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Novel application of pyronin Y fluorophore as high sensitive optical sensor of glucose in human serum

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ABSTRACT

This paper presents a novel, high sensitive, facile and rapid spectrofluorimetric method for the direct assessment of glucose in human serum samples. The strong fluorescence intensity of pyronin Y (PY) is found to be selectively quenched substantially by adding solutions of glucose. Therefore, a new quantitative method to determine glucose could be developed. The noticeable and specific quenching effect of glucose on PY emission could be attributed to the increase in the nonradiative interactions as a result of a charge-transfer excited state with the location of the positive charge at the xanthenes moiety. Various parameters namely, the best working solvent, the proper pH medium in addition to the concentration of PY fluorophore corresponding to maximum fluorescence intensity were closely investigated. Under optimal conditions, the standard curve was linear in the glucose concentration ranges from 5.0×10^{-9} to 1.0×10^{-6} mol L^{-1} (r=0.994). The detection limit (S:N=3) (LOD) is 2.9×10^{-9} mol L^{-1}. Validation of this approach was done by examining glucose concentration in serum samples of twenty patient and ten health donors. Achieved recovery reached 99.63–100.43% and 99.57–100.70%, respectively, in good agreement with those provided from hospital using traditional method. The relative standard deviations (RSD) were estimated between 0.00–0.78% and 0.15–1.91%, respectively.

1. Introduction

In recent years, the development of glucose sensors has been received considerable attention due to the demands of glucose controlling in various food processing, fermentation and biotechnological processes, as well as the continuous glucose self-monitoring for substantially reducing morbidity and mortality among diabetes patients [1–3]. Additionally, glucose and its derivatives could induce leukemia cell K562 to dissociate to huge engulf cell [4]. Apparently, glucose is closely related to malignant tumor, diabetes mellitus [5]. Therefore, the determination of glucose in human serum with high sensitivity and precision has significance in diagnosing and treating human diseases.

To date, many different approaches to develop glucose sensors have been pursued including different electrochemical methods [1,6–9], Fourier-transform infrared spectroscopy [10], UV–vis spectroscopy [11,12], localized surface Plasmon resonance [13], capillary enzyme bioreactor [14] as well as fluorometry [15–20]. Among these approaches, fluorescence-based systems are actively explored and receiving increasing attention encouraged by special advantages of fluorometry for biological analysis [2,15–20]. Moreover, the technique is extremely sensitive; fluorescence measurements cause little or no damage to the host systems and the measurements can be made by different parameters such as fluorescence intensity, fluorescence decay time, etc.; the structure and distribution of biomolecules can also be probed by the phenomenon of fluorescence resonance energy transfer (FRET). Among these fluorescence-based glucose sensors, traditional organic fluorophores (e.g., organic dyes) are widely employed as recognition elements.

Dyes can be classified as anionic (direct, acid, and reactive dyes), cationic (basic dyes) and non-ionic (disperse dyes) [21]. The pyronin Y (PY) cationic dye (Fig. 1) is a water soluble chromogenic xanthenes reagent of extended conjugation that is responsible for its absorption in the longer wavelength, i.e., 540 nm of the visible spectrum [22]. PY has been used to determine species such as As(III), Cr(VI), tannins [23], also in the doping of organic thin films [24], and depositing on a p-type silicon surface to construct diodes [25]. However, it has not attracted much attention for biological applications. Recently, few reports accounts for the use of PY in the determination of ascorbic and uric acids, trace formaldehyde in blood plasma, and in the labeling of proteins and cell organelles [26–29].

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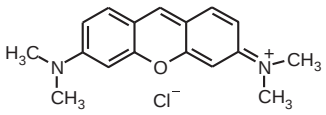

Fig. 1. Chemical structure of PY.

In the present study, we have added a novel functionality of PY xanthenes dye as a highly sensitive spectrofluorimetric probe for glucose with minimal interference. The method based on a selective quenching of PY fluorescence by glucose in acetonitrile at pH 4.2. The proposed method was also performed to the analysis of blood glucose in human serum samples and the results were in good agreement with clinical data provided by the hospital, which indicates that the method presented here is not only simple, sensitive, linear but also reliable and suitable for practical applications.

