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Research article

The study of phenylboronic acid optical properties towards creation of a glucose sensor

Katarzyna Kur-Kowalska, Małgorzata Przybyt, Ewa Miller*

Lodz University of Technology, Institute of General Food Chemistry, Stefanowskiego 4/10, 90-924 Lodz, Poland

* corresponding ewa.miller@p.lodz.pl

Abstract: *The article presents influence of pH and glucose concentration on phenylboronic acid (PBA) fluorescence studied by steady-state and time-resolved measurements. Fluorescence of PBA decreases with growing pH. These changes reflected acid-base equilibrium of PBA and allowed to estimate value of* pK_d *as 9.2, which is comparable with literature data. Fluorescence intensity of phenylboronic acid is quenched in presence of glucose. The effect of quenching is more pronounced with increasing pH. At pH 7 quenching can be described by Stern-Volmer equation, at pH 8 and 9 by modified one. The obtained quenching constants are growing with pH increase. The quenching of phenylboronic acid fluorescence by glucose is a static one, which is confirmed by time-resolved measurements. Two lifetimes were found for fluorescence decay of phenylboronic acid. The lifetimes are practically independent on pH and glucose concentration and also fraction of both lifetimes are nearly the same. The obtained Stern-Volmer constants can be interpreted as apparent equilibrium constants of ester formation between acid and glucose.*

Keywords: *boronic acid derivatives, glucose sensor, optical sensor, fluorescence quenching.*

Introduction

Glucose sensors and biosensors are the most popular ones because of their very broad application area. Most of them are optical or electrochemical ones. Compared to traditional methods of analysis they show a number of advantages: short time and simplicity of measurement, high stability and ability of continuous measurement. Depending on their purpose and construction, they consist of one or several components and can exist as electrodes [1,2] or chips [3]. Due to these advantages, they are used in many areas, especially in medicine. In diabetes care, they are used for the determination of glucose concentration in blood [4,5] as well as in other body fluids [6,7].

Boronic acid derivatives are the subject of interest of many research centers because of their characteristic property - binding diols with high affinity through reversible ester formation with boronic group. Phenylboronic acid and its

derivatives show fluorescence which can be attenuated by sugar binding giving as the result chemical sensor for glucose or other saccharides [8-12]. Such system can be composed from one or several components [8,13,14] and can be characterized by the increase or decrease of fluorescence emission intensity. For example, boronic acid electrostatically bound to cysteine modified gold surface showed a decrease of fluorescence emission at the presence of glucose [15]. Another derivative – 8-quinolineboronic acid at the presence of sugars (fructose, galactose, and arabinose) showed a significant increase of fluorescence emission intensity [16]. Application of boronic acid derivatives as a sensing element could allow constructing a non-invasive sensor, which could be included in contact lens to measure glucose level in tears [17]. Generally, many attempts were done to synthesize sophisticated phenylboronic acid derivatives with increased sensitivity for sugars and improved selectivity for glucose [17-19] but there is little or no data in literature about fluorescence properties of the simple phenylboronic acid and its esters with saccharides.

The aim of this paper is characterization of interactions of the simplest fluorescent boronic acid derivative – phenylboronic acid with glucose at different pHs in aspect of further application in glucose sensing.

Experimental

Phenylboronic acid – ($C_6H_4B(OH)_2 \cdot H_2O$, 95%) was purchased from SIGMA-ALDRICH (Germany). Glucose (anhydrous pure p.a.) was purchased from Chempur (Poland). All other reagents used were of most possible purity. Distilled water was used throughout.

Absorbance spectra were collected using a Nicolet Evolution 300 spectrophotometer (Thermo Scientific, USA).

Steady-state fluorescence measurements were performed using a Fluoromax-4 spectrofluorometer (Jobin Yvon-Spex Instruments S.A., Edison, New Jersey, USA). Fluorescence spectra were measured with 10 mm path-length closed quartz cells. The excitation and emission slits were set at 5 nm each. The increment was set at 1 nm and integration time at 0.5 second. The measurements were carried out at room temperature.

Fluorescence emission decays were measured with a time-correlated single photon counting apparatus from Edinburgh Instruments Co (UK), equipped with hydrogen lamp (nF900 Nanosecond Flashlamp) as an excitation light source. The instrument profile was obtained by replacing the sample with Ludox as a scatter. Data were collected in 1023 channels to 1000 counts in the peak, and the calibration time was 53 ps per channel. The data were analyzed by a least-squares reconvolution procedure using the software package provided by the Edinburgh Instruments.

