chemosensors

Communication

Comparison of Colorimetric and Fluorometric Chemosensors for Protein Concentration Determination and Approaches for Estimation of Their Limits of Detection

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Abstract: Here, we present a direct comparison of different dyes and assays for the determination of protein concentrations. We compared the classical Bradford assay with two modern assays based on the fluorogenic dyes QuDye and ProteOrange and showed that the Bradford reagent achieved excellent results in the determination of protein concentrations as compared with more modern rivals. We also showed that standard approaches for determining the limit of detection (LoD) and limit of quantification (LoQ) may not work correctly with the tested dyes. We proposed a new approach that extends the standard algorithm for LoD and LoQ determination. This approach works well with both classical colorimetric and fluorogenic dyes, as well as with nontrivial fluorescent probes.

Keywords: Bradford reagent; ProteOrange; QuDye; fluorescent dye; protein assay; LoD; LoQ

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1. Introduction

In biochemical studies involving the study of proteins, determining their concentration is one of the most frequent tasks encountered [1,2]. The classic method for determining protein concentrations is the Bradford assay, which utilizes the Coomassie® Brilliant Blue G-250 dye [3,4]. In acidic media, the absorption maximum of the dye shifts from 465 nm to 595 nm when binding to a protein. Such behavior of Coomassie G-250 is explained by the protonation of acidic amino acid residues in an acidic medium, resulting in the protein becoming more hydrophobic and positively charged, thus increasing the binding to the hydrophobic part of the dye; on the other hand, the positive charges of arginine and lysine residues stabilize the anionic form of the dye [5]. Recently, it has become increasingly popular to use fluorogenic dyes to determine protein concentrations [1,6,7]. In this case, the fluorescence intensity increases when the dye binds to protein, which results from the chemical modification of the dye [8] or from a decrease in the vibrational freedom of the dye in the protein-bound state and a consequent increase in the quantum yield of fluorescence [9,10]. In this paper, using two commercially available dyes as examples, we compared the effectiveness of new fluorogenic techniques with the classical colorimetric assay for the determination of protein concentrations.

Fluorescent methods are now widely used for the determination of various types of analytes [11–16]. The most important characteristics related to the sensitivity of fluorescent sensors are the limit of detection (LoD) and the limit of quantification (LoQ), which are the concentrations where the response is most probably (95%) above the noise level (LoD) or can be confidently quantified (LoQ). They are defined as the signal gain over the background noise by factors of 3.3 and 10, respectively [17,18]. The LoD and LoQ are usually defined as $LoD = 3.3\ S/b$, $LoQ = 10\ S/b$, where S is the standard deviation (SD) of the response and b is the slope of the calibration curve. In the present work, we showed that this

traditional approach may produce incorrect results. To properly estimate the LoD and LoQ, we modified the standard algorithm by accounting for the standard deviations of the response at low concentrations and by adding to the equation a term that takes into account deviations of the experimental data from the linear dependence in the low concentration range.

2. Materials and Methods

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions with the defined protein concentrations were obtained by a series of sequential dilutions of 10 mg/mL BSA in phosphate-buffered saline (PBS). The protein concentration was verified by the absorption of the sample at 280 nm (ε280 nm = 43,824 $M^{-1}cm^{-1}$, MW = 66,400 Da). Bradford reagent was used as part of the protein assay kit (Bio-Rad, Hercules, CA, USA); fluorogenic dyes ProteOrange and QuDye were purchased from Lumiprobe (Moscow, Russia). Assays were conducted according to manufacturers instructions. Samples of proteins were diluted with working solutions of the dyes according to Table 1. All samples were prepared in triplicate (for each concentration, three independent dilutions with working solution were produced to take into account possible errors in pipetting and measuring). Measurements were performed in 96-well plates using a plate reader (Tecan Infinite® M1000 Pro, Switzerland).

Table 1. Composition of the assay reaction mixtures.

Dye	Volume of Sample Added, μL	Volume of Working Solution, μL
Coomassie G-250	10	200
ProteOrange	2.5	97.5
QuDye	5	95

For the spectrophotometric measurements of the samples with Bradford reagent, the sample volume was ~200 μL according to the manufacturer's protocol. At this volume, the optical density was close to the optimum 0.3–0.9 [19]. For fluorescent dyes, the final sample volume was 100 μL, instead of the manufacturer's recommended 200 μL. This allowed us to use half of the protein at the same concentration to increase the sensitivity of the method. The plate reader we used had an excitation/detection spot of only 2 mm, so it was not reasonable to take a larger volume. Before measurements, the Z- position of the focusing optics was optimized; settings at which the dispersion of the detected parameters was minimal were used.

