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Compact optoelectronic flow-through device for fluorometric determination of calcium ions

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ABSTRACT

A low-cost and compact complete fluorometric detector dedicated for measurements under conditions of flow injection analysis has been developed. This device is fabricated by integration of light emitting diodes (LEDs) applied in the double role: as the source of light inducing fluorescence and as the detector of light emitted by excited fluorescent analyte. The device is made of three LEDs only, without any additional fibers, filters and lenses. The LEDs are integrated in the form of flow cell of 0.060 mL internal volume. The developed detector has been tested in simple flow injection manifold dedicated for fluorometric determination of calcium using calcein method. The system offers sensitive and selective determination of calcium at ppm levels with relatively high flow throughput (near 60 injections per hour) and satisfactory reproducibility. The practical utility of developed detector has been confirmed by its application for analysis of mineral waters, medicines as well as physiological fluids.

1. Introduction

The most popular methods for fluorometric determination of calcium ions exploit calcein. This compound (Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein) also known as fluorexon, synthesized by the condensation of fluorescein, iminodiacetic acid and formaldehyde, exhibits both fluorescence (at similar excitation/emission wavelengths as fluorescein) and metal ion chelating properties like EDTA. Originally, calcein has been developed as the end-point indicator for complexometric titrations of calcium with EDTA in the presence of magnesium ions [1]. However, it is also useful for fluorometric detection of calcium ions [2], because at high pHs the calcium–calcein complex still exhibits fluorescence, whereas the fluorescence of free calcein (ligand) is quenched. This fluorometric scheme of detection/sensing is easily adapted for conditions of flow injection analysis [3–5], also in the microfluidic format [6], as well as is useful as a platform for optical sensors development [7,8].

One of limitations in widespread application of fluorometry in many fields of modern practical analysis is rather high cost of instrumentation and miniaturization problems. Therefore, one of current trends in analytical spectrometry research is the development of inexpensive, dedicated light sources and detectors for fluorometry based on economic optoelectronics. At present, light emitting diodes (LEDs) are recognized as effective fluorescence inductors, especially in capillary electrophoresis systems [9,10]. LEDs as light sources are received considerable attention due to their excellent stable output with less energy consumption, high intensity, low-cost, small dimensions and a variety of wavelengths in the ultraviolet and visible range of spectrum.

From the practical point of view, more troublesome is the detection of light emitted by induced fluorescent compounds. Sometimes, instead of spectrofluorimeters for fluorescence detection less expensive photomultiplier tubes are applied, However, these fluorometric systems still require additional optical components for light transmission, filtering, collimation and focusing, what significantly increases the cost and complexity of such devices and makes their integration rather bothersome. On the other hand, only recently some analytical communications have been published [11–13] where LEDs are reported as selective fluorescence detectors, when they operate in the reverse mode. These findings open up possibilities for the development of complete fluorometric devices made of LEDs only. In such devices LEDs would be applied in both, complementary functions: as the selective light sources for fluorescence induction and as the selective detectors of light emitted by excited molecules of fluorescent analyte.

It this contribution we present a project of extremely simple, inexpensive, miniature and highly integrated flow-through device fabricated of LEDs only, without any additional optical fibers, lenses, etc. The main goals of this work is the development of such complete and compact fluorometric device dedicated for calcium ions determination and the practical examination of its utility for flow injection analysis of real samples important from pharmaceutical and bioanalytical point of view.

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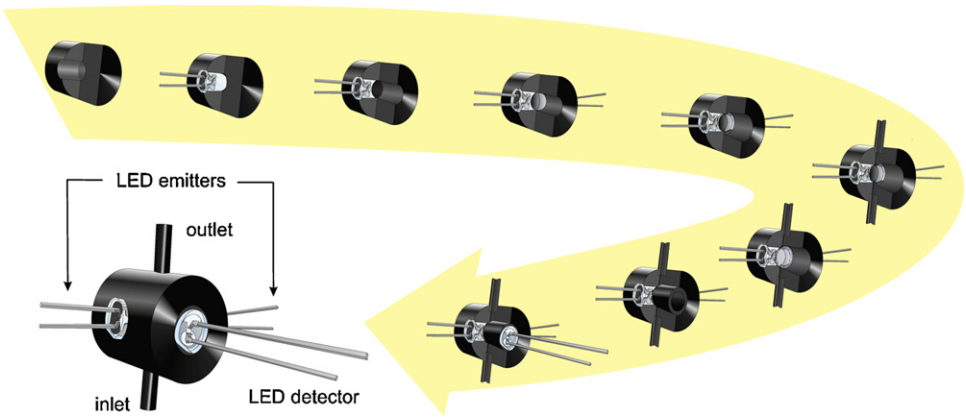

Fig. 1. Step-by-step fabrication of the developed fluorometric device.

