Talanta 96 (2012) 121–126

Contents lists available at SciVerse ScienceDirect

Talanta

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

Low-cost optical detectors and flow systems for protein determination

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ARTICLE INFO

Article history:
Available online 7 February 2012

Keywords:
Proteins
Photometry
Fluorimetry
Flow injection analysis
Integrated flow-through detectors
Light emitting diodes

ABSTRACT

Two miniature, fibreless, compact and highly integrated flow-through optoelectronic detectors dedicated for photometric and fluorimetric determination of proteins have been developed. Both detectors operating according to paired-emitter-detector-diode methodology are constructed only of light emitting diodes and therefore their total cost is extremely low. The photometric detector is dedicated for protein determination according to Bradford method based on detection of protein adduct with Coomassie Brilliant Blue. The fluorimetric detector allows determination of proteins after reaction with fluorescamine. Both developed detectors have been incorporated into economic flow systems constructed of microsolenoid valves and pumps. The resulting multicommutated/multipumping flow analysis systems enable detection of albumins and globulins at ppm levels, thus they are useful for protein determination in diluted samples of physiological fluids.

1. Introduction

Protein determination is a fundamental kind of chemical analysis in biological and biochemical research, biotechnology, food and environmental investigations, clinical diagnostics as well as in many other areas of modern human activity. The significance of protein assay is evidently confirmed by a huge citation number (more than 100.000) of Lowry et al. [1] and Bradford [2] papers. The predominant majority of protein assays is based on optical detection and some of them has been adapted to flow analysis format, which is convenient way for mechanization of analytical procedures. Colorimetric biuret method was applied for total protein assays performed in conventional FIA [3], multi-commutated FIA [4] as well as in flow-batch [5] systems. Additionally, the mentioned above MCFA system [4] and flow-batch analyzer [5] offer photometric determination of albumins using bromocresol green. The Bradford protocol [2] based on photometric determination of protein with Coomassie Brilliant Blue adduct has been adapted for measurements under flow conditions offered by common [6,7] and miniaturized [8] FIA systems. SIA system based on photometric detection of protein complex with eosin Y has been developed for automated albumin determination [9]. Fluorimetric determinations of proteins with stopped-flow mixing technique [10] as well as SIA [11,12] and microfluidic [13] systems have been developed using Bengal Rose [10], fluorescamine [11] and Albumin Blue AB-580 [12,13] as fluorogenic compounds. Spectrofluorimeters are also applied for protein assays performed by the combination of FIA with resonance light scattering detection using bromothymol blue [14], Biebrich scarlet [15] and acid chrome blue K [16] dyes. Flow injection chemiluminescence system for protein determination has been developed using fluorescein derivate [17].

In almost all flow analysis systems mentioned above for the optical detection of respective protein derivatives multiwavelength and multifunctional spectrophotometers and spectrofluorimeters were used. Taking into account that these flow systems are developed for particular kind of analysis performed at particular light wavelengths it is obvious that these rather expensive and sophisticated analytical instruments can be replaced by significantly simpler and cheaper dedicated optical detectors. As we have shown recently [18], a complete dual detection system for contemporary photometric and fluorimetric measurements can be constructed using light emitting diodes only. Such detectors operate according to so-called paired-emitter-detector-diode (PEDD) concept [19]. The prototypes of compact, integrated flow-through devices made of paired LEDs only and designed for photometric [19] as well as for fluorimetric [20] detection of model analytes in flow systems have been also demonstrated. As LEDs are the one of the cheapest optoelectronic elements, the total cost of such fibreless compact device, which is a flow-through cell integrated with dedicated light source and dedicated light detector, is extremely low (less than one euro). Moreover, such low-voltage detectors can be powered with simple battery and for the recording of analytical signal generated by these devices an ordinary, low-budget voltmeter is entirely sufficient [21].