2. Experimental

2.1. Apparatus

The absorption spectra were recorded with a double beam PerkinElmer Lambda 25 UV–visible spectrophotometer fitted with a tungsten halogen lamp for operation in the visible range and a deuterium lamp for operation in the UV range. All luminescence measurements were recorded with a Meslo-PN (222–263000) thermo scientific lumina fluorescence spectrometer in the range (190–900 nm). The pH was measured using a pHs-JAN-WAY 3330 research pH meter.

2.2. Reagents

A stock standard solution of PY (5.0×10^{-3} mol L^{-1}) was prepared by dissolving 37.85 mg of PY (Sigma–Aldrich) in 25 mL bidistilled water. Stock standard solutions (1.0×10^{-2} mol L^{-1}) of glucose (Adwic Co., Egypt) were prepared by dissolving 90.0 mg in bidistilled water and diluting to 50 mL with acetonitrile. All the solutions were kept in the refrigerator and protected from light. Working standard solutions were obtained by making appropriate dilutions of the stock standard solution with acetonitrile. Analytical grade materials of fructose, lactose, galactose, maltose, ascorbic acid in addition to potassium iodide, magnesium sulfate, calcium, zinc and sodium chlorides are used throughout the work.

HCl/KCl buffer 0.2 mol L^{-1} (pH 1.0–2.2) was prepared by mixing appropriate volume of 0.2 mol L^{-1} hydrochloric acid with 0.2 mol L^{-1} potassium chloride. Acetate buffer 0.2 mol L^{-1} (pH 3.5–5.6) was adapted by mixing appropriate volume of 0.2 mol L^{-1} acetic acid with 0.2 mol L^{-1} sodium acetate. In the mean time, borate buffers (pH 6–8.5) were obtained by mixing appropriate volumes of 0.2 mol L^{-1} boric acid with 0.2 mol L^{-1} sodium hydroxide and finally phosphate buffer (pH 8.8–11.0) was prepared by mixing appropriate volume of 0.2 mol L^{-1} K_2HPO_4 with 0.1 mol L^{-1} sodium hydroxide. All reagents used were analytical grade unless otherwise indicated.

2.3. Methods

2.3.1. Preparation of glucose solutions

In 10 mL clean and sterilized measuring flasks, standard solutions of glucose were prepared by the various additions of (1×10^{-2} mol L^{-1}) glucose stock solution to give different concentrations of glucose. The solutions were diluted to the mark with acetonitrile at room temperature. The above method was followed in the subsequent measurements of emission spectra, effect of pH and effect of solvents. The fluorescence intensity was measured at $\lambda_{ex}/\lambda_{em}=548/568$ nm.

2.3.2. Preparation of the PY/glucose solutions

Working solutions containing PY (3×10^{-5} mol L^{-1}) and different concentrations of glucose were prepared in 10 mL measuring flasks. Accurate volume (0.06 mL) of PY (5×10^{-3} mol L^{-1}) was mixed with appropriate volumes of glucose (1×10^{-2} mol L^{-1}). The solution was then completed to the mark with acetonitrile. The pH was adjusted to 4.2 using 2.0 mol L^{-1} acetate buffers, and stored for 5 min at room temperature. The above procedure was used for the subsequent studies of linearity, selectivity, precision, recovery and determination of glucose in human serum samples.

2.4. Validation

2.4.1. Linearity

After the preparation of the different working solutions containing standards of glucose along with PY in acetonitrile as described above, the calibration curve was obtained by plotting the peak intensity at $\lambda_{em}=568$ nm of PY on *y*-axis against 1/[glucose] on *x*-axis. The concentrations of calibration standards were analyzed and the linearity was evaluated by comparing the correlation coefficient (r) between theoretical and back-calculated concentrations of calibration standard samples.

2.4.2. Selectivity

The selectivity was performed on a human serum from twenty individual patient donors and a ten human serum samples from healthy donors receiving no medication for the assessment of potential interferences with endogenous substances at the linear range for the determination of glucose.

2.4.3. Precision

The intra-day precision of optical sensor was evaluated by replicate ($n=3$) analysis of the serum samples containing glucose. It was evaluated at different concentration levels for 3 day. The precision was estimated by the relative standard deviation (R.S.D).

