Talanta 133 (2015) 107–111

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Towards chemiluminescence detection in micro-sequential injection lab-on-valve format: A proof of concept based on the reaction between Fe(II) and luminol in seawater

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ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form
26 May 2014
Accepted 15 June 2014
Available online 10 July 2014

Keywords:
Chemiluminescence
Lab-on-valve
Iron
Luminol
Seawater

ABSTRACT

Micro-sequential injection lab-on-valve (μSI-LOV) is a well-established analytical platform for absorbance and fluorescence based assays but its applicability to chemiluminescence detection remains largely unexplored. In this work, we describe a novel fluidic protocol and two distinct strategies for photon collection that enable chemiluminescence detection using μSI-LOV for the first time. To illustrate this proof of concept, we selected the reaction between Fe(II) and luminol and developed a preliminary protocol for Fe(II) determinations in acidified seawater. The optimized fluidic strategy consists of holding 100 μL of the luminol reagent in a confined zone of the LOV and then displacing it with 50 μL of sample while monitoring the chemiluminescent product. Detection is achieved using two strategies: one based on a bifurcated optical fiber and the other based on a customized detection window created by mounting a photomultiplier tube atop of the LOV device. We show that detection is possible using both strategies but that the window strategy yields significantly enhanced sensitivity (355 ×) due to the larger detection area. In our final experimental conditions and using window detection, it was possible to achieve a limit of detection (LOD) of 1 nmol L^{-1} and to quantify Fe(II) in acidified seawater samples up to 20.00 nmol L^{-1} with high precision (RSD < 6%). These analytical features combined with the long-term stability of luminol solution and the full automation and low reagent consumption make this approach a promising analytical tool for shipboard analysis of Fe(II). The intrinsic capacity of the LOV to operate at a low microliter level and to handle solid phases also opens up a new avenue for chemiluminescence applications. Moreover, this contribution shows that LOV can be a universal platform for optical detection, capable of absorbance, fluorescence and luminescence measurements in a single instrument setup.

1. Introduction

Chemiluminescence is a powerful analytical tool for the determination of organic and inorganic species. This technique, which is based on the emission of light resulting from a chemical reaction, relies on simple and relatively inexpensive instrumentation and can be applied in different areas of analytical and bioanalytical chemistry [1,2]. Chemical assays relying on chemiluminescence require the control of the different reaction parameters in order to ensure a suitable quantum efficiency of the emission. The analytical signal produced has a transient profile after mixing with the reactant species, requiring very reproducible timing between mixture and detection [3].

Flow injection analysis and related techniques are able to fulfill these requirements. These automation tools ensure high precision due to the repeatable dispersion of the sample plug into a carrier stream [4], and for this reason have been extensively used in the development of new chemiluminescence based analytical methods [5]. Recent developments in this field have been focused on redesigning flow cell geometry [6]. The serpentine shape proposed by Francis and co-workers [7] demonstrated a clear improvement in the magnitude of the analytical signal for different chemiluminometric systems, which led to enhanced detection [6–8]. Under flow analysis format, chemiluminescence reactions require the in-line mixture of at least two solutions that are merged at a predefined time [3]. For this task, multichannel propulsion manifolds (e.g. flow injection and multicommutation techniques) have been preferred because the reagent(s) and sample streams can

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http://dx.doi.org/10.1016/j.talanta.2014.06.076
0039-9140/

easily be merged at the inlet of the flow cell, which is impossible using single channel manifolds (e.g. sequential injection). In fact, the classic sequential aspiration of reagent(s) and sample is impractical for most of the chemiluminescence reactions because light will be generated before the reacting mixture reaches the flow cell. As such, novel strategies based on the latest advances in the microfluidics field must be devised.

A candidate to meet this challenge is the micro-sequential injection lab-on-valve technique (μSI-LOV) [9], the miniaturized version of sequential injection analysis. In this approach, it is possible to hold a reagent plug in the integrated flow cell of the device followed by the addition of sample [10]. For chemiluminescence reactions, this means that it is possible to generate light inside the detection area by placing the reagent into the flow cell and then displacing it with sample. Therefore, this strategy expands the applicability of μSI-LOV platform to chemiluminescence detection, even when very fast reaction rates exist.

