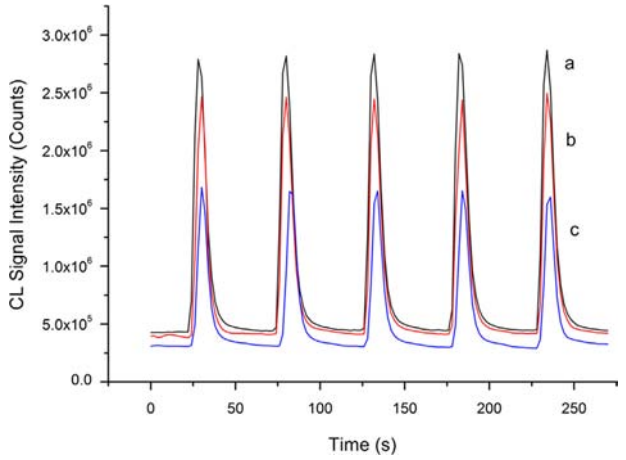

Fig. 3. Comparison of effect of carrier solvent (a) 0.02 M Buffer, (b) 1.0 mM Brij-35 and (c) ACN: Buffer; 30:70 on CL signal intensity of BUS solution (500 ng mL^{-1}) in Ru-Oxidant CL system.

3.3. Effect of the mobile phase pH on the CL intensity

The majority of compounds that elicit chemiluminescence with $Ru(bpy)_3^{3+}$ have an amine group, and aliphatic tertiary amines show the highest response [20]. Therefore, it is expected that pH can play an important role in the CL intensity. In most cases, the ideal pH for separation may not be suitable for maximum CL emission; a compromise is always necessary. The mobile phase pH is critical for efficient chromatographic separation, and in this case, the mobile phase acts simultaneously as the carrier flow system and CL reaction medium. Therefore, the pH was chosen carefully by considering this conflict. The pKa of BUS is 7.6, so we decided to screen a wide pH range (between 4 and 10) by adjusting the pH of the 0.02 M phosphate buffer. The optimum pH for the maximum CL emission was determined using direct injection mode in the absence of Brij-35. It was observed that the CL intensity increased considerably as the pH increased up to 6 and then reduced gradually as the pH increased. It was further optimized in a narrow window between 5.5 and 7.0 at an interval of 0.5 mM with 1.0 mM Brij-35 in the buffer, and the same trend was observed, as shown in Fig. 5. Although at higher pH there was an improvement in total signal strength, the background signal increased considerably; therefore, the effective signal intensity was lower, which can be attributed to the reaction of $Ru(bpy)_3^{2+}$ with hydroxide ions. The optimum condition for the analysis of BUS with of $Ru(bpy)_3^{3+}$ was found to be pH 6.0.

3.4. The effect of the concentration and the flow rate of the chemiluminescence reagents

Rapidly decaying CL emissions can be significantly influenced by the flow rate of the reagents. Usually higher flow rates result in intense emissions. In the case of the $Ru(bpy)_3^{2+}$ CL system, very high flow rates (6.4 mL min^{-1}) have been reported to improve the signal intensity [35]. Here, we studied the effect of flow rates on the CL reagents from 10 μL min^{-1} to 40 μL min^{-1} by recording the peak heights of a 500 ng mL^{-1} solution of BUS in direct injection mode with 1.0 mM Brij-35 in buffer (pH 6, 80 μL min^{-1}). A slight increase in intensity of approximately 6% was observed at 20 μL min^{-1} and considered the optimum flow rate because there was no significant change by further increasing the flow rate. It should be noted that in the proposed microfluidics detector setup no compromise has been made in the flow rate to reduce the reagent consumptions as reported earlier [35]. At high flow rates, the mixing process will be enhanced due to an increase in the Reynolds number, which results in fast CL reactions. However, in the case of normal systems, the major limitations of high flow rates are the excessive usage of expensive chemicals and the generation of toxic waste. Microfluidics systems are an attractive

