БІОФІЗИКА КЛІТИНИ

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NEUTRAL RED PROTONATION BEHAVIOR IN THE COURSE OF ENZYMATIC GLUCOSE OXIDATION IN AQUEOUS AND LIPOSOME MEDIA

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Kinetic analysis of glucose oxidation by glucose oxidase has been performed with a view to gain new information on the catalytic properties of the enzyme adsorbed onto a surface of lipid vesicles. The course of enzymatic reaction has been monitored spectrophotometrically with the pH-indicator neutral red. Neutral, positively and negatively charged lipid vesicles have been prepared from egg phosphatidylcholine (PC), or its mixtures with cetyltrimethylammonium bromide (CTAB) (5 mol %) or cardiolipin (CL) (5 mol %). The apparent dissociation constant of neutral red in these systems in the presence of glucose oxidase has been found. The kinetic parameters of the glucose oxidase interaction with glucose and dioxygen have been found to remain unchanged on enzyme association with liposomes.

KEY WORDS: glucose oxidation, kinetic analysis, neutral red, apparent ionization constant, absorbance-based optical biosensor.

Recently, much effort has been devoted to designing the optical biosensors [1]. One important line of optical biosensor development involves the glucose sensing, and glucose oxidase (GOx) is still receiving considerable attention as a recognition biological element of glucose biosensors [2, 3]. Among various optical biosensors, the simplest seem to be absorbance-based sensors, whose operation is based on measuring the changes in the absorbance of a dye sensitive to a certain biochemical reaction, particularly, to the reaction of glucose oxidation. The organic dye suitable for designing such a glucose sensor is neutral red [4].The shift in equilibrium between deprotonated forms (NR) and protonated (NRH^+) of this dye can be monitored by the absorbance measurements, because the two forms of monomeric neutral red exhibit distinct absorption maxima (450 and 535 nm, respectively) [5].

Immobilization is the key factor in designing a biosensor. Among the various enzyme immobilization protocols, the self-assembled lipid structures such as lipid vesicles or liposomes, are particularly attractive [6, 7]. Liposome can provide a flexible, membrane-like environment allowing the activity of the surface-bound or bilayer-incorporated enzyme to be retained at a high level. The affinity of liposomal membranes for various agents can be modified within a wide range by varying the bilayer composition to prevent undesirable chemical interference [8]. Polymerized phospholipid vesicles contained within gel beads has been reported as a novel immobilization support [9]. Liposomes have long been used as a diffusion limiting membrane for glucose to increase the linear range of enzyme to a level of clinical use [10]. Recently, it was demonstrated that the glucose oxidase-containing liposomes immobilized in solid supports provide a highly stable biocatalysis [11].

According to the second step of dye screening [4], in present study the dye absorbance properties in PC, PC/CTAB or PC/CL liposomal dispersions containing GOx have been compared, with emphasis on the applicability of liposomes of different composition to designing an absorbance-based glucose biosensor.

MATERIALS AND METHODS

Glucose oxidase from *Aspergillus niger* (185 units/mg) was purchased from Fluka (Germany). Neutral red was obtained from Reakhim (Russia). Glucose and cetyltrimethylammonium bromide (CTAB) were from Sigma (Germany). Egg yolk phosphatidylcholine (EPC) and cardiolipin (CL) were from Biolek (Kharkov, Ukraine).

The measurements were performed in 5 mM sodium-phosphate (Na_2HPO_4 and NaH_2PO_4) buffer, pH 6.8 or 7.4, at room temperature. Unilamerllar lipid vesicles were prepared by the ethanol injection [12] or extrusion [13] methods. FAD and neutral red (both NRH^+ and NR forms) concentration was determined spectrophotometrically, using extinction coefficients $\varepsilon_{450} = 1.4 \cdot 10^4 M^{-1} cm^{-1}$, $\varepsilon_{525}^{NRH^+} = 2.64 \cdot 10^4 M^{-1} cm^{-1}$ and $\varepsilon_{525}^{NR} = 2.4 \cdot 10^3 \ M^{-1} cm^{-1}$, respectively. A stock glucose solution (300 mM) was left to mutarotate overnight. The absorbance measurements were carried out at 20°C with SF-46 spectrophotometer at a wavelength of 525 nm.