For basic optical characteristics, PBA was dissolved in 0.01 mol/L phosphate buffer, pH 7. The pH profile of PBA fluorescence was studied at pH ranging from 4 to 10. At pH from 4 to 5 acetate buffers were used, at higher pHs

– phosphate ones. For fluorimetric titration of PBA with glucose 3 mL of $5 \cdot 10^{-4}$ mol/L PBA solution was poured into quartz cuvette and 3 µL of 1 mol/L glucose solution was added. The cuvette was shaken for 1 minute and after that, fluorescence emission spectrum was measured at excitation wavelength 260 nm. Such procedure was repeated 10 times to obtain final concentration of glucose in sample 10^{-2} mol/L. Time-resolved measurements were made for PBA concentration 10^{-3} mol/L. Glucose concentration was varying from 0 to $2 \cdot 10^{-2}$ mol/L. All experiments were done at room temperature.

Results and discussion

Optical characteristics of phenylboronic acid

Phenylboronic acid shows absorbance in UV range with characteristic vibrational structure [20]. The absorbance spectra differ slightly with concentration of PBA (results not shown). For low concentrations ($5 \cdot 10^{-3}$ mol/L and below) the last maximum is settled at 266 nm, for higher at 260 nm. The obtained result is consistent with data found in literature [20].

Fluorescence emission spectra were measured at excitation wavelength 266 nm in concentration range from 10^{-6} to $5 \cdot 10^{-2}$ mol/L to find the limiting concentration for auto-quenching effect. PBA in phosphate buffer shows one sharp and smooth emission band with maximum located at 296 nm, which is similar to results reported in literature [21]. Maximum emission was observed for PBA concentration 10^{-3} mol/L; for higher concentrations, the auto-quenching effect was observed.

The influence of pH on PBA optical properties is shown on Figure 1. The absorbance of PBA measured at 266 nm practically does not depend on pH up to 7.5; for higher pHs is decreasing gradually (Fig. 1C). Springsteen and Wang [22] obtained similar results. They estimated the pK_d of phenylboronic acid as 8.8 [22,23]. Other boronic acid derivatives like 8–quinolineboronic acid showed similar absorbance dependence on pH [16]. Fluorescence pH profile for PBA is similar for that obtained for absorbance (Fig. 1B) but more expressed. Rough estimation of obtained fluorescence results gave value about 9.2, which is similar. To obtain more accurate results more measurements must be done (in broader pH range). Examples from literature [16,22,23] indicate that absorbance of various derivatives of boronic acid have similar pH profile connected with acid-base dissociation equilibrium of borate group (Fig. 2). As it can be seen from scheme shown on Figure 2, there are two forms of phenylboronic acid (neutral and anionic) in the solution. At higher pH (above neutral) the anionic form is predominant. Boronic acid derivatives are the Lewis acids and therefore, they can connect OH^- ion from the solution. In neutral form, the boron atom is in trigonal sp^2 hybridization and is electron-deficient Lewis acid. At alkaline pH electron-rich Lewis base is formed and boron atom is in tetrahedral sp^3 hybridization [7,13]. The equilibrium between these two forms depends on pH. The neutral form is more fluorescent than anionic one. The trigonal form of

boronic acid binds diols (among them glucose) much less than tetrahedral one [24]. Binding of diols is shifting apparent acid-base dissociation constant to lower pH [22]. For glucose ester of phenylboronic acid pK_d is 6.8 [22].