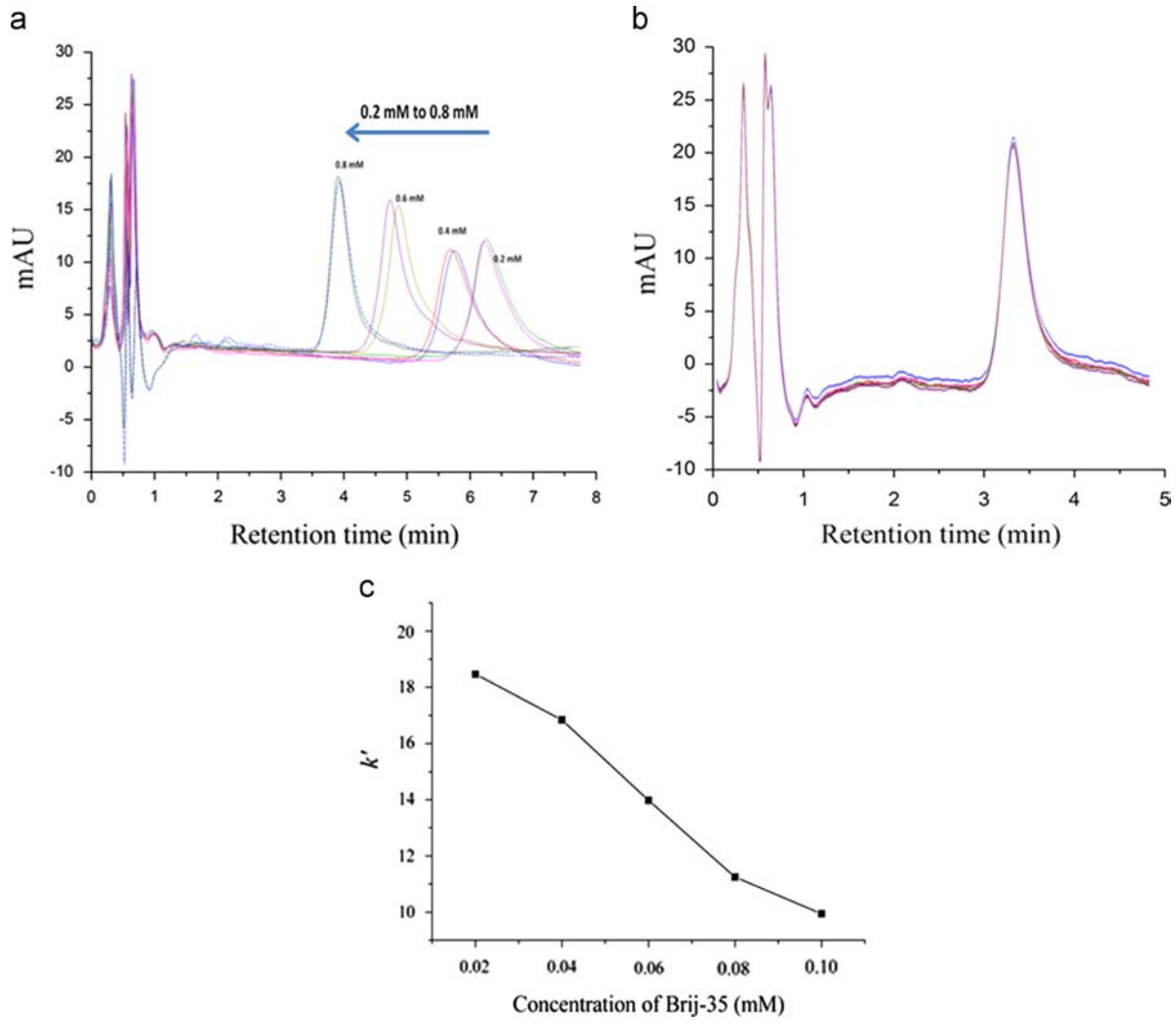

Fig. 4. The effect of surfactant concentration on retention behaviour of BUS hydrochloride (5 μg mL^{-1}), chromatograms at 215 nm (a) obtained from 0.2 mM to 0.8 mM of Brij-35 in mobile phase (b) six replicate injections using 1.0 mM Brij-35 in mobile phase (c) the change in retention factor (k') as brij-35 concentration increases in mobile phase.

alternative that utilize the advantages of high flow rates without consuming larger volumes of reagents because of the short analysis times and unique designs. Moreover, the optimized flow rate for CL reagents in microfluidic chips is compatible with the capillary HPLC flow rate, and it is suitable for post column reactions. This result can be considered a merit of coupling microfluidic CL detectors to microbore or capillary HPLCs.