RESULTS AND DISCUSSION

The apparent ionization constant of neutral red can be represented by:

$$K_a^a = \frac{[H^+][NR]}{[NRH^+]}; \quad pK_a^a = pH + \log\left(\frac{\varepsilon_{525}^{NR} \cdot D_0 \cdot l - A_{525}}{A_{525} - \varepsilon_{525}^{NRH^+} \cdot D_0 \cdot l}\right) \tag{1}$$

where $[H^+]$,$[NR]$ and $[NRH^+]$ denote the concentration of proton, NR, and NRH^+, respectively. A_{525} is the absorbance of neutral red measured spectrophotometrically at 525 nm at a given pH, $D_0 = [NR] + [NRH^+]$, is the total concentration of neutral red, ε_{525}^{NR} and $\varepsilon_{525}^{NRH^+}$ have been mentioned in the second section. l is the optical path length (1 cm).

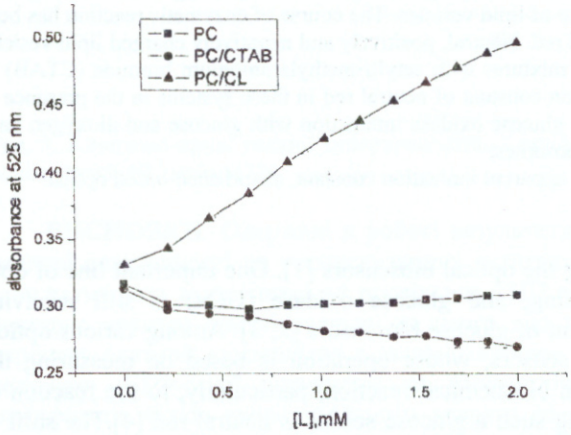

Fig.1. Titration of neutral red with PC, PC/CTAB and PC/CL liposomal suspensions.

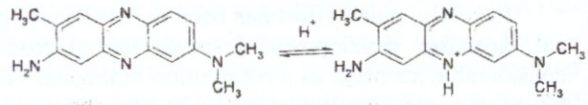

Fig.2. Acid-base equilibrium of neutral red.

Positive charge of the NR protonated form favors its association with GOx, bearing net negative charge at neutral pH (isoelectric point of GOx is 4.2), and NR binding to protein brings about significant increase of the dye absorbance (20-30%) [4]. This indicates that the pK_a^a values of neutral red in GOx solution are higher than the value of pK_a^w (in water). As illustrated in Fig.1, the neutral red absorbance decreases slightly in the zwitterionic (PC) and positively charged liposome (PC/CTAB) suspension, and increases in the negatively charged liposome (PC/CL) medium. This means that the pK_a^a values in various liposome media are also different from that in water similar to the situation in GOx solution. At the same time, in the case of dye titration by zwitterionic (PC) liposomes, the small drop of A_{525} values is more likely attributable to the poor stability of the dye, and the pK_a^a may be quite close to the value of pK_a^w. Two possible explanations of the observed effect seem to be as follows. The first is that the two forms of neutral red have very close affinities upon non-polar interactions with PC bilayers. The second is the lack of liposome-dye interactions. Unlike zwitterionic liposome, in positively charged liposome suspension, the relatively higher extent of A_{525} decrease values indicates a decrease of pK_a^a value suggesting the affinity of NR to lipid bilayer to be higher than that of the NRH^+ form. Meanwhile, in negatively charged liposome media, relatively higher affinity of positively charged protonated form of neutral red to phospholipid vesicles is likely to increase the pK_a^a value with respect to pK_a^w, which results in drastic absorbance increase.