2. Experimental

Calcein (product no. C0875) was purchased from Sigma (USA). Other reagents of analytical grade were obtained from POCh (Poland). Standards for calibration were prepared of $Ca(NO_3)_2{\cdot}4H_2O$. Doubly distilled water was used throughout. Real samples were dissolved and diluted with water. Reference calcium determinations were performed by complexometric titration with EDTA in the presence of ammonium purpurate (murexide) used as end-point indicator. Medicines and mineral waters were purchased in the local drugstore. Human urine and serum samples with determined calcium levels were obtained from the Department of Laboratory Diagnostics (Medical University of Warsaw).

All analytical measurements were performed in a simple two-channel flow-injection manifold constructed using common FIA equipment: a peristaltic pump (model Minipuls 3 from Gilson, France), manual rotary injection valve (model 5020 from Rheodyne, USA) and 0.8 mm ID Teflon tubing (Cole–Palmer, USA). The injection valve for calibrants and samples was placed into water line. Calcein content in KOH solution was 0.5 g/L. This solution was stored in refrigerator for 24 h and then used for measurements. Both, water and KOH channels were connected and after the Y-shaped connector a 30 cm long mixing coil and detector were mounted. The common flow rate and injection volume applied in the course of all measurements were 4.05 mL/min and 0.35 mL, respectively.

Both kinds of LEDs applied for the detector fabrication have common shape, 5.0 mm diameter, transparent lens and 30° viewing angle. 475 nm blue LEDs (product no. OSUB5131A-PQ) and 630 nm red LEDs (product no. OSHR5131A-QR) were obtained from Optosupply (China). LEDs operating as light emitters were powered with home-made low-voltage circuit based on L272 chip, which contains two operational amplifiers and can independently supply two emitters. The solderless board and all electronic components and were purchased from TME (Poland). As an analytical signal, the electromotive force generated by LED playing the role of light detector was applied [14]. The voltage signal without any additional amplification was measured and recorded using ordinary multimeter (model UT70B from UNI-T, China) operating as voltmeter. This multimeter was connected with data storage computer using RS232 interface.

The stepwise fabrication of developed detector is illustrated by Fig. 1. The flow-cell is made from polymeric cylinder-shape block in which 4.9 mm diameter crosswise aperture are drilled and two identical 5 mm blue LED emitters are placed. In the next step, in the center of cylinder 5 mm diameter hole is milled from both sides for enlarging it to 7 mm to a depth of LEDs. Then another crosswise perpendicularly to LED aperture is made to place inlet and outlet tubes. Finally, two 7 mm diameter acrylic windows are placed in central whole and clamp with two 7 mm bushes having 5 mm lengthwise opening, adequate to place the LED acting as fluorescence detector. In the result, the total internal volume of the flow cell and the distance between chips of LED emitters are 0.06 mL and 5 mm, respectively. The real size of complete device in the final form with flow input/output and mounted LEDs is shown in Fig. 2.

3. Results and discussion

As mentioned in Section 1, the spectral properties of fluorescein and calcein are similar and therefore LEDs selected for induction and detection of fluorescence of calcium–calcein complex are the same as previously [12]. In the presented fluorometric device blue LEDs are applied for the fluorescence excitation, whereas red LED acts as the fluorescence detector. Because the increase of LED luminance intensity with the current has similar to

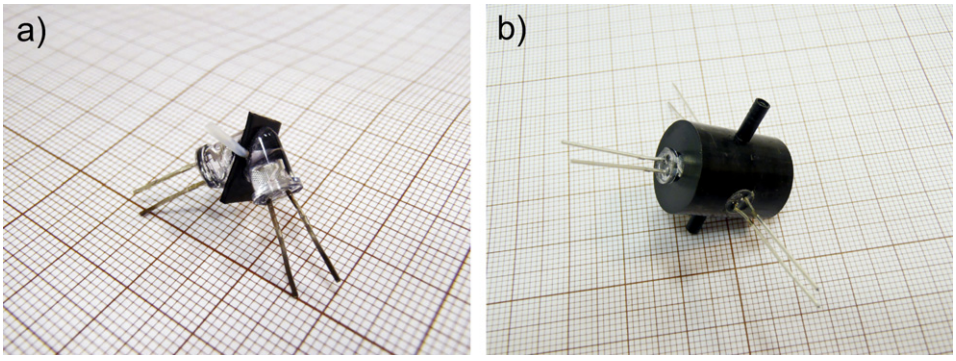

Fig. 2. Photos of the prototype of "standard" 2-LED based detector (A) and the developed "improved" 3-LED-based detector (B).