The effect of oxidant ($S_2O_8^{2-}$) and $Ru(bpy)_3^{2+}$ concentrations on the chemiluminescence signal intensity was investigated by changing the concentration from 0.5 mM to 2.0 mM to 0.5 mM to 5.0 mM, respectively, by holding one of the reagent concentration constant. The CL signal intensity of 500 ng mL^{-1} of BUS was recorded using direct injection mode with 1.0 mM Brij-35 in buffer (pH 6, 80 μL min^{-1}), and the CL reagent flow was maintained at 20 μL min^{-1}. The maximum CL emission was observed with a solution that had 1.0 mM of oxidant and 2.0 mM of $Ru(bpy)_3^{2+}$.

3.5. Evaluation of the method performance

The optimized micellar chromatography conditions and chemiluminescence parameters were utilized to evaluate method performance by undertaking the analysis of standard preparations of BUS. The chromatogram of the standard solution of BUS (0.5 μg mL^{-1}) was recorded using CL detection under aqueous-organic (30:70; acetonitrile: buffer) and micellar mode (1.0 mM Brij-35 in buffer) as shown in Fig. 6a and b. To ensure the retention behavior and selectivity in micellar mode of separation using surfactant Brij-35 modified phenyl stationary phase, fexofenadine, a pro drug that is used along with BUS to study the enzyme and transporter activity in pharmacokinetics evaluations [36] which exhibits CL response in Ru-Oxidant system was selected and analyzed as per the optimized conditions. As seen in Fig. 6c, the two compounds were well separated, and the calculated resolution factor was 4.28. The tailing factor (t) and peak width at half height was calculated for BUS at 0.5 μg mL^{-1} as 1.51 and 0.31, respectively. Under the optimized CL conditions, the linear response of BUS was established with limits of detection and quantitation. The coefficient of determination (r^2) was found to be 0.9986 over the range of 5–500 ng mL^{-1}. The limit of detection (LOD, $3\sigma/s$) and quantification (LOQ, $10\sigma/s$) were calculated based on the standard deviation of response (σ), obtained by injecting sample matrix and determined from noise, and slope of the calibration curve (s). It was found to be 0.27 ng mL^{-1} and 0.89 ng mL^{-1} respectively. The accuracy and instrumental precision was assessed by calculating the mean and %RSD of residual standard concentration, peak area, retention time and peak height from six consecutive injections of standard solution at three