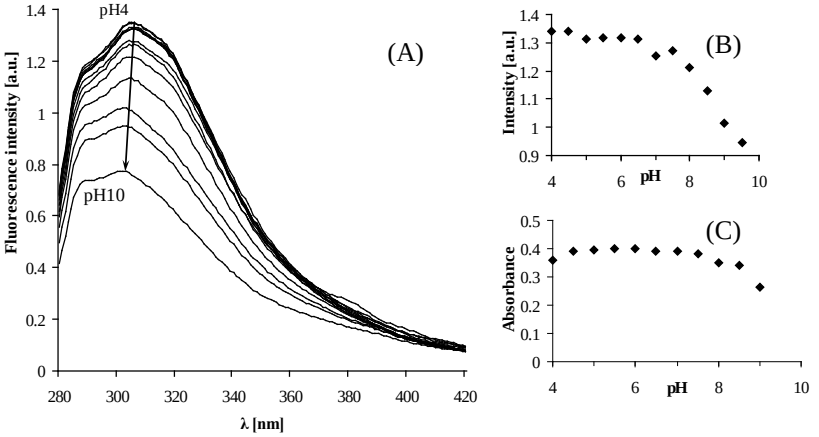

Figure 1. A – Fluorescence emission spectra of phenylboronic acid ($5 \cdot 10^{-4}$ mol/L) at different pHs; λ_{exc} = 266 nm; B – Fluorescence intensity at maximum (λ_{em} = 303 nm) of phenylboronic acid ($5 \cdot 10^{-4}$ mol/L) as a function of pH ; C – Absorbance at 266 nm of phenylboronic acid (10^{-3} mol/L) as a function of pH

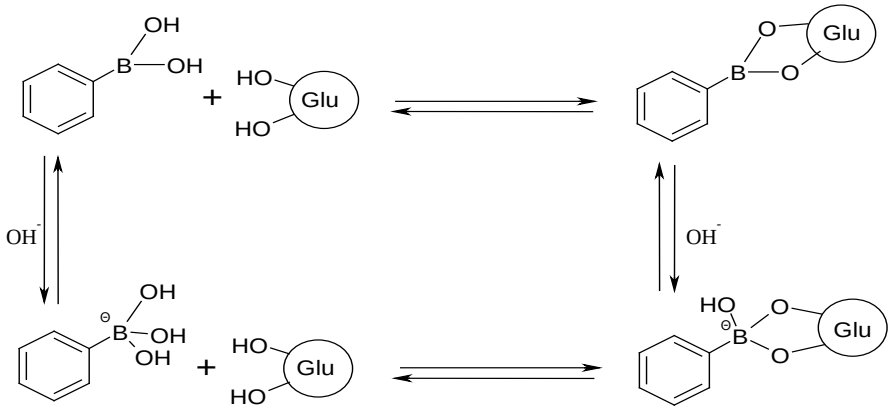

Figure 2. Acid-base and glucose binding equilibria of phenylboronic acid

Interaction of PBA with glucose

Boronic acids are known to bind diols with high affinity. Between acid and diol (like saccharides) an ester bond is formed. Formation of ester bond is reversible (Fig. 2). This reaction is characteristic for all boronic acid derivatives, and makes them the object of study on construction of an optical glucose sensor, because formation of ester causes the changes of phenyl boronic acid derivatives

fluorescence [7,17-19]. Boronic acid derivatives anion in sp^3 hybridization better binds sugar molecules as compared with neutral form [24]. Binding of sugar is causing the decrease of fluorescence intensity of phenylboronic acid [21]. As the apparent binding constant of sugars with PBA is growing with increasing pH, the quenching effect should also be more expressed [23].

Quenching of PBA fluorescence intensity by glucose was studied by fluorimetric titration at pH 7, 8 and 9 as described in experimental part. Results for pH 7 are shown on Figure 3A. One can see that with increasing concentration of glucose emission of fluorescence is decreasing. The effect is more pronounced with increasing pH. In the presence of glucose, the emission maximum is shifted by 2-4 nm to lower wavelengths and the shape of emission spectrum is changed (Fig. 3B).

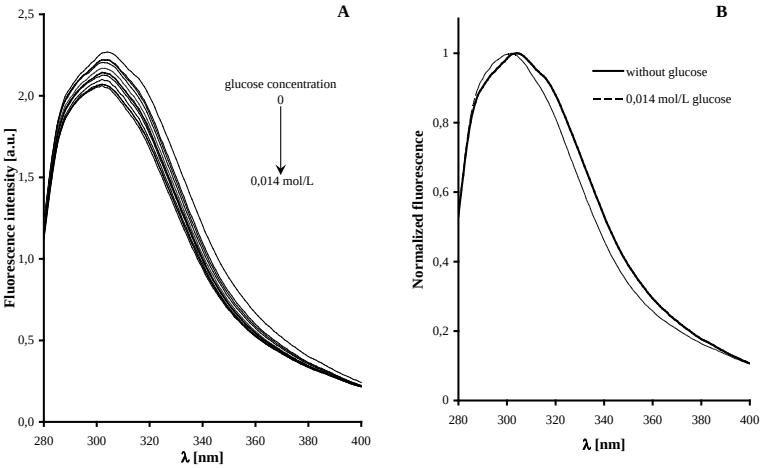

Figure 3. A – Fluorescence titration traces of PBA solution ($5 \cdot 10^{-4}$ mol/L) at pH 7 by glucose; $\lambda_{exc} = 260$ nm; B – normalized fluorescence emission spectra of PBA in the absence and presence of glucose at pH 7