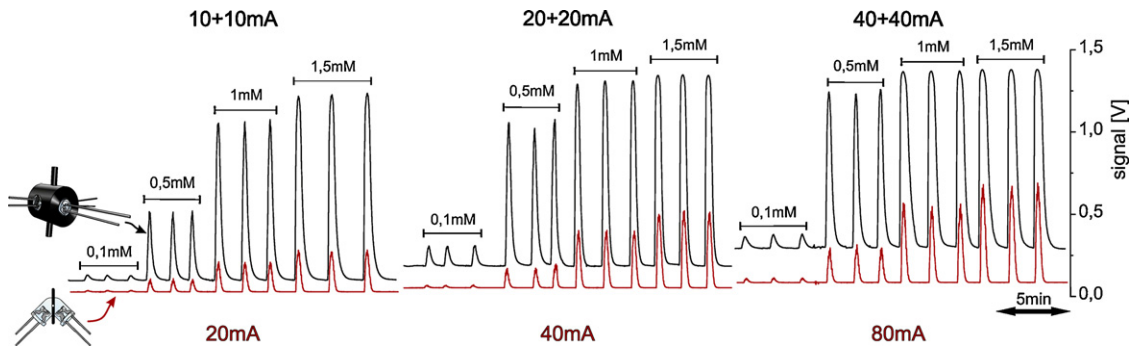

Fig. 3. Recordings for FIA measurements performed with "standard" and "improved" detector (both detectors are depicted). The currents supplying devices are given in the figure.

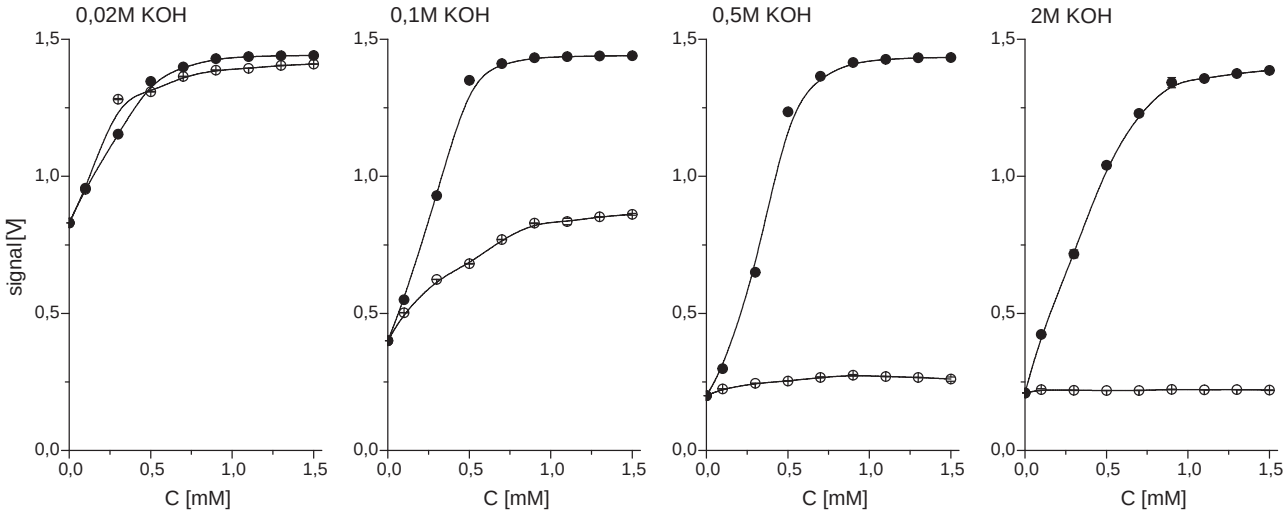

Fig. 4. Calibration graphs for calcium (filled points) and magnesium (open points) ions obtained at different concentrations of potassium hydrogen in the carrier of calcein (given in figure).

logarithmical character, for high currents the saturation of emitted light is observed. Therefore, much more efficient lighting is resulting by the use of two LED than the one powered with two times greater current. Moreover, at high currents the risk of LED failure (burnout) significantly increases. Hence, the first goal of this work is the development of fluorometric flow-through cell with two LED emitters.