2.4.4. Recovery

The average recoveries of glucose were evaluated at different concentration levels, each concentration was repeated three times. Recoveries were evaluated using the formula: . Thus, from the peak intensity of assayed samples compared to the one of reference standards prepared in acetonitrile, % recovery could be calculated.

2.5. Determination of glucose in serum solution

3 mL of citrate solution was added to 4.0 mL plasma of healthy or patient donors. The solution was centrifuged for 15 min at 4000 r/min to remove proteins. Then, the serum sample was placed in 10 mL calibrated volumetric flasks containing 0.06 mL of 5×10^{-3} mol L^{-1} PY fluorophore. After that, acetate buffer was added to adjust the pH at 4.2 then completed to the mark with acetonitrile to give the test solution. The fluorescence intensity of the PY fluorophore was measured alone and after addition of the test solution. The change in the fluorescence intensity was used for determination of glucose in serum sample.

3. Results and discussion

3.1. Spectral characteristics

The absorption spectrum of PY (1×10^{-5} mol L^{-1}) at pH 4.2 in acetonitrile shows intense broad band in the visible region attributed to π–π^* transition, spectrum 1. Moreover, the emission spectrum of PY exhibits intense emission peak at 568 nm (spectrum 2) which was quenched substantially by adding glucose in acetonitrile solutions at pH 4.2, spectrum 3 (Fig. 2).

3.2. Effect of solvent

The effect of different solvents on the fluorescence intensity of PY (1×10^{-5} mol L^{-1}) was studied. In Fig. 3, the highest fluorescence intensity of the PY solution was observed in aprotic solvents like acetonitrile that may be due to the formation of highly emissive anhydrous solvates of the PY species. Also, in DMF, PY exhibits a highly intense emission band close to that in acetonitrile. Thus, the poisonous effect of acetonitrile could be ignored by working in DMF. On the contrary, a much lower intensity of PY emission was obtained in protic solvent like water and ethanol. This could be attributed to the efficient quenching of the excited state of PY by interactions with high-energy vibration oscillators like O–H groups [30].

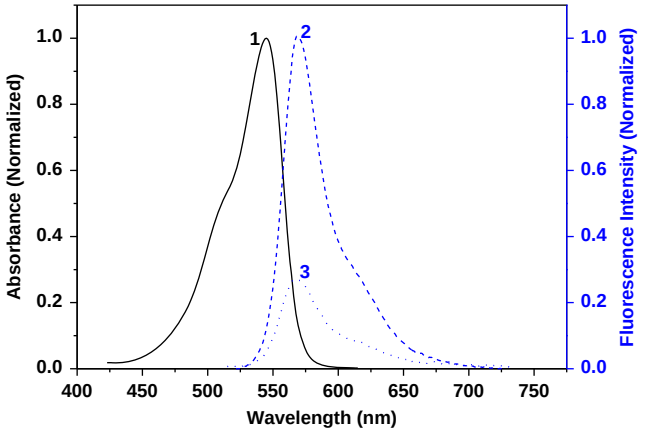

Fig. 2. The absorption spectrum of PY (curve 1) and the emission spectra of PY in absence and presence of glucose (curves 2 and 3, respectively). (Experimental conditions: solvent=acetonitrile, [PY]=1×10^{-5} mol L^{-1}, [glucose]=2×10^{-5} mol L^{-1}, λ_{ex}=548 nm).

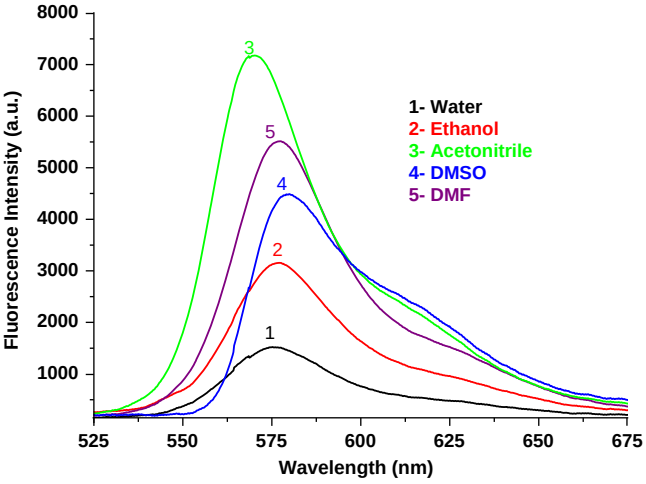

Fig. 3. The fluorescence intensities of 1×10^{-5} mol L^{-1} PY at λ_{ex}=548 nm, in presence of different solvents.