In this work, we establish the proof of concept for fast chemiluminescence using the μSI-LOV technique combined with two different strategies for photon collection, based on optical fibers or using a customized detection window. To achieve this goal, we chose the reaction between luminol and Fe(II) as a model, seeking to determine Fe(II) in seawater samples at nanomolar levels.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared using ultra high purity (UHP) water (18.2 MΩ) obtained from a Nanopure system (Barnstead International, Dubuque, IA, USA), and were stored in acid washed high-density polyethylene bottles and handled in a Class-100 laminar flow hood.

The 1.0 mmol L^{-1} luminol solution was obtained by dissolving the solid in 25 mmol L^{-1} NaOH, previously prepared from the dissolution of NaOH monohydrate (TraceSelect, Fluka Analytical, St. Louis, MO, USA) in UHP water. An aqueous solution containing 0.025% (v/v) Brij (Sigma-Aldrich, ST. Louis, MO, USA) was used as carrier. Hydrochloric acid (HCl) 6 mol L^{-1}, purified by single distillation in a quartz-finger sub-boiling sill from a commercial solution (12 mol L^{-1}) (Fisher Scientific, Certified A.C.S. Plus, Pittsburgh, PA, USA), was used to prepare all acid solutions.

Low iron surface seawater, collected during a research cruise in the Southern Ocean using trace metal clean protocols [11], was filtered through a 0.2 μm capsule filter (Acropak, Pall Life Sciences, MI, USA). All seawater samples were acidified to 6.0 mmol L^{-1} HCl by the addition of 6 mol L^{-1} sub-boiled HCl.

Fe(II) standard solutions were prepared in UHP water or seawater (containing 1 mmol L^{-1} HCl) by stepwise dilution of a commercial solution with a concentration of 1000 mg L^{-1} (Fisher Scientific).

2.2. Micro-sequential injection lab-on-valve (μSI-LOV) manifold

All experiments were conducted using a μSI-LOV system (microSIA, FIAlab instruments, Bellevue, WA, USA). This instrument comprised a 1000 μL glass syringe, and a customized opaque LOV (containing a PMT tube holder) mounted atop of a 6-port multiposition valve (VICI Valco, Houston, TX, USA) (Fig. 1). All tubing connections, including the holding coil with a capacity of 500 μL, were made with 0.8 mm (i.d.) polytetrafluoroethylene (PTFE) tubing (Optimize Technologies, Oregon City, OR, USA). Emitted light was detected using a photomultiplier (PMT) (FIAlab instruments) through a bifurcated optical cable with polyetheretherketone (PEEK) sheath terminations on the flow cell ends (FIAlab Instruments) or by placing a PMT tube directly in front of the LOV flow cell (Fig. 1, orange circle). During analytical operation, the manifold was covered by a black plastic box to minimize the influence of the external light on the analytical signal. The control of the fluidic protocol and collection of analytical data were performed using FIAlab for Windows 5.9.3 (FIAlab instruments). Peak height measurements were used as the analytical signal. To minimize contamination, the μSI-LOV manifold was soaked overnight at the end of each analytical day with 24.0 mmol L^{-1} HCl and rinsed before and after the experiments with the same solution.