For the Bradford assay, the background absorbance of the samples at 800 nm was subtracted from the absorbance at the absorbance maximum of 595 nm. To measure the fluorescence signal of fluorogenic probes, samples were excited at 480 nm, emission was detected at 590 nm, and the slit was set to 10 nm. Absorption and fluorescence emission spectra were measured using the same plate reader. Linear regression was performed using Origin 2021 software (OriginLab).

3. Results and Discussion

We used BSA as a reference protein and prepared a series of dilutions with concentrations ranging from ~10 mg/mL to 0.07 μg/mL (5 orders of magnitude). Then, we measured the response of various dyes to the protein and generated calibration curves (Figure 1). We also measured the absorption and fluorescence emission spectra of the samples for the visual comparison of the dyes properties. For a better representation of the regions of low concentrations, the calibration curves were also plotted in logarithmic scale (Figure 2).

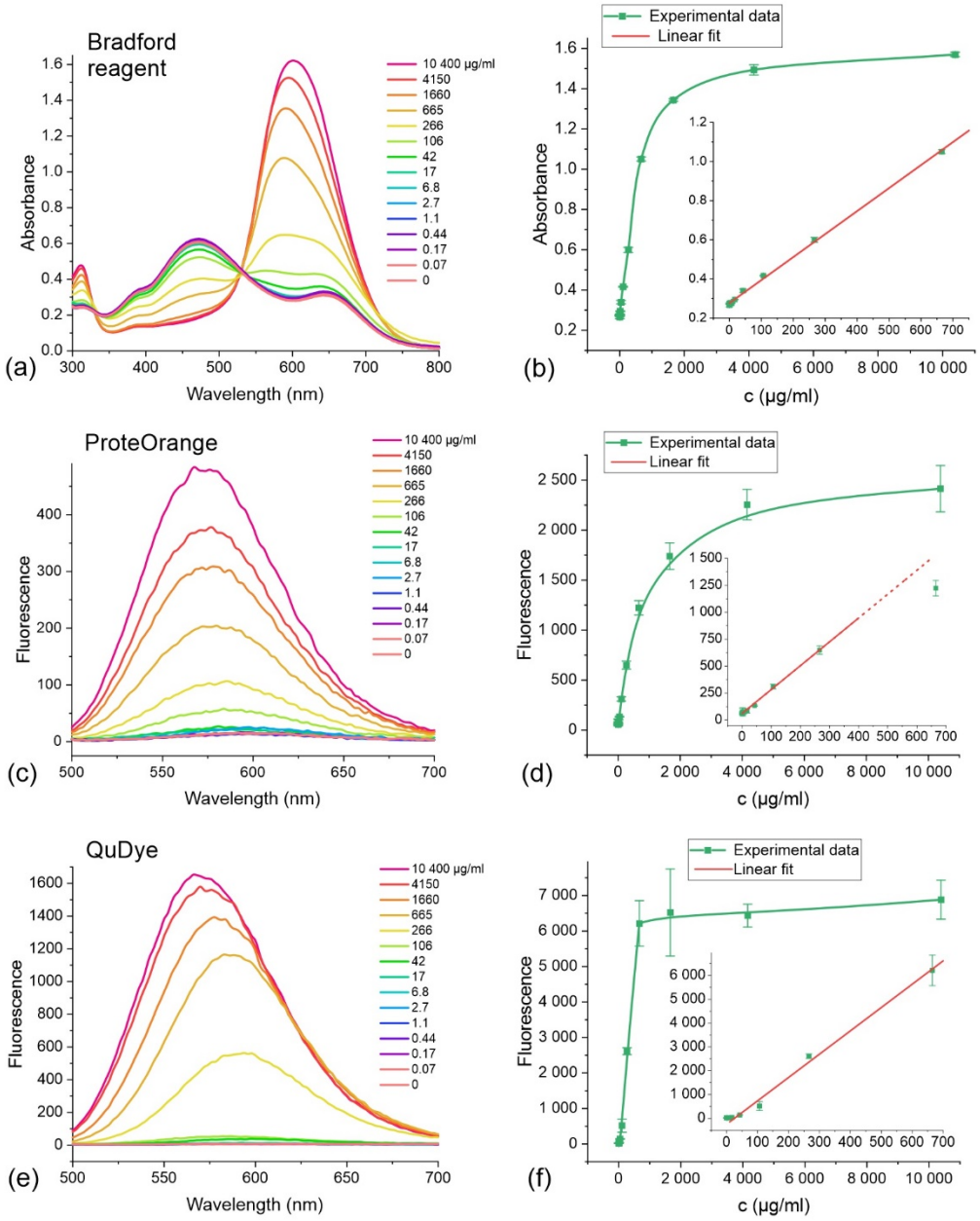

Figure 1. Response spectra of dye to the presence of protein (**a**,**c**,**e**) and calibration curves (**b**,**d**,**f**) for Bradford reagent (**a**,**b**), ProteOrange (**c**,**d**), and QuDye (**e**,**f**). Concentrations of the stock solutions are presented. For each concentration, the experiments were performed in triplicate. The error bars are the SD.

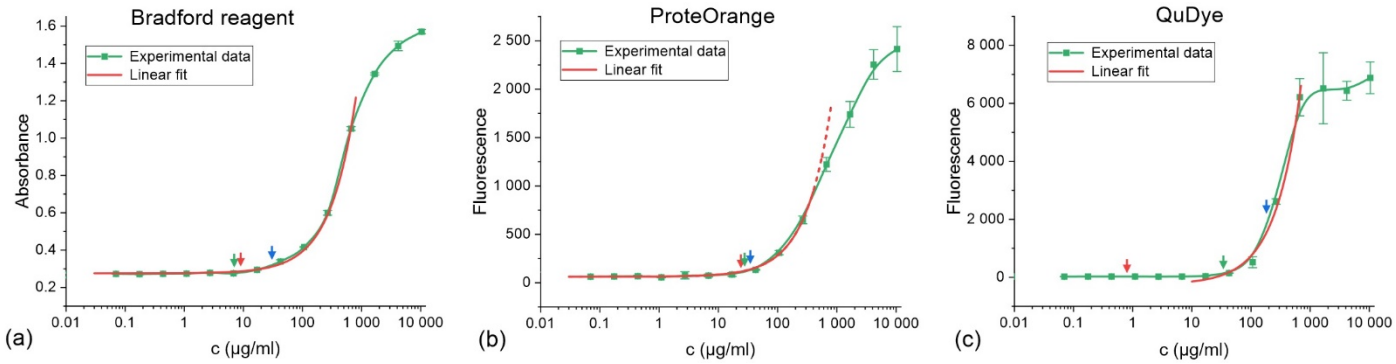

Figure 2. Calibration curves in logarithmic scale for Bradford reagent (**a**), ProteOrange (**b**), and QuDye (**c**). The arrows indicate the LoDs calculated using Equations (1) (in blue), (2) (in red), and (3) (in green).