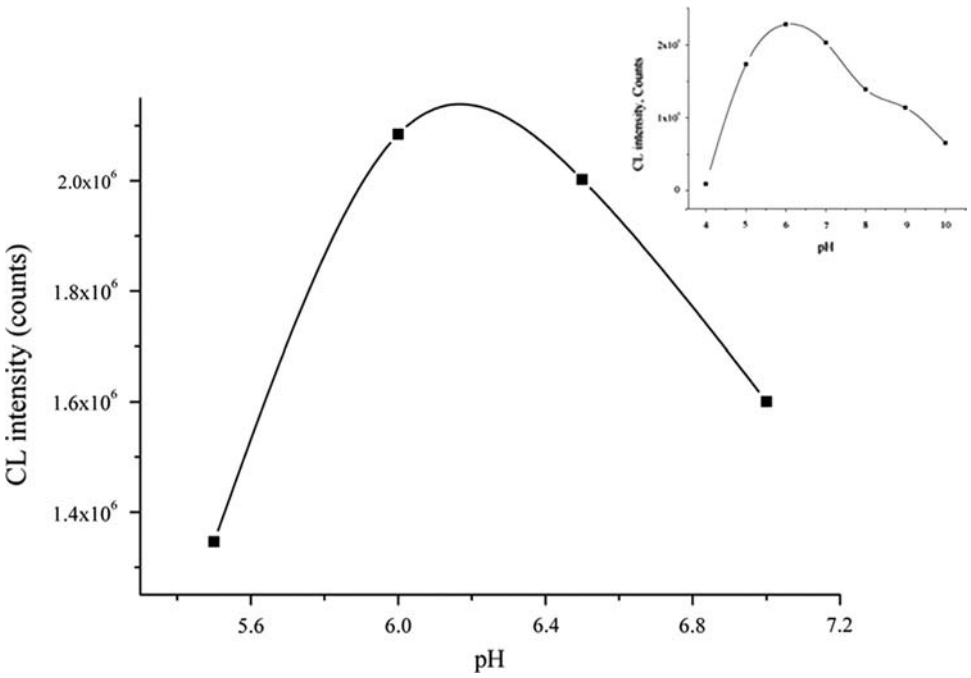

Fig. 5. The influence of the pH of phosphate buffer on the chemiluminescence signal intensity using 0.1% Brij-35 in 0.02 M phosphate buffer (pH 6) as the eluent. Insets: pH screened over 4–10 using 0.02 M phosphate buffer.