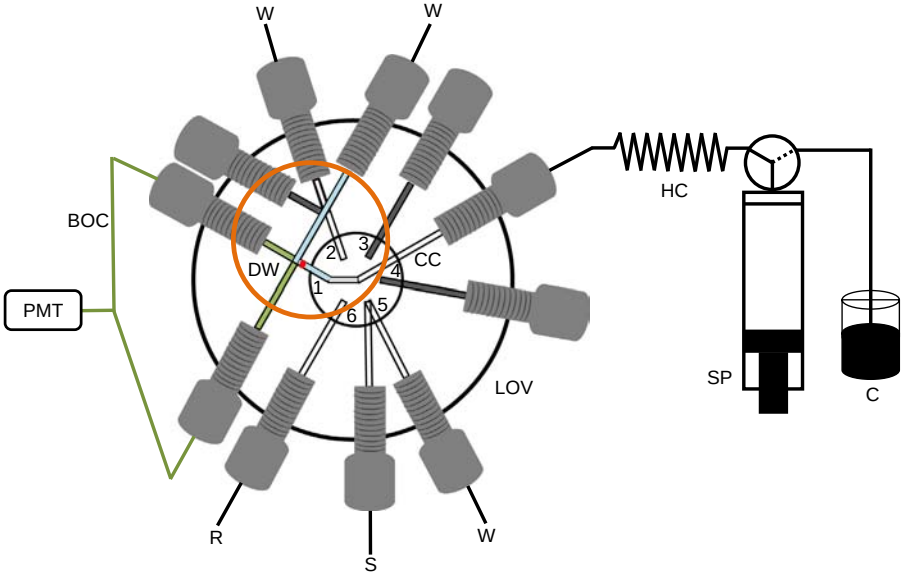

Fig. 1. Schematic of the manifold used for the determination of Fe(II) in seawater samples using luminol chemiluminescence under μSI-LOV format. LOV: lab-on-valve, SP: syringe pump, PMT: photomultiplier tube, BOC: bifurcated optical cable (illustrated by the green lines), CC: central channel, DW: detection window (area inside the orange circle that is covered by PMT tube), HC: holding coil, C: carrier solution (0.025% (v/v) Brij), R: reagent (1.0 mmol L^{-1} luminol), S: sample/standard solution, and W: waste. Closed channels, luminol–Fe(II) reaction area and reaction's interface are gray, blue and red marked, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Protocol sequence for the determination of Fe(II) in seawater under μSI-LOV format.

Step	Description	Pump direction	LOV position	Volume (μL)	Flow rate (μL s^{-1})
1	Fill syringe with carrier	Aspirate	–	750	300
2	Flush flow cell	Dispense	Flow cell	500	250
3	Refill syringe	Aspirate	–	500	300
4	Aspirate luminol	Aspirate	Reagent	100	25
5	Fill flow cell with luminol	Dispense	Flow cell	120	25
6	Remove excess of reagent	Dispense	Waste	300	150
7	Aspirate sample	Aspirate	Sample	50	25
8	CL reaction and detection	Dispense	Flow cell	480	40

2.3. Protocol sequence

The chemiluminometric determination of iron in seawater included eight steps (Table 1). In brief, 750 μL of carrier solution was aspirated into the syringe and the flow cell was then flushed with 500 μL for 2 s. Next, the syringe was refilled with 500 μL of carrier and 100 μL of luminol, which was aspirated into the holding coil. The flow direction was then reversed and the luminol plug was parked in the LOV flow cell after a piston displacement of 120 μL. The holding coil and LOV central channel were then flushed with 300 μL carrier to remove excess reagent. Finally, 50 μL of sample/standard was inserted into the holding coil and subsequently propelled to the flow cell using a flow rate of 40 μL s^{-1} until the syringe was completely emptied. During the last step, the PMT detection (integration time 200 ms) was activated and the analytical signal was recorded.

3. Results and discussion

3.1. Design of μSI-LOV for chemiluminescence detection and flow cell geometry

To minimize contamination, we placed the μSI-LOV instrument inside a Class-100 laminar flow hood and used optical cables with PEEK sheath terminations for light collection. Additionally, and to prevent trapping of air bubbles in the holding coil and LOV channels, the carrier solution contained Brij at a concentration of 0.025% (v/v).

Our fluidic strategy took advantage of programmable flow, and consisted of immobilization of the reagent plug in a confined zone of the LOV (Fig. 1, blue area), followed by sample perfusion. This was necessary because the chemiluminescence of Fe(II) and luminol is a fast reaction [12], and in a single channel configuration it is impossible to merge the reagents in front of the flow cell, in contrast with the multichannel approach offered by other flow analysis techniques [5]. Furthermore, instead of "direct aspiration" (where the different plugs are sequentially aspirated in the direction of the flow cell without flow reversal) [7], we pushed the sample directly into the existing reagent plug, with no reversal of the pump pressure. This technique allowed us to use higher flow rates with a negligible risk of introducing bubbles into the system and promoted effective mixing between reagent and sample. The protocol sequence also included two cleaning steps (Table 1): the first (step #2, Table 1) ensured cleanup of the manifold between consecutive injections and the second (step #6, Table 1) prevented reaction between sample and any potential residue of luminol that could still be present in the holding coil.