While glucose was added, increasing absorbance shows that deprotonated form of neutral red (NR) is changed to the protonated form (NRH^+) (Fig.2). The kinetics of the absorbance changes was followed by measuring the dye absorbance at the wavelength of 525 nm (Fig. 3). Under aerobic conditions, when oxygen consumption in solution is followed by its uptake from air, the following reactions proceed in the system under study:

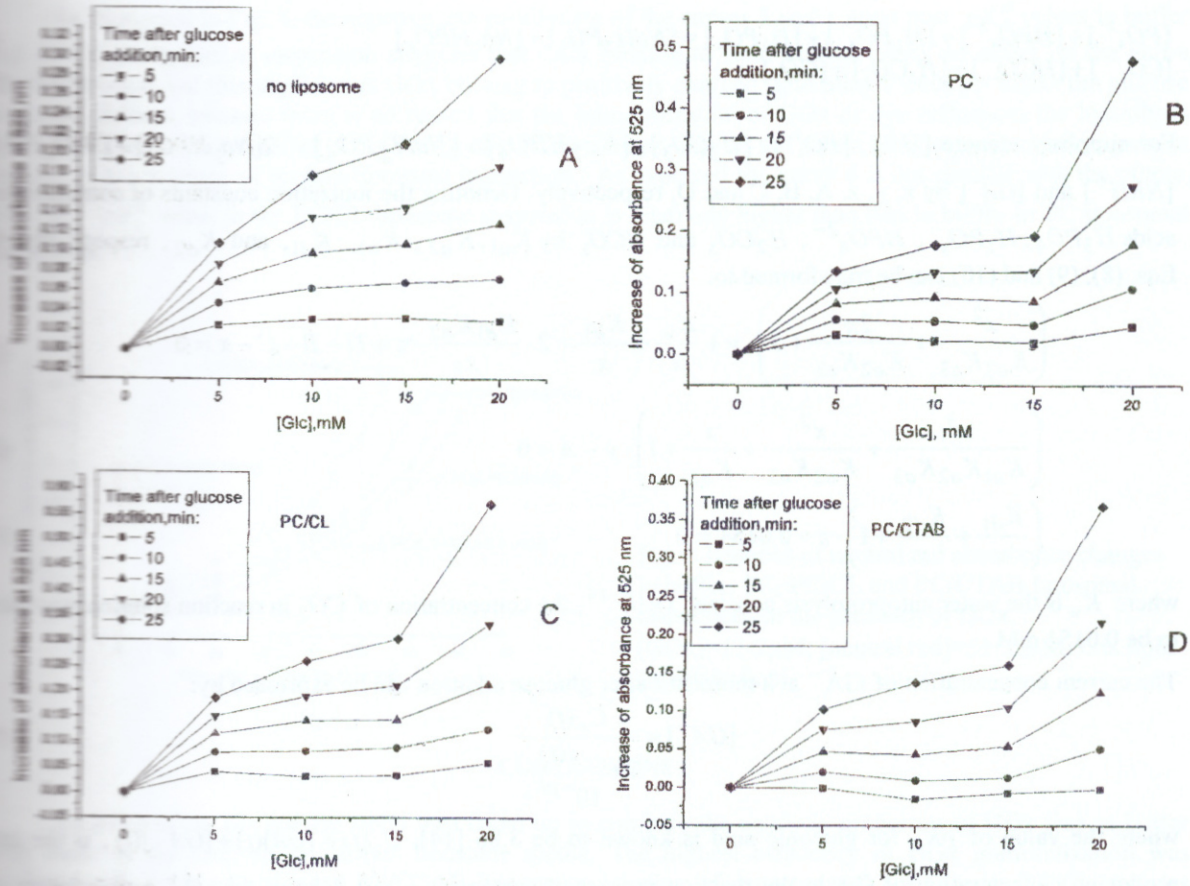

Fig.3 Absorbance changes of neutral red detected at varying glucose level in A) buffer, B) PC ([PC]=0.28mM), C) PC/CL([PC/CL] =0.28mM.) and D) PC/CTAB ([PC/CTAB]=0.11mM) liposomal dispersions and [GOx]=11.2μM, [neutral red]=51.35μM.