Bradford's reagent (Coomassie G-250) showed a linear dependence of the growth of light absorption with increasing protein concentration in the range of 0 to ~670 μg/mL. ProteOrange showed linear fluorescence growth in response to the protein in the range of up to 350 μg/mL. It was also clearly visible that the standard deviations increased with increasing concentrations (Figure 1d). In the case of QuDye, a linear response to the protein was observed at concentrations of 40 to 670 μg/mL. The high threshold of the response may indicate the complex character of dye–protein interactions; this was also confirmed by the hypsochromic shift of the emission maximum at higher concentrations (Figure 1e).

To determine the LoD, a calibration line is usually built using linear regression in the range of the linear response of the dye to the analyte; then, the LoD is determined using the following equation:

$$LoD = 3.3\,\frac{S}{b} \quad (1)$$

where S is the standard deviation of the response (intercept with intensity axis) and b is the slope of the calibration curve.

According to Equation (1) and the results of the linear regression for the Bradford reagent, ProteOrange, and QuDye, the LoDs were 30.5, 34.6, and 182 μg/mL, respectively (Table 2). However, one can see that, at these values, the dye response could reach significant values (Figure 2, blue arrows). In the case of QuDye, it was about one-third of the dynamic range, while the deviations of the signal in the region of "noise" were quite small. In other words, the calculated LoDs seemed to be overestimated.

Table 2. Calculated parameters from the linear regression and blank responses as well as the LoDs calculated by using these parameters and Equations (1)–(3).