The LOV device used in this work for chemiluminometric detection presents the same relative arrangement of the flow cell and lateral channels previously used for spectrophotometric [13] and/or fluorimetric [13,14] detection. This is in contrast to the previous approach reported by Yang et al. [15], where a Z-type flow cell was attached to the LOV unit, in a manifold architecture closer to the classic sequential injection manifold. The unique transformation of the typical μSI-LOV design was a customized front panel that held a PMT tube in front of the reaction area, creating a detection window (Fig. 1). Therefore, the photons generated by the reaction could be collected with two distinct strategies: using optical fibers or by placing the PMT in front of the reaction area. In the first case, using the native ability of the LOV to accommodate optical fibers, a bifurcated fiber was placed in a perpendicular position at the entrance of the flow cell (in the same relative position used for fluorescence detection [14]) and the emitted light was directed to an external enclosed PMT detector (Fig. 1, green lines). In the second case, a PMT tube was mounted atop of the LOV, creating a detection window in front of the reaction area (Fig. 1, area covered by the PMT window corresponds to the orange circle). Hence, our flow cell was the LOV section where photons could be generated, corresponding to the blue channels of Fig. 1.

3.2. Implementation of Fe(II)–luminol chemiluminescence reaction under μSI-LOV format

We adopted the reaction between luminol and Fe(II) as a case study for our proof of concept. The chemiluminescence of luminol in the presence of Fe(II) (and in the absence of an oxidizing agent (e.g. H_2O_2)) is particularly suited for this determination due to the fast oxidation kinetics of Fe(II) over other metal redox pairs (e.g. Co (III/II) or Cu(II/I)) [12]. This leads to an enhanced selectivity combined with high sensitivity, characteristic of chemiluminescence based assays. In fact, this chemistry has been successfully applied for the quantification of Fe(II) in seawater at nano to picomolar levels [16–20].

We selected the chemical parameters – luminol concentration and pH – based on reported methodologies [16,20], keeping in mind the complexity of the reaction [21] and aiming at a simple and straightforward protocol. Hence, we used a luminol concentration of 1.00 mmol L^{-1} [20] and bypassed the use of an oxidizing agent [12,21,22]. Regarding pH, luminol was dissolved in 25 mmol L^{-1} NaOH in order to guarantee the alkalinity necessary for the reaction. Although the theoretical pH value of this solution (~12.4) was higher than the optimum pH for the reaction (10.4) [16,20,21], the acidified standard/sample plug that penetrates into the luminol plug ensures a final pH in the optimum range, an approach similar to that reported by Bowie et al. [16]. This strategy also made the system simpler, and minimized potential contamination originating from the chemicals used (e.g. buffer) [12].

Another important parameter to ensure a suitable mixture is the flow rate applied to the sample to perfuse the reagent plug retained in the flow cell. Considering the laminar flow pattern present in most of the flow analysis techniques, mixing is improved by axial dispersion, which is positively correlated with tube length, tube radius and flow rate [4]. In the particular case of μSI-LOV, the characteristics of the device enhance axial dispersion because the LOV channels have a high internal diameter (i.d.) (1.6 mm instead of the typical 0.5–0.8 mm i.d. of tubing used in flow manifolds) and the syringe pump allows precise control of flow rates. Based on this background, we selected a flow rate of 40 μL s^{-1} to burst the sample into the luminol, combining the maximum axial dispersion within an appropriate time frame for detection (the reaction between luminol and Fe(II) occurs in 300–700 ms [12] and the applied flow rate ensured a residence time inside the detection area of 1200 ms).