The prototype of fluorometric paired emitter detector diode reported recently [12] and the improved 3-LED-based fluorometric detector presented in this contribution are shown in Fig. 2. Both detectors have been compared in the same manifold for flow injection analysis. The measurements were performed at various currents supplying LED emitters. The FIAgrams obtained in the course of both detector calibrations are shown in Fig. 3. As expected, the improved detector offers significantly higher sensitivity although the baseline signal only slightly increased. In consequence calibration graphs for the improved detector are shifted towards lower concentrations. The upper determination limit (not observed in case of 2-LED-based detector) is caused by self-quenching of fluorescence and by the saturation of LED acting as the fluorescence detector. Additionally, the FIAgrams shown in Fig. 3 well illustrate good reproducibility and high throughput (near 60 injections per hour for 3-LED-based device and around 80 injections per hour for 2-LED-based device) offered by FIA systems based on both kinds of detectors.

The calcein method has been originally developed for determination of calcium ions in the presence of magnesium [1]. However, dependently of experimental conditions, the method can be applied for determination of calcium only [3,5], sum of calcium and magnesium [4,6] as well as for other alkali metal ions [2]. Therefore in the developed FIA system the effect of potassium hydroxide concentration has been investigated. As shown in Fig. 4, the increased KOH concentration causes significant decrease of baseline signal due to quenching of fluorescence from free calcein. As the fluorescence of calcium–calcein complex is almost the same, the sensitivity of calcium determination increases. Moreover, the increase of alkalinity eliminates the sensitivity on magnesium, due to its precipitation (as hydroxide). However, for extremely concentrated solutions of potassium hydroxide the decrease of sensitivity towards calcium ions is observed. Additionally, it can be supposed that the effects from alkaline cation impurities in KOH reagent are manifested by the increase of baseline signal. Thus, for further measurements 2 M KOH was applied as a working solution. Due to its dilution in the FIA system with water applied as the

Table 1
Results of mineral waters analysis.

Product	Ca content declared	Ca content referenced	Ca content found
Żywiec Zdrój	42.6 mg/L	42.7 ± 0.4 mg/L	40.0 ± 0.8 mg/L
Kropla Beskidu	44.1 mg/L	48.7 ± 0.5 mg/L	48.9 ± 0.9 mg/L
Nałeczowianka	110.2 mg/L	111 ± 1 mg/L	110 ± 1 mg/L
Cisowianka	131.3 mg/L	132 ± 1 mg/L	129 ± 4 mg/L
Muszynianka	180.9 mg/L	179 ± 2 mg/L	182 ± 4 mg/L

Table 2
Results of pharmaceutical products analysis.

Product	Manufacturer	Main component(s)	Ca content declared	Ca content referenced	Ca content found
Ringer's solution	Fresenius Kabi	Electrolytes	88 mg/L	88.8 ± 0.8 mg/L	88.0 ± 0.8 mg/L
Polyelectrolyte isotonic saline solution	Fresenius Kabi	Electrolytes	80 mg/L	84 ± 3 mg/L	88.0 ± 0.8 mg/L
Calcium Pliva *effervescent tablet*	Pliva	Calcium lactate	177 mg/tablet	176 ± 2 mg/tablet	174 ± 2 mg/tablet
Calcium z folii Polfa *effervescent tablet*	Polfa	Calcium lactate	180 mg/tablet	181 ± 2 mg/tablet	179 ± 2 mg/tablet
Calcium Gluconicum *tablet*	Hasco Lek	Calcium gluconate	45 mg/tablet	43.5 ± 0.4 mg/tablet	43.0 ± 0.4 mg/tablet
Calcium Sandoz forte *effervescent tablet*	Sandoz	Calcium lactate-gluconate, calcium carbonate	500 mg/tablet	503 ± 5 mg/tablet	505.3 ± 0.2 mg/tablet
Sanosvit calcium *syrup*	Nycomed	Calcium glubionate, calcium lactobionate	114 mg/5 mL	113.0 ± 0.9 mg/5 mL	115.8 ± 0.3 mg/5 mL
Calcium z Vit. C forte *effervescent tablet*	Biotter	Ascorbic acid, calcium carbonate	300 mg/tablet	300 ± 1 mg/tablet	299.8 ± 0.3 mg/tablet
Rutokal C plus *effervescent tablet*	NP Pharma	Rutoside, Ascorbic acid, calcium carbonate	100 mg/tablet	100 ± 1 mg/tablet	100.6 ± 0.1 mg/tablet
Zdrovit Alercal *effervescent tablet*	Zdrovit	Quercetin, calcium carbonate	300 mg/tablet	300 ± 2 mg/tablet	294.7 ± 0.3 mg/tablet