3.3. Effect of the amount of PY

In acetonitrile preparations, the fluorescence intensity of PY was noticeably enhanced upon increasing its concentration gradually until optimal value at 3×10^{-5} mol L^{-1}. Subsequently, it decreased markedly at higher concentrations (Fig. 4). The increase in fluorescence intensity could be attributed to higher number of emissive species. In contrast the decrease may be due to self quenching accompanied to concentrations higher than 3×10^{-5} mol L^{-1}.

3.4. Effect of pH

The pH of the medium had a great effect on the fluorescence intensity of PY. Therefore HCl/KCl, acetate, borate and phosphate buffers provide the pH range 1–11 were prepared and used throughout this study. Monitoring the fluorescence from PY at different pH (Fig. 5) reveals an increase as well as a decrease in the fluorescence intensity. The maximum fluorescence intensity (λ_{em}=568 nm) was found at pH 4.2 in presence of acetate buffer.

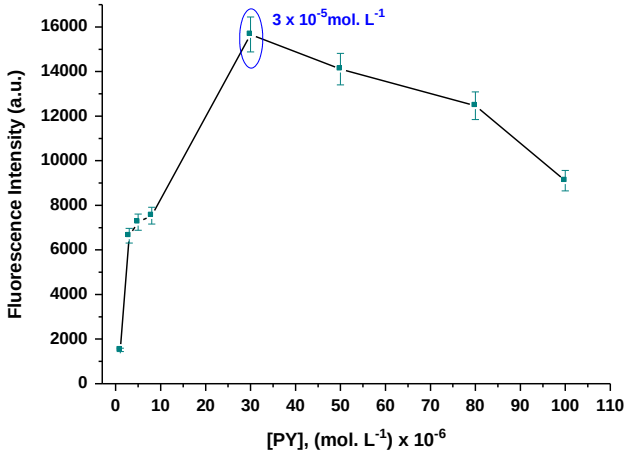

Fig. 4. The effect of different concentrations of PY on its fluorescence intensity at λ_{ex}=548 nm and in presence of acetonitrile.

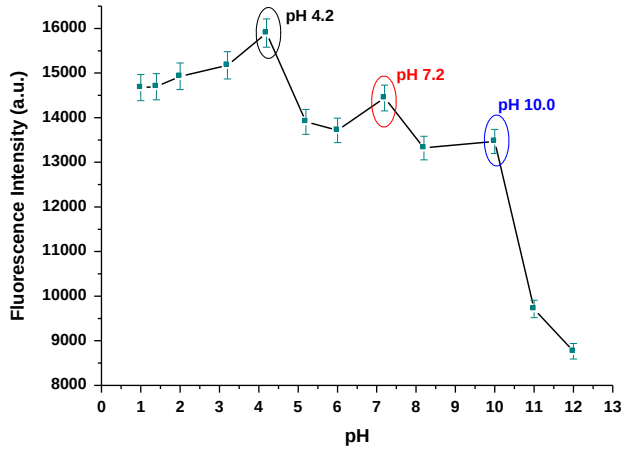

Fig. 5. The fluorescence intensities of PY at different pH, (experimental conditions: solvent=acetonitrile, buffer=HCl/KCl, acetate, borate and phosphate buffer, [PY]=3×10^{-5} mol L^{-1}, λ_{ex}=548 nm).

This could be related to a different pH-assisted charged formula of PY including the highly emissive species at pH 4.2 (Fig. 5).

3.5. Reagents and time stability test

The effect of the order of reagent addition on the fluorescence intensity of the working system was examined. Addition order of PY, glucose solutions and buffer, gave the optimal performance and therefore was chosen for further studies. The optimized system showed constancy in the exerted fluorescence in 5 min. and remained stable for at least 6 h.