Considering our fluidic approach to the chemistry and the limited time frame for detection, we assessed the effect of two physical variables of the μSI-LOV system that could have a major

impact on the sensitivity of the assay: the volume of luminol necessary to fill the flow cell and the volume of sample.

In the first case, the volume of the channels between the exit of the selection valve and the exit of the flow cell (connection to the waste line) (Fig. 1, blue channels) comprised a total volume of approximately 54 μL, which limited the maximum volume of luminol that could be used in each determination. To find the optimal volume needed to place the luminol plug into the flow cell (Table 1, step #5) after aspirating 100 μL of luminol into the holding coil (a volume large enough to guarantee a complete replacement of the carrier by the reagent), we assessed its influence on sensitivity (in the range 40–140 μL) by establishing calibration curves with Fe(II) (in concentrations up to 500.0 nmol L^{-1}, prepared in 1 mmol L^{-1} HCl). Since the dispensed volume of luminol that provided maximum sensitivity (120 μL, Fig. 2) was higher than the volume aspirated in the previous step (100 μL), we hypothesized that the location of the reaction interface had a significant influence on the magnitude of the analytical signal. Our fluidic protocol formed a reaction interface between luminol and Fe(II) (Table 1, step #8), and its displacement changed, simultaneously, the relative concentrations of luminol and pH (due to dispersion), as well as its location relative to the center of the detection window. It was expected that volumes between 50 and 100 μL would ensure maximum sensitivity because minimum dispersion of the luminol plug (~54 μL) could be achieved in this range, guaranteeing an efficient replacement of the carrier solution and an excess of reagent for the reaction with Fe(II) present in the sample plug. However, the results (Fig. 2) showed a different behavior, with an increase in the sensitivity of the determination for dispensing volumes up to 120 μL, which reflected the importance of the location of the reaction interface. Considering the volumes applied in our protocol sequence, the reaction interface was approximately located near the entrance of the channel corresponding to the typical LOV spectrophotometric flow cell configuration [9] (Fig. 1, red mark). Furthermore, the fast reaction kinetics (the maximum emission is reached between 300 and 700 ms [12]), the instantaneous mixture between reagent and sample and its displacement along the detection channel (s) favored light emission close to the center of the PMT window, resulting in the highest sensitivity. As the volume of luminol delivered to the flow cell is increased beyond 120 μL (140 μL, Fig. 2), we observed a decrease in sensitivity because the interface containing the optimal luminol concentration is moved closer to the exit of the detection window. In addition, once the sample is injected into the flow cell, the zones with greatest sample and luminol concentrations are located at each end of the detection window. This separation impedes adequate zone overlap, produces suboptimal levels of sample and luminol at the reaction interface resulting in a drop in sensitivity. We also found a similar behavior when we used acidified seawater (in this case with concentrations of Fe(II) up to 30.00 nmol L^{-1}).

We assessed the influence of the sample volume on the sensitivity of the determination by establishing calibration curves (up to Fe(II) concentrations of 30 nmol L^{-1}) using volumes of seawater between 25 and 200 μL. We fixed our initial volume at 25 μL in order to keep a significant portion of the sample in an undiluted form (this fact was important due to the large i.d. (1.6 mm) of the central channel of the LOV that facilitates dispersion). We found higher slope values when smaller sample volumes were applied (Fig. 3), indicating our most suitable detection conditions. After establishing the interface point by adjusting the location of the luminol plug one would expect that, after reaching a plateau, the analytical signal would remain constant. However, the results demonstrated a clear drop in sensitivity when the sample volume exceeded 50 μL, which can possibly be explained by our fluidic protocol associated with the need to maintain a strict optimum reaction pH [16,20,21]. When the sample was directed towards the luminol plug it did not immediately reach the highest concentration of luminol, modification of the sample volume may influence the degree of overlap of the two plugs, which would in turn change the location of the reaction interface and also the number of target species available for the reaction. Furthermore, and in contrast to luminol that is always present in large excess, the capacity to buffer the H^+ from the sample plug was limited. For example at the reaction interface, considering a sample volume of 25 μL (without any dispersion effect), the ratio of luminol:Fe(II) is approximately 45,000 whereas the ratio OH^-:H^+ is only 34. When we used sample volumes larger than 50 μL, this combination between mixture and pH control modified the reaction conditions and moved the pH to values outside the optimum value of 10.4 [16,20,21], resulting in a drop of sensitivity (Fig. 3). For volumes higher or equal to 100 μL, we found that the reaction interface remained unchanged because the extra volume of sample never reacted with the luminol.