The main goal of this work is the development of LED-based flow-through detectors for protein determination and their implementation into economic flow analysis systems based on low-voltage (12 V), computer-controlled microsolenoid valves and

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doi:10.1016/j.talanta.2012.01.061

pumps. The developed photometric flow system is based on dedicated detector for Bradford assay [2], while in case of fluorimetric flow system the detector designed for fluorescamine-based method [22] is developed.

2. Experimental

2.1. Chemicals

Proteins: bovine serum albumin (BSA, product no. A7906), human serum albumin (HSA, product no. A9511), γ-globulins isolated from bovine blood (BBG, product no. G7516) and γ-globulins isolated from human blood (HBG, product no. G4386) were purchased as lyophilized powders from Sigma–Aldrich (USA). Calibrators (proteins standards, products no. 5-116 and 5-117) and human serum standards (products no. 5-172 and 5-173) were obtained from Cormay (Poland) as aqueous solutions and lyophilisates for reconstitution with water, respectively. Analyzed samples of real human serum and urine were from Medical University of Warsaw (Department of Laboratory Diagnostics).

Coomassie Brilliant Blue G-250 (CBB, product no. 27815) and fluorescamine (FA, Fluram®, product no. 47614) were purchased from Sigma–Aldrich (USA). Other reagents of analytical grade were obtained from POCh (Poland). Doubly distilled water was used throughout.

The solutions used in flow systems for Bradford assay were: 8.5% phosphoric acid and 100 mg/L CBB in water solution containing 5% ethanol and 8.5% phosphoric acid. The working solutions used for fluorimetric assay were: 0.05 M borate buffer, pH of 10 and 400 mg/L FA in acetone.

2.2. Instrumentation

Microsolenoid devices: pumps (product no. 120SP1210-4TE) and three-way valves (product no. 100T3MP12-62-5) were purchased from Bio-ChemValve Inc. (Boonton, NJ USA). The solenoid pumps operated with 2 Hz frequency and indicated stroke volume of 16 μl. These devices were computer-controlled by the KSP Measuring System (Poland). Flow manifolds were constructed using PTFE Microbore tubings (ID 0.8 mm) from Cole-Palmer (USA).

Photometric detector was constructed using 595-nm yellow LEDs (product no. LL-503UYC-Y24DC) obtained from LuckyLight (China). Fluorimetric detector was constructed using 405-nm ultraviolet LEDs (product no. OSSV5111A) and 630-nm red LEDs (product no. OSHR5131A-QR) purchased from Optosupply (China). All applied LEDs have common shape, 5 mm diameter and transparent lens.

The body of photometric flow-through cell was made from cylindrical nontransparent polymeric block using micromilling machine. Main channel in the size of 2 mm determined the aperture of the cell. Optical path was limited by two transparent acrylic windows inserted to the main block and tighten with two bushes. Unmodified LEDs were placed in the bushes. Inlet and outlet stubs were placed in the holes in main block for connecting to the flow system. The fluorimetric flow-through cell was made in the similar way as photometric one described above. LEDs acting as emitters were inserted tightly in the large channel perpendicular to the axis of symmetry of the cell and then micromilled as a one block. The details of detector fabrication are given elsewhere [23].

LEDs operating as light emitters were powered with stable over time currents using home-made low-voltage circuit based on two operational amplifiers L272 chip. All typical electronic components and solderless board were purchased from TME (Poland) Electromotive force generated by LEDs operating as light detectors (treated as an analytical signal [19,21]) was measured and recorded with UNI-T multimeter (model UT70B, China) functioning as voltmeter and connected with data storage computer via RS232 interface.

The primary, reference and supporting optical measurements were performed using UV–vis spectrophotometer (Shimadzu, model PC 2401, Japan) and fiber optic spectrofluorometer (Ocean Optics Inc., model USB2000FLG, USA).