3.6. Proposed mechanism

Addition of glucose solutions to PY fluorophore, results in a selective and a significant decrease in the PY fluorescence intensity. To explain this atypical behavior, the resonance structures of PY were proposed in Fig. 6A. In the resonance structures on the right, a nonemissive charge transfer (CT) excited state of PY is formed where the positive charge is located at the xanthene ring, and a structural change of the amino groups has taken place [31]. Moreover, the high electron density due to the oxygens in the pyranose structure of glucose would specifically provide an effective stabilization of the positively charged xanthene, favoring the formation of the CT state [31]. Furthermore, the noticeable quenching effect of glucose on PY emission could be related to the increase in the nonradiative interactions because of a charge-transfer excited state with the location of the positive charge at the xanthenes moiety, Fig. 6B. An important effect of the glucosidic oxygens on the nonradiative deactivation of PY has been previously reported [31].

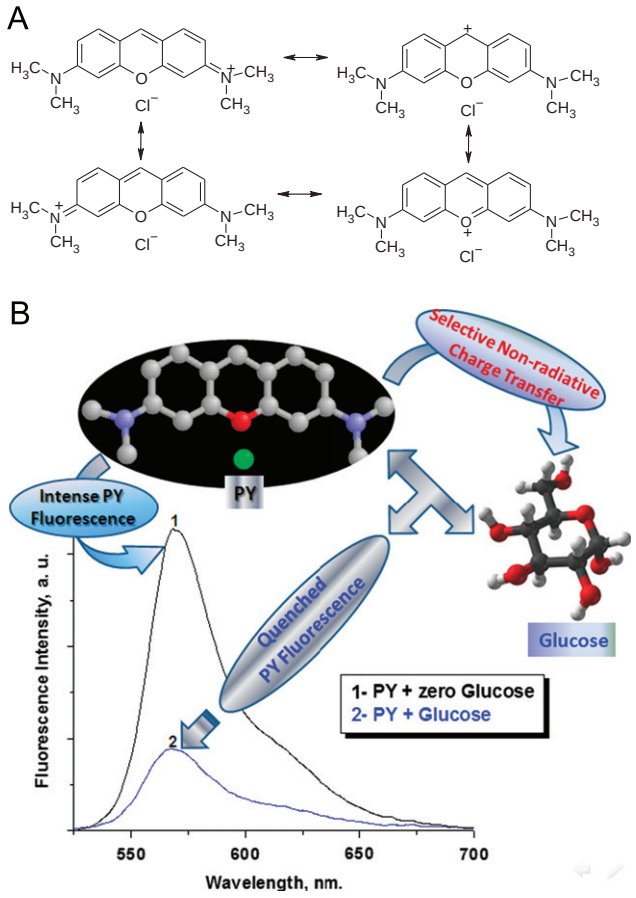

Fig. 6. Resonance structures of PY (A) and the diagrammatic presentation of the quenching mechanism (B).

4. Analytical application

4.1. Linear range and limit of detection

The effect of wide variations of glucose concentrations on the fluorescence intensity of PY is shown in Fig. 7. The figure reveals a substantial decrease in the fluorescence intensity of PY signal at $\lambda_{em}=568$ nm. Under the optimal conditions, the fluorescence intensity of PY decreased linearly with the concentrations of glucose over the range 5.0×10^{-9} to 1.0×10^{-6} mol L^{-1} ($r=0.994$). The detection limit ($S:N=3$) (LOD) is 2.9×10^{-9} mol L^{-1} and the limit of quantification (LOQ) is 8.8×10^{-9} mol L^{-1}, (Fig. 8 and Table 1).

4.2. Accuracy and precision of the method

To compute the accuracy and precision, the methods described under "general procedures" were repeated three times within the day to determine the repeatability (intra-day precision) and three times on different days to determine the intermediate precision (inter-day precision) of the method. The results of this study are