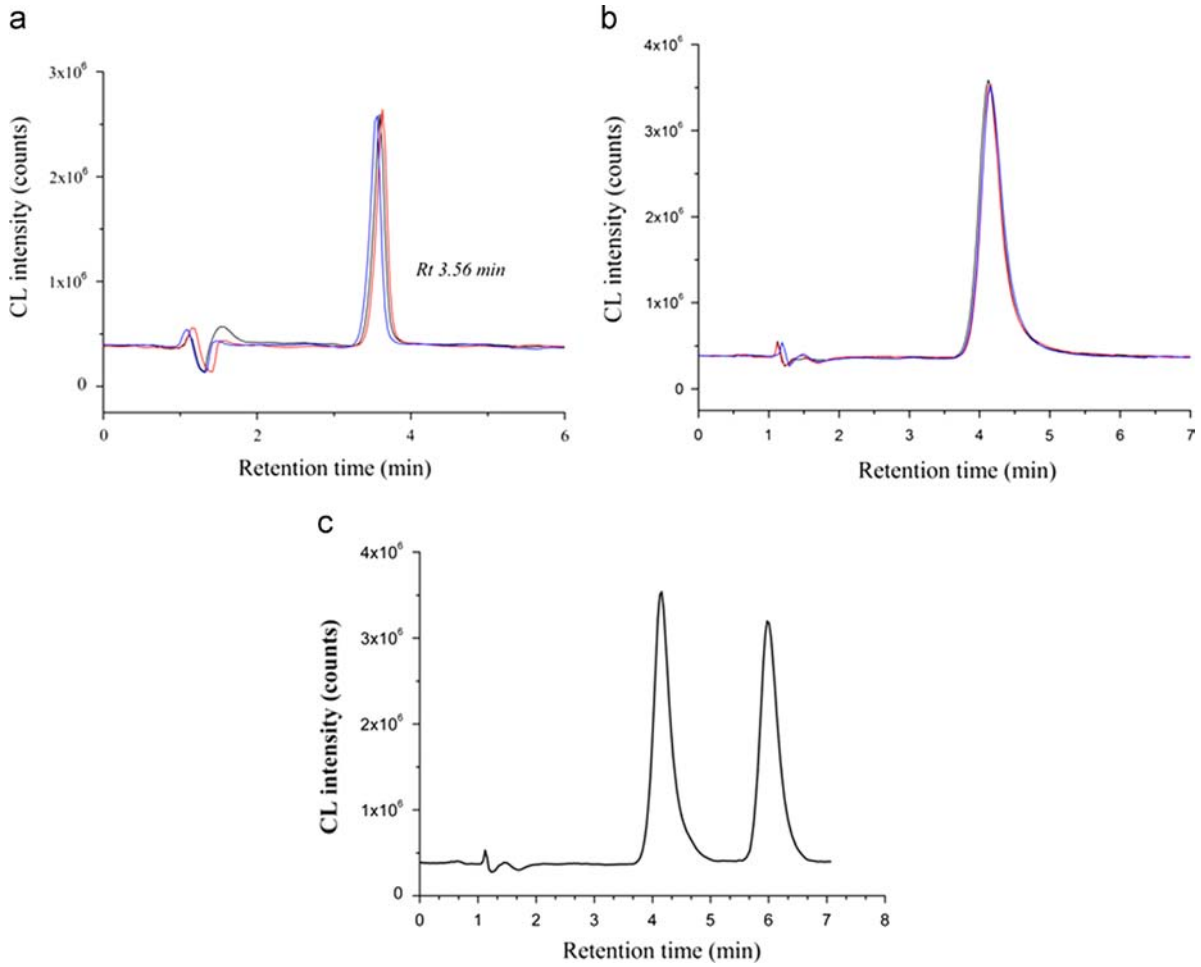

Fig. 6. The chromatogram obtained using microfluidic CL detection and capillary HPLC for 0.5 μg mL^{-1} of BUS using (a) Acetonitrile: buffer; 30:70 (*Rt*, 3.56′ ± 0.01), (b) Brij-35 (*Rt*, 4.13′, ± 0.02), (c) the selectivity of BUS under optimised micellar mode of separation-a mixture of BUS and fexofenadine (0.2 μg mL^{-1}, *Rt*, 5.97′) using 1.0 mM Brij-35 as the mobile phase.

Table 1
The accuracy and precision of critical chromatographic measures for BUS at three different concentrations using *micellar capillary HPLC-microfluidic CL* system.

Nominal conc. (ng mL^{-1}) (n=6)	%Residual conc. (recovery)		Mean peak height (in counts) (%RSD)	Mean peak area (%RSD)	Retention time	
	Mean (%)	%RSD			Mean RT (min)	%RSD
5	97.8	1.0	31,178.0 (5.5)	11,807.8 (2.0)	4.15	1.07
100	95.1	1.8	625,820.8 (1.1)	225,338.8 (1.7)	4.10	0.60
500	101.4	1.7	3167,481.5 (1.5)	1266,884.9 (1.6)	4.13	0.97

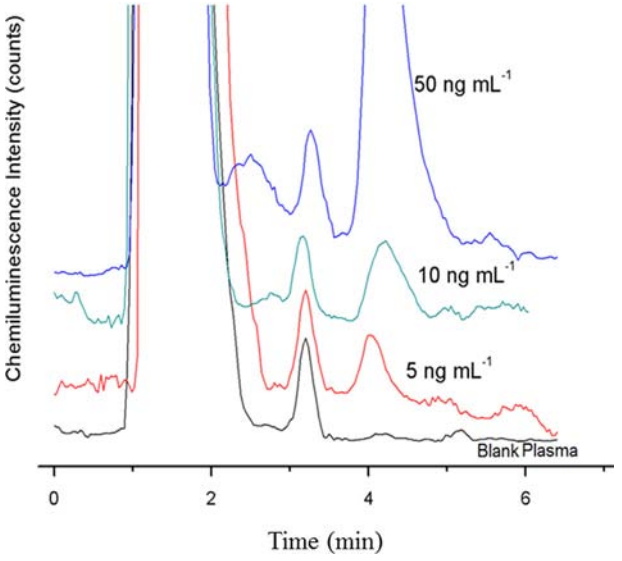

Fig. 7. The direct determination of BUS in human plasma using *micellar capillary HPLC with microfluidic CL detection* from chromatograms of the blank and spiked human plasma samples at three different concentration levels.

different concentration levels (5 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1}). The results are summarized in Table 1, which shows that calculated values are acceptable for all of the critical measures of chromatography. The retention time of BUS was found to be 3.56′ ($\pm$0.01) in acetonitrile condition and 4.13′ ($\pm$0.02) in micellar mode. Satisfactory results were obtained at these concentration levels (5 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1}) for % residual concentration, peak area and peak height, and the calculated relative standard deviations (n=6) were within the limits. These results were sufficient to utilize the developed method to apply for the recovery studies of drug from spiked biological samples.