3. Results and discussion

3.1. Photometric system

The colorimetric protein assay developed by Bradford [2] is based on the absorbance shift of CBB dye in which under acidic conditions the red form of the dye is converted into its bluer form by binding to protein. As shown in Fig. 1, the protein-bound form of dye (P-CBB) has an absorption spectrum maximum near 600 nm,

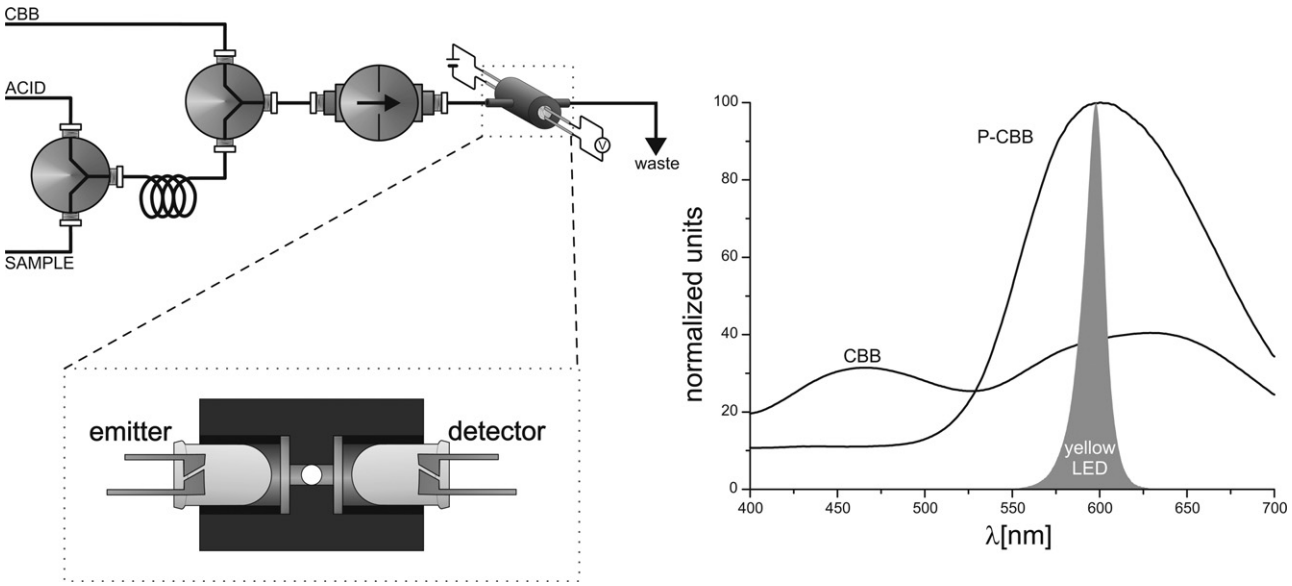

Fig. 1. FIA manifold with the photometric LED-based detector, its cross-section and normalized absorption spectra of CBB and its complex with protein (P-CBB). Additionally, the emission spectrum of LED used for the detector construction is shown.

whereas free form of dye (CBB) is brownish-red. The binding of the dye to the protein stabilizes the blue form. The increase of absorbance at this range of wavelength is proportional to the concentration of protein in the sample. The reaction is relatively fast and therefore easy to adapt for measurements under flow conditions.

The simple flow system with photometric flow-through PEDD developed for protein determination using this methodology is depicted in Fig. 1. Additionally, the cross-section of the flow-through detector is shown. As LED-emitter a yellow LED (595 nm) has been chosen, because the maximum of its emission spectrum is fully compatible with the maximum of absorption spectrum of protein–dye adduct. Both spectra are shown in Fig. 1. Several LEDs were tested as potential detectors in the developed PEDD and the yellow LED, identical as the LED emitter, was found to be the most promising. The orange (610 nm) LED and red (630 nm) LED have exhibited approximately 80% and 70% of the yellow LED sensitivity, respectively. As predicted, the sensitivities of yellow-green (570 nm), green (525 nm), bluish-green (505 nm) and blue (475 nm) LEDs were negligibly low (less than 10% of yellow LED sensitivity), because LEDs can play a detector role only for light of higher energy than the light emitted by them [18,19]. The effect of PEDD powering in the 5–40 mA range was also investigated. With the current decrease, the range of maximal, quasi-linear sensitivity is shifted towards lower concentrations, however for very low energies the decay of sensitivity is observed. Such dependence stays in agreement with earlier findings and was discussed elsewhere [21]. The PEDD supplying currents in the 13–16 mA range were found to be optimal.