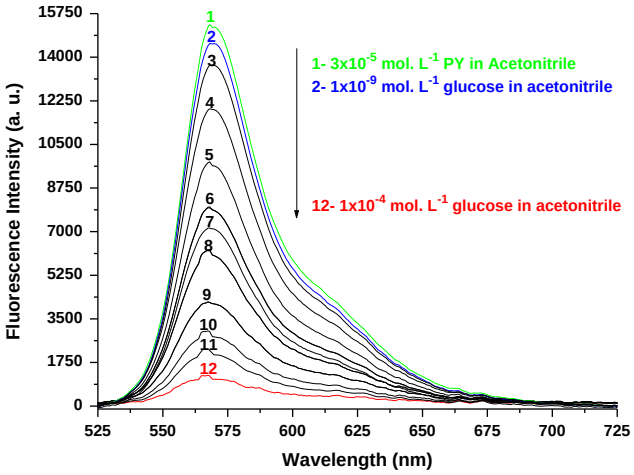

Fig. 7. Fluorescence spectra of 3×10^{-5} mol L^{-1} PY in presence of different concentrations of glucose in acetonitrile at $\lambda_{ex}=548$ nm and pH 4.2.

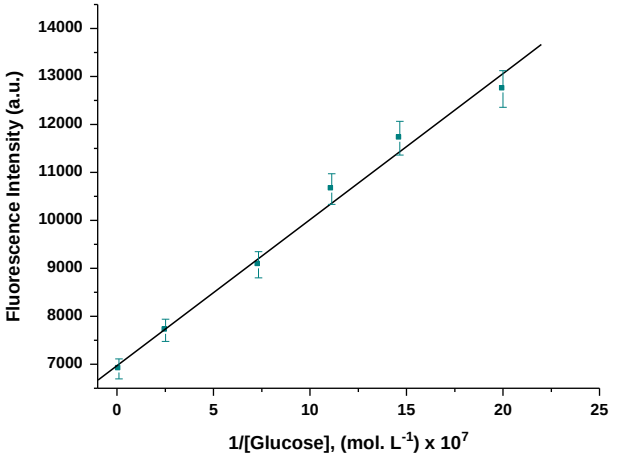

Fig. 8. Linear relationship of the different molar concentrations of glucose in acetonitrile and the fluorescence intensity of PY (3×10^{-5} mol L^{-1}) at $\lambda_{ex}=548$ nm.

summarized in Table 2. The percentage relative standard deviation (%RSD) values were 0.15–1.9% (intra-day) and 0.24–2.1% (inter-day) indicating high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the taken concentrations of glucose. *Bias* {*bias%*=[(*Concentration found*−*known concentration*)×100/*known concentration*]} and was calculated at each concentration and these results are also presented in Table 2. Percent relative error (%RE) values of 0.09–1.17% (intra-day) and 0.3–3.22% (inter-day) demonstrates the high accuracy of the proposed method.

4.3. Interference study

For further evaluating the detection selectivity of PY fluorophore for glucose determination, the investigation was carried out at a glucose concentration of 1×10^{-6} mol L^{-1} with various coexistence substrates added. Moreover, monitoring the emission intensity of PY in presence of different interfering ions can offer an evidence for the previously mentioned mechanism. As shown in Table 3, the results exhibited that the tolerable concentration ratios of coexisting substances to 1×10^{-6} mol L^{-1} glucose was over 300-fold for Na^+, K^+, Zn^{2+}, 100-fold for Mg^{2+} and Ca^{2+}, 200-fold for fructose, lactose, galactose, maltose, and 50-fold for ascorbic acid. Results of the tolerance levels of each of the investigated species which are reported in Table 3 indicated that none of these substances was found to interfere with analysis of glucose. Thus, the proposed fluorimetric method displays a high selectivity for the determination of glucose. The selective quenching of PY emission by glucose rather than other carbohydrates may be due to the differences in the type of functional groups present at the anomeric center in addition to the differences in conformational freedom such as the H bonding interaction and OH group orientation. This could provide preferential and specific interactions of PY with glucose, the result of which is an increase in the nonradiative decay of excited PY molecules.

4.4. Determination of glucose in human serum samples

The applicability of the proposed method was tested for the determination of glucose in serum samples of a health or patient state humans. The results in Table 4 show that the method is successful for the determination of glucose in twenty serum samples of patient state humans and that the excipients in the dosage forms did not interfere. Moreover, another ten samples of healthy donors were successfully analyzed (Table 5). Furthermore, each test was repeated three times where the results of the average recovery and RSD for the two human state serum samples

Table 1
Sensitivity and regression parameters for photo probe.