$$GOx - FAD + Glucose \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} GOx - FAD - Glucose \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} GOx - FADH_2 + Gluconolactone \tag{2}$$

$$GOx - FADH_2 + O_2 \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} GOx - FAD + H_2O_2 \tag{3}$$

$$Gluconolactone + H_2O \overset{k_4}{\leftrightarrow} Gluconic\ Acid\ (GA) \tag{4}$$

$$GH \overset{k_a}{\leftrightarrow} G^- + H^+ \tag{5}$$

$$NR + H^+ \overset{k_a}{\leftrightarrow} NRH^+ \tag{6}$$

$$O_2\ uptake\ from\ air\ (k_5) \tag{7}$$

During the reductive half-reaction (2) two electrons and two protons are transferred to FAD, resulting in the conversion of the yellow oxidized flavin from ($\lambda_{max} = 450$ nm) to colorless reduced form $FADH_2$ and glucose oxidation to gluconolactone. Then $FADH_2$ is regenerated (oxidized) by O_2 (reaction 3), which is simultaneously reduced to hydrogen peroxide. After the gluconolactone hydrolysis (reaction 4), gluconic acid dissociates yielding free protons, which protonate the NR to NRH^+ and induce the absorbance increase. To determine the effective kinetic constants of reactions (2), (3) and (5), the analytical process is shown below.

Given the material balance and charge balance equations the following relationships hold:

$$[H_2PO_4^-] + 2[HPO_4^{2-}] + 3[PO_4^{3-}] + [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] + [GA^-] =$$
$$= 2[Na_2HPO_4] + [NaH_2PO_4] + [H^+] + [NRH^+] \tag{8}$$

$$[PO_4^{3-}]+[HPO_4^{2-}]+[H_2PO_4^-]+[H_3PO_4]=[NaH_2PO_4]+[Na_2HPO_4]$$
$$[CO_3^{2-}]+[HCO_3^-]+[H_2CO_3]=[CO_2]$$

For simplisty, denote $[H^+]$, $[PO_4^{3-}]$, $[H_2CO_3]$, $[Na_2HPO_4]+[NaH_2PO_4]$, $2[Na_2HPO_4]+[NaH_2PO$ $[NRH^+]$ and $[GA^-]$ by x, y, z, A, B, C and D, respectively. Denoting the ionization constants of correspond acids H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, H_2CO_3 and HCO_3^- by K_{a1}, K_{a2}, K_{a3}, K_{d1}, and K_{d2}, respectively, Eqs. (8), (9) and (10) can be transformed to:

$$\left(\frac{x^2}{K_{a2}K_{a3}}+\frac{2x}{K_{a2}K_{a3}}+3\right)\cdot y+\frac{K_w}{x}+\frac{K_{d1}}{x}+2\cdot\frac{K_{d1}K_{d2}}{x}\cdot z+D-B-C-x=0$$

$$\left(\frac{x^3}{K_{a1}K_{a2}K_{a3}}+\frac{x^2}{K_{a2}K_{a3}}+\frac{x}{K_{a3}}+1\right)\cdot y-A=0$$

$$\left(\frac{K_{d1}}{x}+\frac{K_{d2}}{x}+1\right)\cdot z-0.0154=0$$

where K_w is the water autoprotolysis constant, 1×10^{-14}, the concentration of CO_2 in reaction solution was tak to be 0.0154 mM.

The current concentration of GA^- at a moment t after glucose addition can be expressed by:

$$[GA^-]=\frac{C_v(t)}{1+\frac{x(t)}{10^{-pK_a}}}$$

where the value of pK_a for gluconic acid is known to be 3.86 [14], $C_v(t)=[GA](t)+[GA^-](t)$, is the to producing concentration of GA in the reaction process at a moment t, $x(t)$ denotes the H^+ concentration moment t.

Analogously, the concentration of NRH^+ (t) can be given by:

$$[NRH^+]=\frac{D_o\cdot x(t)}{x(t)+10^{-pK_a^a}}$$

It is noteworthy that the systems under study have somewhat different pK_a^a, the values of which in PC/CTA GOx, GOx, PC-GOx and PC/CL-GOx reaction systems were estimated to range from 7.04 to 7.40.