Fig. 2 presents the recordings from calibration of the developed MCFA system on BSA (top) and BBG (bottom). The developed flow system allows protein determination in the ppm range of concentrations with high flow throughput (36 injections per hour). It is worth to notice that both measurement cycles were performed using single protein standard, because as reported elsewhere [24] the first microsolenoid valve together with mixing coil and pump can play the role of flow module useful for precisely controlled dilution of calibrant. In Fig. 2 the corresponding calibration graphs for albumins and globulins are shown. Additionally, the results from calibrations of MCFA system on HSA and HBG, performed in the same way as in case bovine-originated proteins, are given.

CBB binds to basic and aromatic side chains amino acids in proteins and this causes that various proteins respond differently to this assay. Peptides rich in arginine, lysine, phenylalanine, tyrosine and tryptophan give higher signals than those with lower content of mentioned amino acids [25] and therefore, as can be seen from Fig. 2, the sensitivity for albumins is higher than for globulins. Since albumins and globulins have different responses, some errors could occur using either BSA or BBG as standards for total protein determination. The use of proteins standards (a mixture of albumins and globulins with the composition corresponding to the proportions of proteins in blood serum, but without non-protein components) solves this problem. On the other hand, as shown in Fig. 2, the differences in sensitivities for the same classes of proteins originated from different sources are negligible. This lack of difference is expected because for example BSA and HSA are homologous, single-chain proteins having similar sequence, conformation and folding [26]. Taking into account the costs of proteins isolated from human and bovine tissues the analytical confirmation of this statement is important from economic point of view. The results shown in Fig. 2 evidenced that BSA and HSA as well as BBG and HBG can be applied alternatively. The presented MCFA system (calibrated with proteins standards prepared from bovine blood) has been applied for determination of total protein in human serum standards. Results of analysis for 44.0 g/L and 67.0 g/L certified sera were 44.2 ± 0 .8 g/L and 66.0 ± 1.1 g/L, respectively. Such accuracy and precision of protein determination is sufficient for the needs of clinical diagnostics.

3.2. Fluorimetric system

The fluorimetric detection investigated in this work is based on the reaction of FA with proteins [22]. A significant enhancement of fluorescence intensity observed with the increase of protein concentration establishes the basis of assay. As shown in Fig. 3, the blue-green fluorescent derivative formed in the course of this reaction has the excitation and emission wavelength maxima at 390 nm and 480 nm, respectively (additionally, absorption spectrum of FA is shown). The reaction is fast and therefore convenient for flow measurements. The multipumping flow system with fluorimetric flow-through detector developed for protein determination using this methodology is shown in Fig. 3. Similarly as in the system for photometric measurements (Fig. 1), the valve (optionally added to this multipumping manifold) together with adjacent pump enables precisely controlled dilution of calibrant and therefore it can be applied as the module for single-standard calibration under flow