3.3. Analytical performance

The characterization of this novel analytical strategy for the determination of Fe(II) considered its linear range, limit of detection (LOD), sensitivity and precision, and exploited two different detection configurations: the use of a bifurcated optical fiber and the detection through a PMT mounted atop of the LOV device.

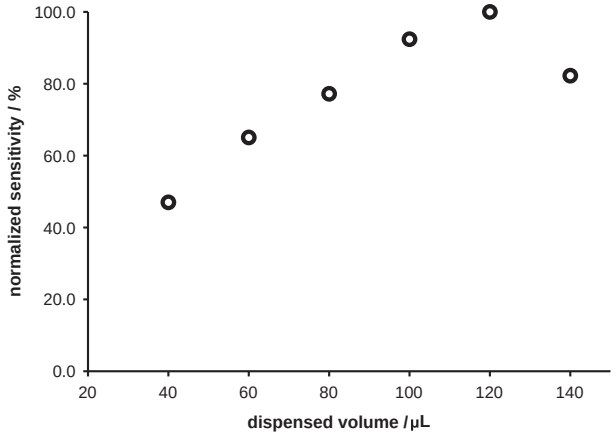

Fig. 2. Influence of the dispensing volume of the luminol plug (step #5, Table 1) on the sensitivity of Fe(II) determination. Analytical signals were collected using the window detection strategy and Fe(II) solutions were prepared in acidified UHP water.

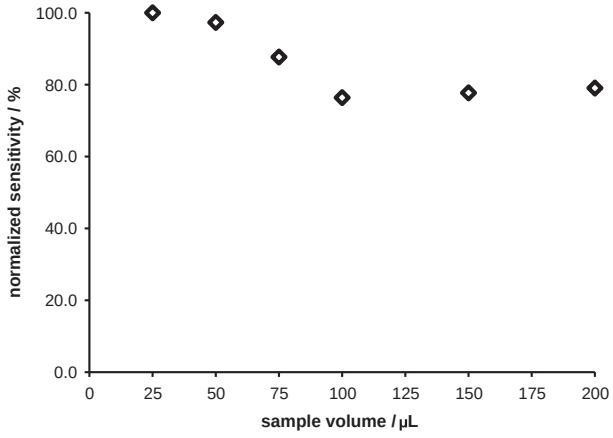

Fig. 3. Influence of sample volume (acidified seawater) on the sensitivity of the assay. Analytical signals were collected using the window detection strategy.

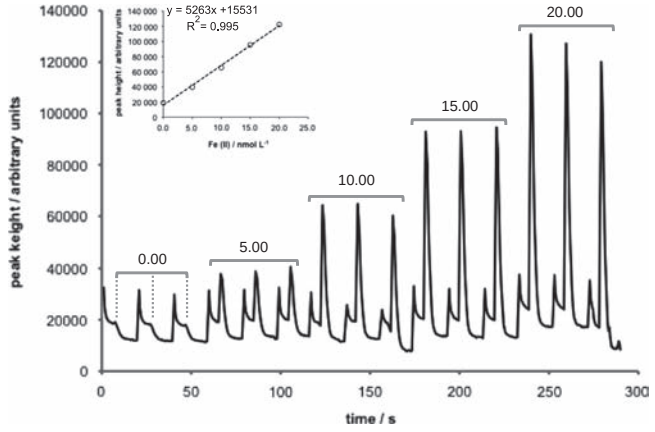

Fig. 4. Analytical output and calibration curve (inset) obtained after processing acidified seawater samples spiked with Fe(II) at concentrations up to 20.0 nmol L^{-1}. Analytical signals were collected using the window detection strategy and peak labels indicate Fe(II) concentrations in nmol L^{-1}.