Because of the quenching effect of glucose on PBA emission intensity, it was tried to check if the obtained data fit to Stern-Volmer equation:

$$\frac{F_o}{F} = 1 + K_{SV}[Q] \tag{1}$$

where: F_o – initial fluorescence intensity at the absence of a quencher,
F – fluorescence intensity at the presence of a quencher,
K_{SV} – Stern-Volmer constant,
$[Q]$ – quencher concentration.

Stern-Volmer equation is describing dynamic (collisional) quenching. In case of static quenching, when fluorophore and quencher form non-fluorescent complex in ground state, derivation is leading to the same form of equation but the constant denoted as K_S has the meaning of equilibrium constant of complex

formation [25]. One can suspect that as PBA and glucose form ester less fluorescent than free acid the results of quenching experiments could be presented in Stern-Volmer coordinates [25].

Because binding of glucose caused changes of shape PBA emission spectrum for presentation data in Stern-Volmer coordinates integrated (in range 280 – 350 nm) fluorescence intensities were taken into account. As it is shown on Figure 4A the obtained results did not fit to Stern-Volmer coordinates. The plots of F_o/F against glucose concentration are underlinear. Therefore, a modified Stern-Volmer equation was applied. This equation is describing the case when only a part of fluorophore molecules is accessible for quenching. There is an assumption that in the solution there are two populations of fluorophore. One of them is available (*a*) for the quencher while the other is unavailable or buried (*b*) [25].

$$\frac{F_o}{\Delta F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \tag{2}$$

$$\Delta F = F_o - F \tag{3}$$

where: K_a is the Stern-Volmer quenching constant of the accessible fraction

f_a is the fraction of initial fluorescence which is accessible to quencher

$$f_a = \frac{F_{oa}}{F_{oa} + F_{ob}} \tag{4}$$

F_{oa} and F_{ob} are the initial fluorescence intensities of the fraction *a* and *b.*

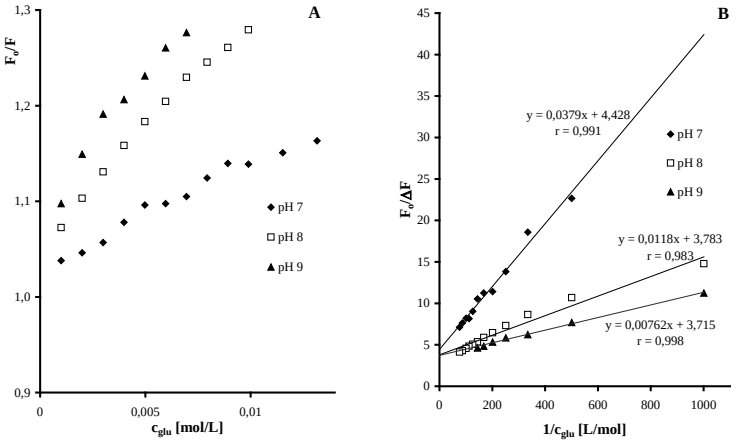

Figure 4. Application of two models of quenching to results of fluorimetric titration of BPA by glucose: A – Stern-Volmer coordinates (1); B – modified Stern-Volmer equation (2)

On Figure 4B, one can see that the results of fluorimetric titration of PBA with glucose can be fitted to the modified Stern-Volmer equation. The obtained K_a constants values are 117 [L/mol], 320 [L/mol] and 488 [L/mol] at pH 7, 8 and

9 respectively and f_a is about 0.23, 0.26 and 0.27. In this case, K_a can be interpreted as apparent equilibrium constant of ester formation and f_a as the fraction of anionic form. The obtained results are quite reasonable as with increasing pH the amount of anionic form is increasing. The scheme of PBA acid –base equilibrium shown on Figure 2 indicates that in the solution there are two forms (fractions) of PBA, and that in the alkaline solution the anion, in which boron atom is in sp^3 form, is predominant. This form binds diol much stronger than neutral form of acid [24]. The obtained constants are quite reasonable and can be compared with equilibrium constant for ester formation between glucose and PBA anion [24].