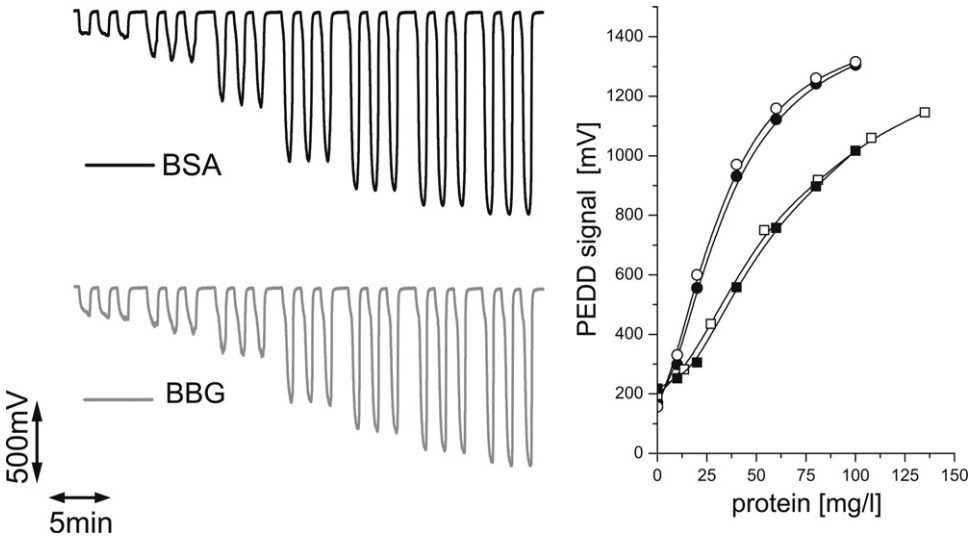

Fig. 2. FIAgrams for photometric detection of BSA and BBG and corresponding calibration graphs with open circles and squares, respectively. Additionally, data for HSA and HBG (filled circles and squares, respectively) are shown.