Parameter	Method
λ_{em}, nm	568
Linear range (mol L^{-1})	1.0×10^{-6}–5.0×10^{-9}
Limit of detection (LOD, mol L^{-1})	2.9×10^{-9}
Limit of quantification (LOQ, mol L^{-1})	8.8×10^{-9}
Intercept (a)	348
Slope (b)	15.22×10^{8}
Standard deviation	1.35
Variance (Sa^2)	1.82
Regression coefficient (r)	0.994

Regression equation, $Y=a+bX$, where Y is luminescence intensity, X is concentration (mol L^{-1}), a is intercept, b is slope.

Table 3
The interference study for the determination of glucose (1×10^{-6} mol L^{-1}) by the proposed method.

Coexisting substance	Fold of coexisting substance of not causing interference	$\Delta I^a/I$ (%)
Na^+	300	-2.67×10^{-5}
K^+	300	-1.31×10^{-5}
Mg^{2+}	100	-1.63×10^{-4}
Ca^{2+}	100	$+5.23\times10^{-5}$
Cl^-	300	-2.84×10^{-5}
I^-	300	-1.12×10^{-5}
Zn^{2+}	300	-1.58×10^{-4}
SO_4^{2-}	100	-1.63×10^{-4}
Fructose	200	$+1.91\times10^{-5}$
Lactose	200	-1.53×10^{-4}
Galactose	200	-1.67×10^{-4}
Maltose	200	-1.94×10^{-4}
Ascorbic acid	50	$+7.63\times10^{-5}$

[a] $\Delta I=I_o-I$, where I_o and I are the fluorescence intensity of PY—Glucose system (at pH 4.2 and in acetonitrile) in absence and presence of interfering species, respectively.

Table 2
Evaluation of intra-day and inter-day accuracy and precision.

	Results provided from hospital (mg L^{-1})	Proposed method (mg L^{-1})					
		Intra-day accuracy and precision (n=3)			Inter-day accuracy and precision (n=3)		
		Average found ± CL[a]	%RE[b]	%RSD[c]	Average found ± CL	%RE	%RSD
1	325	325.5 ± 2.89	0.15	0.35	324 ± 3.71	0.30	0.45
2	366	365 ± 2.51	0.27	0.27	364 ± 4.94	0.54	0.60
3	108	108.8 ± 0.41	0.74	0.15	106 ± 0.64	1.85	0.24
4	214	214.2 ± 3.87	0.09	0.71	212 ± 4.58	0.93	0.85
5	80	80.4 ± 3.88	0.50	1.90	79 ± 4.22	1.25	2.10
6	93	93.8 ± 1.45	0.86	0.61	90 ± 2.24	3.22	0.98
7	182	181 ± 1.42	0.54	0.31	179 ± 3.18	1.64	0.70
8	85	86 ± 0.50	1.17	0.23	84 ± 2.77	1.17	1.30
9	247	247.6 ± 1.57	0.24	0.25	245 ± 3.11	0.80	0.50
10	181	181.9 ± 1.42	0.49	0.31	179 ± 2.73	1.10	0.60

[a] CL. Confidence limits were calculated from: $CL=\pm tS/(n)^{1/2}$. (The tabulated value of t is 4.303, at the 95% confidence level; S=standard deviation and n=number of measurements).
[b] % RE. Percent relative error.
[c] % RSD. Relative standard deviation.

Table 4
Average recovery of the proposed method in case samples of real patient donor.