Time-resolved measurements

There is no data in the literature about fluorescence decay of phenylboronic acid therefore it was interesting to make time-resolved measurements for free acid and for its ester with glucose. Example of obtained results is shown at Figure 9. One can see that there is very little difference between decay at the absence and presence of glucose. Due to apparatus limitation, the measurements could be done up to 1000 counts in a peak. As the result, calculated lifetimes are not very precise and the statistics is quite poor.

The best fit to obtained fluorescence decays was double exponential in case of all studied pHs and glucose concentrations. The obtained lifetimes are collected in Tables 1, 2 and 3. In all tables, $\boldsymbol{\tau_i}$ is lifetime, $\mathbf{f_i}$ is fraction of given lifetime and $\boldsymbol{<\tau>}$ - mean lifetime calculated according to the formula:

$$< \tau >= \Sigma f_i \cdot \tau_i \tag{5}$$

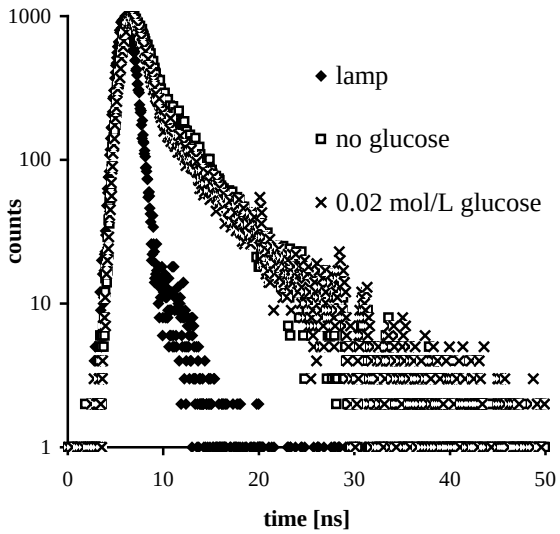

Figure 5. Example of fluorescence decay of phenylboronic acid; pH 7; PBA concentration 10^{-3} mol/L; λ_{exc} = 275 nm

Table 1. Parameters describing fluorescence decay for PBA at pH 7

c_{glu}, mol/L	τ_1, ns	f_1	τ_2, ns	f_2	χ^2	$<\tau>$, ns
0.000	0.81 ± 0.02	0.57	5.52 ± 0.07	0.43	1.26	2.84 ± 0.07
0.002	0.81 ± 0.02	0.57	5.50 ± 0.06	0.43	1.23	2.84 ± 0.07
0.005	0.87 ± 0.02	0.57	5.46 ± 0.07	0.43	1.17	2.85 ± 0.07
0.009	0.97 ± 0.02	0.55	5.34 ± 0.06	0.45	1.22	2.95 ± 0.07
0.012	1.13 ± 0.03	0.55	5.26 ± 0.07	0.45	1.05	3.00 ± 0.07
0.015	1.18 ± 0.03	0.52	5.08 ± 0.07	0.48	1.08	3.06 ± 0.07
0.018	1.14 ± 0.03	0.50	4.97 ± 0.06	0.50	1.03	3.05 ± 0.07
0.022	1.14 ± 0.03	0.49	4.80 ± 0.06	0.51	1.07	3.00 ± 0.07

Table 2. Parameters describing fluorescence decay for PBA at pH 8

c_{glu}, mol/L	τ_1, ns	f_1	τ_2, ns	f_2	χ^2	$<\tau>$, ns
0.000	0.60 ± 0.02	0.51	5.58 ± 0.05	0.49	1.68	3.06 ± 0,06
0.002	0.62 ± 0.02	0.51	5.54 ± 0.05	0.49	1.74	3.05 ± 0,06
0.005	0.67 ± 0.02	0.52	5.64 ± 0.06	0.48	1.53	3.03 ± 0,06
0.009	0.64 ± 0.02	0.50	5.60 ± 0.05	0.50	1.57	3.11 ± 0,06
0.012	0.62 ± 0.02	0.50	5.50 ± 0.05	0.50	1.66	3.04 ± 0,06
0.015	0.64 ± 0.02	0.50	5.49 ± 0.05	0.50	1.52	3.05 ± 0,06
0.018	0.64 ± 0.02	0.51	5.45 ± 0.05	0.49	1.51	2.99 ± 0,06
0.022	0.65 ± 0.02	0.51	5.49 ± 0.05	0.49	1.61	3.04 ± 0,06