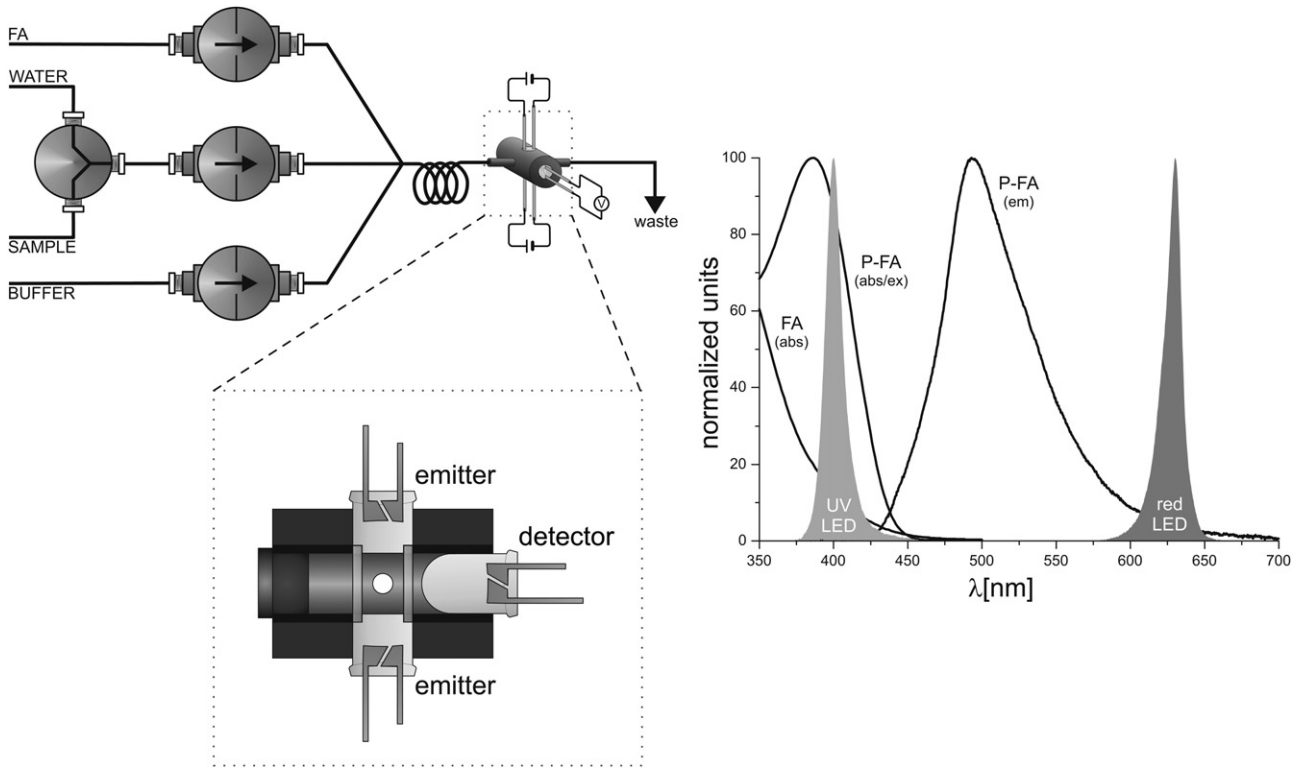

Fig. 3. FIA manifold with the fluorimetric LED-based detector, its cross-section and excitation/emission spectra fluorescamine-BSA derivative. Additionally, absorption spectrum of fluorescamine and emission spectra of LEDs used for the detector construction are shown.

conditions [24]. Two next micropumps deliver fluorogenic reagent (FA) and borate buffer providing the maximal sensitivity of fluorescence detection [11,22]. Due to instability of FA in water solutions, the reagent dissolved in anhydrous acetone is used.

The cross-section of fluorimetric flow-through detector is depicted in Fig. 3. The LED playing the role of fluorescence detector is mounted perpendicularly between two LED used as emitters intended for fluorescence induction. UV-LEDs have been chosen as emitters, because the maximum of their emission spectrum (405 nm) is compatible with the maximum of absorption spectrum of fluorescent protein derivative (Fig. 3). Both LED-emitters were powered with the maximal recommended current (25 mA). Several LEDs were tested as potential detectors of fluorescence under stationary conditions using very simple experimental setup shown elsewhere [18]. In the course of these primary experiments the red (630 nm) LED was found to be the most promising (its emission spectrum is shown in the Fig. 3). The red (660 nm), orange (610 nm), yellow (595 nm) and yellow-green (570 nm) LEDs exhibited approximately 60%, 50%, 50% and 30% of red (630 nm) LED sensitivity, respectively. As predicted, the sensitivities of green (525 nm), bluish-green (505 nm), blue (475 nm) and UV (405 nm) LEDs were negligibly low and their responses, contrary to mentioned above longer wavelength LEDs, were strongly influenced by incidental light from applied LED-emitters.

In general, the reported above partial selectivity of light detection with LED used in reverse mode (as light detector, not emitter) is important advantage. Light detection with photodiode and LED is based on the same mechanism (internal photoelectric effect), however in most cases photodiodes are non-selective in the wide range of UV–vis–NIR spectrum. LEDs operating in the reverse mode can detect light in the narrow spectrum range corresponding only to slightly higher energies than the light emitted by them (average tens of nanometers shift between wavelength maxima for emission and detection). In the present study, this advantage turned out important for proper operation of the developed flow-through detector due to so-called Schlieren phenomenon. This effect, predominantly reported as a problem in case of photometric flow measurements, is a consequence of light inflexions by formation of optical artefacts (lenses and mirrors) inside the flowing segment. These artefacts are generated by concentration or/and refractive index gradients [27]. In the present flow manifold, acetone (used as a FA solvent) is a source of refractive index gradients causing inflexion of light exciting fluorescence. As a result, some fraction of light emitted by LED fluorescence inductors is directed to detector, what could be a source of significant spectral interferences. This phenomenon is easily observed with fiber optic of spectrofluorimeter located in the flow cell instead of LED-detector. As shown in Fig. 4A and B, the noisy and non-specific signal measured at 405 nm

Table 1
Results of fluorimetric determination of total protein level in human physiological fluids.