Dye	B [1]	SE_{slope} [1]	I_0 [1]	S [1]	I_{blank} [2]	S_{blank} [2]	LoD (1)	LoD (2)	LoD (3)	m (ng)
Coomassie G-250	0.00117	8.6×10^{-6}	0.277	0.0108	0.275	0.00328	30.5	9.24	7.20	72
ProteOrange	2.22	0.046	59.6	23.27	67.1	16.4	34.6	24.3	27.7	69.3
QuDye	9.9	0.43	−305	546	25.5	2.43	182	0.81	34	170

[1] Parameters derived from linear regression; [2] Parameters derived from the response at low concentrations; b—slope of the calibration curve; SE_{slope}—standard error of the slope; I_0—intercept of the calibration curve; S—standard deviation of the intercept; I_{blank}—average response at low concentrations (background noise at concentrations <7 μg/mL); S_{blank}—standard deviation of the response at low concentrations; LoD—limit of detection in μg/mL as calculated using Equations (1), (2), or (3); m (ng)—amount of the protein in the sample corresponding to the LoD (3)

It was noted above that the standard deviation of the response increased with increasing concentration, especially for fluorogenic dyes, while the SD of the response in the

low-concentration region (near the actual LoD) was much lower. Therefore, to estimate the LoD, it was more appropriate to use the SD of the response at low concentrations, or the concentrations at which the response did not exceed the background level (S_{blank}). For the tested dyes, it was the area of concentrations below 7 μg/mL. In this case, the detection limit was determined as:

$$LoD = 3.3\,\frac{S_{blank}}{b} \tag{2}$$

According to Equation (2), the LoDs for Bradford reagent, ProteOrange, and QuDye were 9.24, 24.3, and 0.81 μg/mL, respectively. The use of S_{blank} expectedly resulted in lower values of the determined LoDs. However, in the case of QuDye, the LoD was in the region of background noise. This could be explained, on one hand, by the low SD in the noise region and, on the other hand, by the significant discrepancy between the intercept of the regression line and the actual response of the "blank" samples (Table 2).

When considering the basis for the LoD definition, Equation (1) arises from the assumption of Gaussian distribution for the response to an analyte, and that the probability of occurrence of the response values in the background noise did not exceed 5%. At this assumption value of the response at the concentration of the LoD defined as the mean value of the background signal (I_{blank}) increased by 3.3 (rounding of 3.29 [20]) standard deviations of the response in the background (S_{blank}) [17,18,21]. That is,

$$I_{LoD} = I_{blank} + 3.3S_{blank}$$

If we take the equation of linear regression of the response from the concentration

$$I = I_0 + b[C]$$

the concentration at the limit of detection is expressed as

$$[C]_{LoD} = \frac{I_{LoD} - I_0}{b} = \frac{I_{blank} + 3.3S_{blank} - I_0}{b} = \frac{3.3S_{blank}}{b} + \frac{I_{blank} - I_0}{b}$$

thus, the LoD in the general case is expressed by the equation:

$$LoD = 3.3\frac{S_{blank}}{b} + \frac{I_{blank} - I_0}{b} \tag{3}$$

where S_{blank} is the standard deviation of the signal at concentrations below the LoD, i.e., at concentrations at which the response becomes less than the noise; I_{blank} is the mean value of the response at these concentrations; and I_0 and b are the parameters of linear regression of the calibration line (intercept and slope, respectively). For clarity, these parameters are shown in Figure 3 using ProteOrane as an example.