Table 3. Parameters describing fluorescence decay for PBA at pH 9

c_{glu}, mol/L	τ_1, ns	f_1	τ_2, ns	f_2	χ^2	$<\tau>$, ns
0.000	0.64 ± 0.02	0.52	5.64 ± 0.06	0.48	1.53	3.06 ± 0.06
0.002	0.64 ± 0.02	0.51	5.61 ± 0.06	0.49	1.49	3.07 ± 0.06
0.005	0.70 ± 0.02	0.50	5.33 ± 0.05	0.50	1.38	3.01 ± 0.06
0.009	0.74 ± 0.02	0.48	5.12 ± 0.05	0.52	1.42	3.02 ± 0.06
0.012	0.85 ± 0.03	0.47	5.10 ± 0.05	0.53	1.28	3.09 ± 0.06
0.015	0.82 ± 0.03	0.45	4.79 ± 0.05	0.55	1.20	2.99 ± 0.06
0.018	0.89 ± 0.03	0.44	4.63 ± 0.05	0.56	1.11	2.97 ± 0.06
0.022	0.93 ± 0.03	0.42	4.47 ± 0.05	0.58	1.24	2.99 ± 0.06

Phenyl boronic acid shows two fluorescence lifetimes, the first $\boldsymbol{\tau_1}$ less or equal about 1 ns and second $\boldsymbol{\tau_2}$ longer varying in range from 4.6 to 5.6 ns. The fraction of each time is near to 0.5. The influence of pH and glucose concentration on fluorescence lifetimes is weak. At pH 7 one can observe that $\boldsymbol{\tau_1}$ at the absence of glucose is a bit greater as at pH 8 and 9, and $\boldsymbol{\tau_2}$ nearly the same at all pHs. At pH 7 the first lifetime is increasing with increase of glucose concentration and the second one is decreasing. Simultaneously the fraction of $\boldsymbol{\tau_1}$ is decreasing. As the result, the mean lifetime calculated from equation (5) is slightly increasing. At pH 9, the direction of changes of individual lifetimes is the same as at pH 7 but the mean time is practically the same. At pH 8, no influence of glucose concentration on PBA fluorescence decay is observed. The changes of $\boldsymbol{\tau_1}$ and $\boldsymbol{\tau_2}$ are so weak that they suggest that pH and presence of glucose at studied range of

concentration has no influence on PBA fluorescence decay. This is rational suggestion from the point of view that PBA quenching by glucose is caused by ester formation so it is a static one.

Conclusions

Phenylboronic acid is a weakly fluorescing compound. With the increase of pH its fluorescence decreases. These changes are connected with acid-base equilibrium. From fluorescence-pH profile value of pK_d was estimated as 9.2 which is comparable with values obtained by different methods [22,23]. Fluorescence intensity of phenylboronic acid is quenched by the presence of glucose. The effect of quenching is more pronounced with increasing pH. Quenching in this case can be described by modified Stern-Volmer equation and obtained quenching constants are interpreted as apparent equilibrium constants of estrification. Quenching of phenylboronic acid by glucose is caused by ester formation between boronic moiety and diol (glucose). This reaction is more effective at higher pH when boronic anion is formed and boron is in sp^3 hybridization. Due to steric fit, the ester formation equilibrium constant for sp^3 form is much greater than for sp^2. Therefore, the apparent equilibrium constant would increase with growing pH, which was confirmed by experiment. The obtained results indicate that acid-base and estrification equilibria can be studied by fluorescence measurements giving results comparable with those obtained by absorbance measurements. Time-resolved measurements allowed to find lifetimes of PBA. Two lifetimes were found for fluorescence decay of phenylboronic acid. The lifetimes are practically independent on pH and glucose concentration and also fraction of both times are nearly the same.

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