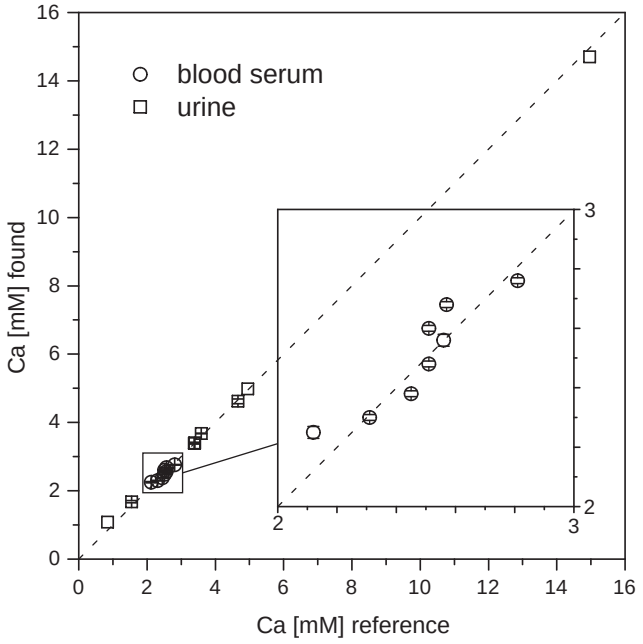

Fig. 5. Results of physiological fluids analysis.

sample carrier the final concentration of KOH in the detection zone was 1 M. As can be seen from Fig. 4, under such conditions the FIA system allows determination of calcium in the ppm range of concentration without influences from magnesium ions. The detection limit and the sensitivity in linear range are 0.24 ppm (0.006 mM) and 39 mV/ppm, respectively.

The presented FIA system has been examined as a tool for analysis of several kinds of real samples. In Table 1 the results for analysis of selected mineral waters are shown. The calcium contents determined using developed detector and procedure are fully comparable with declared and reference values. As can be seen from Table 2, similarly well-correlated results between the developed and reference methods have been obtained also in case of analysis of polyelectrolyte infusion fluids and some popular medicines (effervesced tablets and syrup) containing calcium in various forms.

Finally, the developed detection system has been applied for calcium determination in samples of human serum and urine. The physiological calcium levels in human serum vary from 2 to 3 mM, whereas hypercalcemia and hypocalcemia are both serious medical disorders [15]. The physiological urine calcium levels are similar but strongly influenced by calcium intake. Their knowledge is useful for biomedical diagnostic of renal calculosis (nephrolithiasis). The results shown in Fig. 5 clearly confirm the utility of the developed detector for determination of calcium ions in physiological fluids. The results of analysis serum and urine analysis are fully comparable with those obtained using method and laboratory equipment recommended for clinical diagnostics (the photometric calcium-o-cresolphtalein complexone, CPC method). The obtained regression coefficient for analysis 9 samples of urine and 8 samples serum is $r^2 = 0.9994$ ($n = 17$).

4. Conclusion

In this short communication we have demonstrated that three ordinary LEDs only are just sufficient for the construction of complete and compact fluorometric detector useful for determination of calcium under non-stationary flow conditions in the ppm concentration range. The simplicity and economic aspects of the developed detection system should be emphasized. The developed device is fabricated using only light emitting diodes without any additional optical parts. In consequence, the total cost of complete detector does not exceed one euro. Moreover, the detector is supplied with economic, low-voltage circuit. Finally, for the measurement of analytical signal generated by this device an ordinary low-budget voltmeter can be applied. Despite this simplicity, the developed detector is useful for real, practical analysis. As demonstrated, the simple FIA system based on the developed device has been successfully applied for analysis of mineral waters, simple and complex pharmaceuticals as well as human physiological fluids.

It is supposed, that similar optoelectronic devices operating according to the same concept and fabricated according to the technical project presented in this article can be developed for fluorometric detection of many other substances, only if respective LED emitters and detectors, compatible with the fluorescence properties of target analyte(s), will be selected. Investigations of analogous flow bioanalytical systems dedicated for determination of some metabolites, vitamins and proteins are in progress.

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