Sample	Serum	Serum	Serum	Serum	Serum	Serum	Urine	Urine	Urine
Protein content by reference method	77.1 g/L	71.8 g/L	64.0 g/L	62. g/L	61.4 g/L	60.3 g/L	27.1 g/L	3.8 g/L	3.0 g/L
Protein content by proposed method	77.1 ± 0.1 g/L	70.6 ± 0.5 g/L	62.7 ± 0.5 g/L	64.1 ± 0.1 g/L	58.9 ± 0.5 g/L	62.1 ± 0.1 g/L	29.1 ± 0.3 g/L	3.60 ± 0.04 g/L	2.90 ± 0.03 g/L

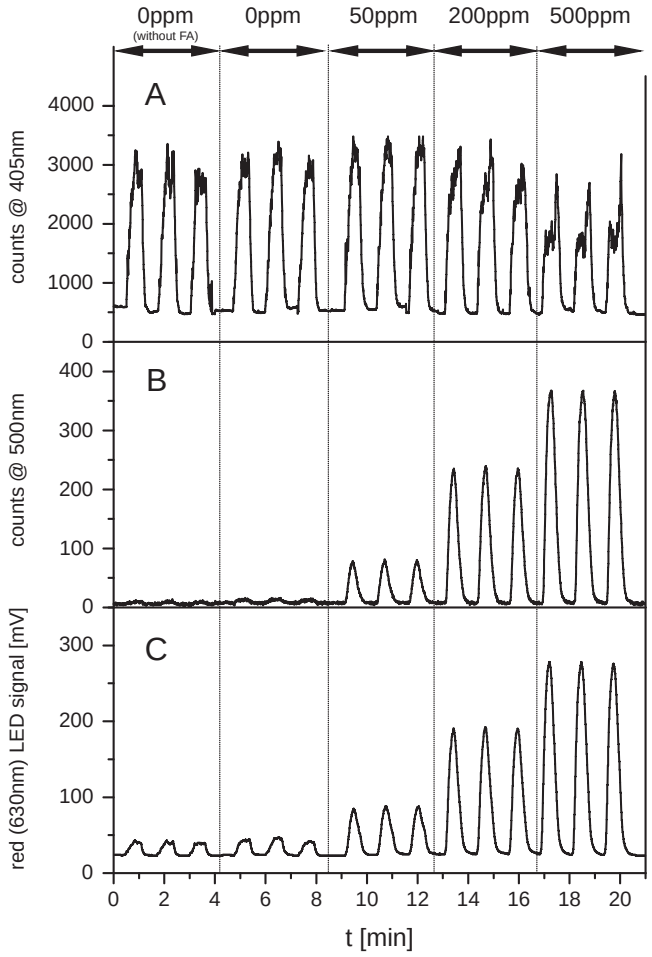

Fig. 4. Simultaneous recordings of light detected in the course of FIA system calibration using fiber spectrofluorimeter at 405 nm (A), 500 nm (B) and red LED (C) located in the fluorimetric flow-through cell at the detector position. BSA concentrations are given in the figure.

(wavelength of fluorescence excitation) is significantly higher than the specific signal measured at 500 nm (wavelength of fluorescence emission). Moreover, this non-specific signal decreases with the decrease of exciting light intensity caused by its absorption by increased concentration of fluorescent agent (P-BSA). As can be seen from Fig. 4C, the red LED applied as the fluorescence detector is almost insensitive on 405-nm wavelength light, thus the measurements are well reproducible.

Fig. 5 presents the results from calibration of the developed flow system on BSA (top) and BBG (bottom). The system allows protein determination in 0–500 μg/mL range of concentration with the flowthroughput higher than 30 injections per hour. The corresponding calibration graphs for BSA and BBG as well as for HSA and HBG are also given in Fig. 5. FA is known to react almost instantaneously with primary and secondary amines to give fluorescent pyrrolinones and non-fluorescent (but interfering) aminoenones, respectively [22,28]. Therefore, the moderate protein-to-protein variation in the sensitivity of FA-based assay is expected. Similarly as in case of the Bradford method, the sensitivity for albumins is higher than for globulins, but no significant differences in the sensitivities for human and bovine proteins are observed. Similar lack of differences was also reported for other fluorimetric assays recently developed of protein determination [29,30].