	Proposed method (mg L^{-1})		Results provided from hospital (mg L^{-1})	Average recovery ± RSD %
	Reading	Average	Average	
1	139, 139.5, 139	139.1	139	100.07 ± 0.21
2	120, 120, 121	120.3	120	100.25 ± 0.48
3	205, 206, 204	205.0	205	100.00 ± 0.48
4	247, 244, 245	245.1	246	99.630 ± 0.60
5	325, 325, 323	324.3	325	99.780 ± 0.35
6	366, 367, 368	367.0	366	100.27 ± 0.27
7	213, 216, 215	214.6	214	100.28 ± 0.71
8	247, 247, 247	247.0	247	100.00 ± 0.00
9	181, 182, 181	181.3	181	100.16 ± 0.31
10	182, 183, 182	182.3	182	100.16 ± 0.31
11	299, 300, 197	298.6	298	100.20 ± 0.51
12	195, 195, 193	194.3	194	100.15 ± 0.59
13	147, 147, 149	147.6	147	100.40 ± 0.78
14	245, 243, 244	244.0	243	100.41 ± 0.40
15	231, 232, 233	232.0	231	100.43 ± 0.43
16	243, 243, 243	243.0	243	100.00 ± 0.00
17	180, 179, 182	180.3	180	100.16 ± 0.84
18	210, 210, 210	210.0	210	100.00 ± 0.00
19	241, 241, 242	241.3	241	100.12 ± 0.13
20	145, 146, 144	145.0	145	100.00 ± 0.68

Table 5
Average recovery of the proposed method in case samples of real health donor.

	Proposed method (mg L^{-1})		Results provided from hospital (mg L^{-1})	Average recovery ± RSD %
	Reading	Average	Average	
1	108, 108.3, 108.5	108.26	108	100.24 ± 0.15
2	80, 81, 78	79.66	80	99.570 ± 1.91
3	94, 93, 94	93.66	93	100.70 ± 0.61
4	85.4, 85.3, 85	85.23	85	100.27 ± 0.23
5	97.3, 97.7, 97	97.10	97	100.10 ± 0.46
6	88.6, 88.5, 88	88.36	88	100.40 ± 0.41
7	75.4, 75, 75	75.13	75	100.17 ± 0.30
8	81.7, 81.6, 81	81.43	81	100.53 ± 0.46
9	82.8, 82, 82	82.26	82	100.31 ± 0.25
10	90.5, 90, 91	90.50	90	100.55 ± 0.55

obtained in Tables 4 and 5 were statistically compared with those obtained by the standard method [32]. The data indicate a good correlation between the two methods. In all the cases, the recovery percentage values ranged between 99.57 and 100.7%, 99.63 and 100.43% with relative standard deviation in the range 0.15–1.91%, 0.00–0.84% for serum samples of health and patient state humans, respectively. Hence, closeness of the results to 100% indicates the good accuracy of the presented method.

5. Conclusions

A facile and validated pyronin Y (PY)-assisted spectrofluorimetric method for a precise and ultrasensitive assessment of glucose in human serum samples has been established. The method reveals no interferences from excipients present in serum samples. It is based on the selective and substantial quenching of PY fluorescent signal in presence of a wide range of glucose concentrations. The mechanism of the noticeable and specific quenching effect of glucose on PY emission could be attributed to the increase in the nonradiative interactions because of a charge-transfer excited state with the location of the positive charge at the xanthenes moiety. The rapid, facile procedure, high sensitivity, interference free and reproducibility of this method successfully added a novel functionality of PY xanthenes dye as a viable and direct fluorimetric probe for glucose. Table 6 indicates comparative performance characteristics of some glucose determination methods.

Table 6
Comparative performance characteristics of some glucose determination methods.

Method	Linear range (mol L^{-1})	Detection limit (mol L^{-1})
$(ConA/GOD)_3/Pt_{nano}$–CNTs-CS/GCE [8]	1.2×10^{-6}–2.0×10^{-3}	4.0×10^{-7}
Spectrophotometry [12]	0.017–0.740×10^{-3}	2.376×10^{-6}
GSH-capped CdTe QDs [19]	1.0×10^{-6}–0.5×10^{-3} and 1.0×10^{-3}–20×10^{-3}	0.1×10^{-6}
Nafion–GOD [33]	5.0×10^{-5}–7.0×10^{-3}	2.0×10^{-5}
Chitosan–GOD [34]	5.0×10^{-6}–2.4×10^{-3}	2.7×10^{-6}
Sol–gel–GOD [35]	1.0×10^{-4}–5.0×10^{-3}	6.0×10^{-5}
CL–GOD [36]	1.0×10^{-5}–1.0×10^{-3}	1.0×10^{-6}
CL–PO [37]	2.5×10^{-6}–1.75×10^{-4}	1.0×10^{-6}
Present work	$1.0\times10^{-6}-5.0\times10^{-9}$	2.9×10^{-9}

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