In the optical fiber detection configuration, linearity ($r^2 > 0.995$) was established up to an Fe(II) concentration of 120.0 nmol L^{-1} whereas for window detection the upper value decreased to 20.00 nmol L^{-1}. Calibration curves resulted from the triplicate analysis of 4 or 5 Fe(II) standard solutions with concentrations equally distributed along the linear working range (Fig. 4). The LOD of 1 nmol L^{-1} for both detection strategies was calculated as the Fe(II) concentration equivalent to the analytical signal obtained by adding the blank signal to 3 times the corresponding standard deviation [23]. Sensitivity was strongly related to the detection strategy because the surface area monitored by the PMT was significantly greater using the window scheme (at least 30 times higher). For window detection, the slope of a typical calibration curve (5263 ± 286 arbitrary units $nmol^{-1}$ L, Fig. 4) was 355 times higher than the bifurcated optical fiber strategy (14.8 ± 0.2 arbitrary units $nmol^{-1}$ L). Nevertheless, the background signal (calibration curve intercept value) was also higher for window detection ($15{,}531 \pm 3164$) than for optical fiber detection (116 ± 14) though significantly lower than the gain in sensitivity (The values in parenthesis corresponded to the slope and intercept values and respective limits of the 95% confidence levels intervals.) In our final experimental conditions, we observed intra and inter-day slope variations below 10% of the values presented here.

Relative standard deviation (RSD%) measured the precision of the determination. For both optical fiber and window detection strategies, RSD values were under 6% for triplicate measurements of each seawater standard solution. We also analyzed seawater samples (originally containing Fe(II) below our LOD of 1 nmol L^{-1}) spiked with different levels of Fe(II). In the optical fiber detection configuration, we found apparent recoveries [24] between 98.5% and 100.1%, for Fe (II) concentrations of 40.00 and 80.00 nmol L^{-1}, respectively. When we used window detection, the apparent recovery ranged between 91.6% and 101.5%, for Fe (II) concentrations of 5.00 and 15.00 nmol L^{-1}, respectively. Although a full validation of the analytical method is beyond the scope of the present work, these values highlight the potential of the two detection strategies for the quantification of Fe(II) in seawater at nanomolar levels using μSI-LOV methodology.

In conclusion, we demonstrated for the first time the use of chemiluminescence detection using μSI-LOV methodology thus establishing the proof of concept for the development of a plethora of chemiluminescence applications based on this technique. We developed here a streamlined determination of Fe(II) in seawater samples at trace levels using luminol as chemiluminometric agent simply by taking advantage of the μSI-LOV design and adjusting the programmable flow protocol. This work also made possible a fast chemiluminescence reaction in a single channel manifold, removing mandatory merging of the different reagents at the inlet of the flow cell.

Another relevant aspect is the dual-mode detection strategy. The selection between optical fibers or detection window can be made according to the requirements of each particular application. The introduction of chemiluminescence capabilities also kept the original LOV design. This means that all new applications will benefit from the intrinsic characteristics of this analytical platform such as the operation at low microliter level and the possibility of handling solid phases (bead injection). This definitely opens a new avenue for research using chemiluminescence detection, which will certainly impact the bioanalytical and nanotechnology fields.

Regarding our particular case study, the long-term stability of the luminol solution and its minimal consumption per determination (100 μL), combined with the rapid analytical throughput (116 determinations per hour), make this approach well suited to shipboard analysis with potential for autonomous operations. Finally, this contribution transforms LOV into a universal platform for optical sensing, able to accommodate absorbance, fluorescence and chemiluminescence detection in the same instrumental setup.

Acknowledgments

This work was supported by NSF, USA grants OCE08-49830 and OCE-0962393 to CIM. We would like to thank Chief Scientists Jim Swift and Alex Orsi for providing shipboard space and time to obtain the Southern Ocean seawater samples used in this development work, and also Bill Landing for the valuable discussions about the luminol–Fe(II) reaction.

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