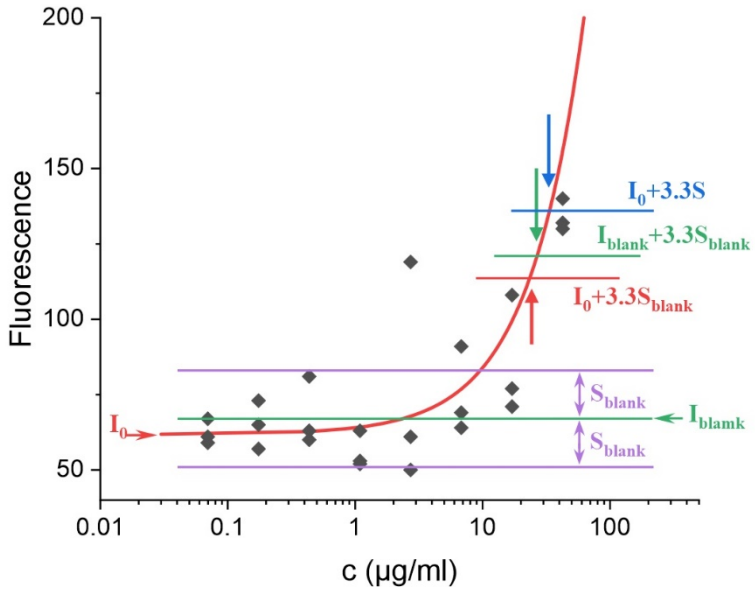

Figure 3. The enlarged region of low concentrations of the ProteOrange calibration curve. Experimental points are shown as black diamonds. The linear regression is shown by the red line. The mean value of I_{blank} and its standard deviation S_{blank} were calculated from the points at concentrations below 7 µg/mL. The fluorescence signal corresponding to the LoD determined by Equation (1) is indicated by the blue line and arrow, by Equation (2) by the red line and arrow, and by Equation (3) by the green line and arrow.

In determining LoD, when the response is measured over the entire dynamic range of the dye, but the impact of nearly zero concentrations is neglected, the I_{blank} values can be assumed to be equal to I_0 and Equation (3) degenerates into Equation (2). If the standard deviations are assumed to be equal in the entire range of measurements, Equation (2) is transformed into Equation (1). Such assumptions are reasonable when the deviations of the response are comparable over the whole range of measurements and the linear regression does not deviate significantly from the experimental data in the region of "blank" samples. In other cases, oversimplifications can result in artifacts that can lead to incorrect LoD determinations. As shown, the deviations can reach orders of magnitude, as in the case of QuDye, for which the I_0 value was even below zero.

The limits of protein detection by Bradford reagent, ProteOrange, and QuDye, as calculated by Equation (3), were 7.20, 27.7, and 34 µg/mL, respectively. When multiplying these values by the volume of sample tested (Table 1), 72, 69.3, and 170 ng of the protein in the sample could be detected by the corresponding methods, respectively. Thus, for determining the protein concentration in solution, the classic Bradford reagent was not worse than newer dyes in terms of the LoD. It also had a large range of linearity of the response in the area of high concentrations. For the measurements, it was necessary to use spectrophotometric equipment, rather than the more expensive spectrofluorimetric equipment, as in the case of fluorescent dyes.

Summing up the results of the LoD determination of the tested dyes, for the proper determination of the LoD of an analyte by dye, linear regression in the region of considerable signals is not enough. It is also necessary to determine the mean value of the signal and its standard deviation at near-zero concentrations. This allows for Equation (3) to be used for a more accurate determination of LoD. Additionally, it should be noted that the results of calculations should be critically interpreted, and the determined parameters should be checked visually using plots of the response dependence on the concentration in the logarithmic scale.

An important characteristic of chemosensors is also the limit of quantification (LoQ). The same linear regression equation as that used for the LoD is usually used to determine

the LoQ, but the multiplication factor of the standard deviation of the background is usually 10 instead of 3.3 [18,22]. That is, the LoD is the concentration at which the signal exceeds the noise at this point by 10 times [20]. Analogous to LoD,

$$LoQ = 10\frac{S_{blank}}{b} + \frac{I_{blank} - I_0}{b}$$

However, it is worth noting that, in the case of significant differences in the standard deviations of the response value in the area of the obtained LoQ from S_{blank}, more complex calculations may be required.

It is also worth noting that most programs used to calculate linear regressions (such as Origin or GraphPad Prism), in the results of the calculations display not the standard deviations of the determined parameters, but the standard errors (SE), which are linked to each other by the equation

$$SE = \frac{SD}{\sqrt{N}}$$

where N is the number of points used in the parameter calculation.

It is correct to use SE in specifying the error of a parameter in the form "mean ± SE", but when calculating the LoD and LoQ, the obtained standard errors must be multiplied by $\sqrt{N}$ to obtain the standard deviations.

4. Conclusions

We have shown that the classical Bradford method allows the determination of protein concentrations and is indeed not inferior to contemporary methods. In addition, we showed that the use of standard approaches, including the use of linear regressions to determine the LoD and LoQ, may not work correctly for the tested dyes. We have refined the commonly used algorithm for analyzing experimental data so that it takes into account possible artifacts. The described protocol can be used not only for protein concentration assays using colorimetric and fluorogenic dyes, but also for other systems with deviations in the experimental data from standard linear regressions.

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Abbreviations

BSA—Bovine serum albumin; LoD—limit of detection; LoQ—limit of quantification; SD—standard deviation; SE—standard error.

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