The utility of developed MPFA system for real analysis was checked with human serum and urine samples. In case of serum analysis the system was calibrated using suitably diluted control sera. Serum samples were 200-fold diluted before injections. For urine analysis the system was calibrated using BSA as standard. The samples were ten times diluted, only for the sample with extremely pathologic protein level the measurements were repeated for 100-fold diluted urine. The samples were simultaneously analyzed in the clinical diagnostic laboratory using conventional biuret assay. The results of analysis collected in Table 1 clearly confirm the usefulness of developed detector and system for the needs of biomedical analysis. The Student's *t*-test was used for the comparison of means of reference and calculated values of total protein concentration. The *t* value (calculated for 95% probability of correct statement) has confirmed no statistically significant differences between results obtained by presented and reference method.

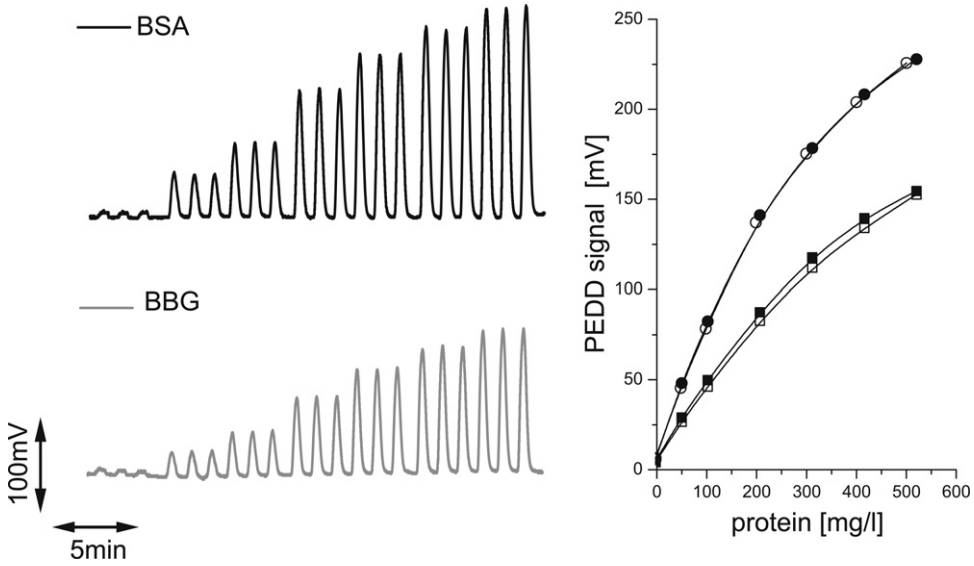

Fig. 5. FIAgrams for fluorimetric detection of BSA and BBG and corresponding calibration graphs with open circles and squares, respectively. Additionally, data for HSA and HBG (filled circles and squares respectively) are shown.

4. Conclusion

The results presented in this contribution clearly confirm that extremely simple optical detectors made of light emitting diodes only are sufficient for both, photometric as well as fluorimetric measurements under conditions of flow analysis. Moreover, as demonstrated, the developed flow systems based on such detectors and economic microsolenoid devices only can be useful as effective analytical tools for practical uses connected with clinical diagnostics.

In our opinion, both developed detectors and flow manifolds can find important applications in more advanced and sophisticated bioanalytical systems. Two the most promising directions for further investigations are flow systems dedicated for detection of selected proteins (for example after on-line separation and optional preconcentration as it has been demonstrated elsewhere [7,8]) and multianalyte flow systems based on arrays of detectors fabricated according to PEDD concept (for example biparametric flow systems for biomedical diagnostics of albuminuria [9,13]).

Acknowledgements

The first author (MP) kindly acknowledges scholarship program "The scientific potential as support to the Mazovia economy" co-financed by the European Union in the frame of European Social Fund Operational, Human Capital Program. These investigations were supported by the Polish Ministry of Science and Higher Education in the frame of Inventus Plus program (project no. IP2011 057371).

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