By combining of Eqs. (11)-(15), and substituting $x(t)$ by 10^r (r denotes "-pH"), a function of $pH(t)$ and C_v can be written by:

$$f(r,C_v)=\left(\frac{10^{3r}}{K_{a1}K_{a2}K_{a3}}+\frac{10^{2r}}{K_{a2}K_{a3}}+\frac{10^r}{K_{a3}}+1\right)\left\{\frac{K_{a2}K_{a3}\left[10^{2r}+B10^r-K_w-(K_{d1}+2K_{d1}K_{d2})\frac{0.0154\cdot10^r}{K_{d1}+K_{d1}K_{d2}+10^r}-\frac{K_aC_v10^r}{K_a+10^r}+\frac{D_010^{2r}}{K_a^a+10^r}\right]}{10^{3r}+2\cdot10^r+3\cdot10^r\cdot K_{a2}K_{a3}}-A\right\}$$

$C_v(t)$ can be obtained from numerical solution of the differential equations (data not shown) corresponding the reactions (2)–(7). The root of the Eq. (16) yields the value of r, i.e. the time dependency of the react medium pH related to that of A_{525} through Eq. (15).

The above model was employed to recover effective kinetic constants for GOx reaction in buffer a liposome media. The Michaelis constant K_{MS} for glucose and effective kinetic constant k_2 and k_5 were taken 30 mM [16], 800 s^{-1} [17] and $2.1\cdot10^{-3}$ s^{-1} [15], respectively. In our systems the kinetic constants k_1, k_3 and were found to be ca. $(1.2\pm0.7)\cdot10^6M^{-1}s^{-1}$, $(1.9\pm0.6)\cdot10^{-6}M^{-1}s^{-1}$, and $(9.6\pm0.2)\cdot10^{-3}M^{-1}s^{-1}$ respectively. Note that the dye absorbance increase is controlled mainly by the kinetic constant k_3, and clear based on the spectrophotometric measurements, it is impossible to determine k_1, k_3 with high precision.

The association of GOx with PC/CTAB liposomes has been demonstrated [15]. In the process of titrat of PC-dye system by GOx, no increase has been observed (data not shown). This may suggest a lack of prote PC-liposome binding.

As illustrated in Fig. 4, the approximate parallelism of the curves 3 and 1 upon near pK_a^a values in buffer and PC/CTAB liposome suspension suggests that GOx binding to lipid bilayer exerts no effect on the entire reaction process and this implies that GOx binding to positively charged lipid bilayer does not affect the glucose oxidation process, because there is no reason that the immobilization of GOx or dye influences the hydrolysis process of gluconolactone (Eq. (4)). Clearly, the PC liposomes also do not affect the glucose oxidation process because of the absence of protein-liposome interaction. As expected the curve 4 is not parallel with the others, since the pK_a^a value in the PC/CL liposome suspension is relatively higher than that in buffer or PC liposomal suspension.

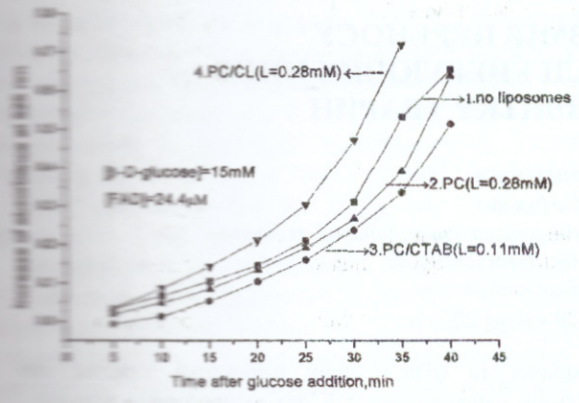

Fig.4. Kinetics of neutral red absorbance changes in buffer, PC, PC/CL and PC/CTAB liposomal dispersions in the presence of GOx, [GOx]=11.2μM, [neutral red]=51.35μM, L is lipid concentration.

CONCLUSIONS

To summarize, the present study was taken up to compare the catalytic properties of GOx free in buffer with those of the enzyme in various liposome media. The highest efficiency of GOx immobilization was achieved with positively charged PC/CTAB liposomes. Binding of deprotonated NR form to PC/CL vesicles resulted in the slight decrease of the dye absorbance. In the PC or PC/CL liposome media GOx was found to remain free in solution, the protonated associated with negatively charged PC/CL bilayer, as judged from significant increase of the dye absorbance. Kinetic analyses showed that GOx retains its catalytic activity on the formation of protein-lipid complexes. Probing the glucose oxidation with neutral red revealed no differences in the reaction process in buffer and in various liposome media. The results obtained may prove of significance in designing the lipid-based glucose optical sensors.

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