3.6. Direct on column injection and analysis of BUS in human plasma

The applicability of the newly developed method to determine BUS directly in biological fluids was confirmed by its analysis in spiked plasma at three different lower concentration levels. An aliquot of the sample diluted in a 1:1 ratio with micellar mobile phase was analyzed using the *micellar-capillary HPLC-microfluidic CL detector* according to the optimized conditions. Fig. 7 shows the chromatograms of BUS spiked plasma samples at three different concentration levels and the blank chromatogram of human plasma used. Reasonable levels of recoveries, as shown in Table 2, were obtained for BUS at three different concentration levels ranging from 88.38% to 96.22% with RSD 1.99–5.31%. The obtained recoveries at clinical concentration levels prove that analytically acceptable levels of drug were released from the plasma in the micellar medium. Hence, the combination of capillary micellar liquid chromatography and microfluidic CL

Table 2
Recovery for BUS from human plasma at three different concentrations determined by direct injection in micellar mode with microfluidic CL.

Nominal conc. (ng mL^{-1}) (n=3)	Recovery	
	Mean (%)	RSD (%)
5	88.4	2.0
10	95.8	5.3
50	96.2	3.2

detection is useful for estimating BUS and similar drugs at lower levels in biological fluids by direct on column injection. The analyte peak was well-separated from proteins and other endogenous components of plasma as it can be seen from the blank chromatogram in Fig. 7. The blank chromatogram proved that no interference from the biological matrix, which shows the excellent specificity of the method for the determination of BUS with direct injection of plasma samples. Therefore, the proposed method is suitable for the specific, accurate and sensitive determination of BUS in human plasma.

The total analysis time per sample was less than 6 min including the sample processing time, which makes it faster and easier to use than conventional chromatographic and mass spectrometric techniques. The direct injection of biological fluids in conventional reverse phase chromatography decreases the column performance due to the irreversible adsorption of proteins and other biomolecules on the stationary phase. Moreover, direct on column injection of biological samples in aqueous-organic system may not release bound drug molecules from proteins, which can significantly affect the recovery. In this work, by employing the combination of micellar mode of separation and sensitive detection technique, the biological sample containing BUS was analyzed directly by avoiding laborious and complicated sample pretreatment, extraction procedures.

4. Conclusions

A new, highly sensitive and selective detection approach using microfluidic CL reaction is introduced for the first time in combination with micellar liquid chromatography for direct determination of drugs in biological fluids. The results confirm that CL detection is well suited for micellar liquid chromatography, and the LOD for BUS was comparable or lower than conventional or advanced techniques. The highly sensitive nature of the CL detector provides a simple alternative to advanced techniques such as mass spectrometry, which is normally not suitable for MLC applications. Furthermore, the micellar mobile phase expedites the chemiluminescence emission and provides lower levels of detection within a short time including sample processing. The great flexibility in fabrication of micro channel of microfluidic chips has been exploited for enhanced on-chip CL emission. The microfluidic chip having dual purpose was successfully interfaced

with capillary HPLC system as a *microflow cell* and *micromixer*. The combination of capillary HPLC and microfluidic on-chip detection eliminates the limitations associated with the high flow rates in conventional CL systems and provides a method for cost-effective analysis. The adaptation of microfluidic CL methods to micellar mode of separation can give new dimensions and more possibilities to this "*green analytical*" technique by strengthening its capabilities with sensitive detection. However, the appropriateness of the surfactants and micelles with specific CL reactions are not yet fully understood. Moreover, selecting a suitable surfactant compatible with both the CL reaction and chromatographic separation will be challenging. Further investigations are necessary in this area to broaden the scope of the micellar liquid chromatography with microfluidic chemiluminescence detection systems.

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