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**HEMOGLOBIN BINDING TO PHOSPHOLIPID MEMBRANES AS REVEALED BY
PYRENE FLUORESCENCE STUDY****O.K. Kutsenko, G.P. Gorbenko, V.M. Trusova***V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077*

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In this work hemoglobin (Hb) association with lipid bilayers was investigated using fluorescent probe pyrene. Model membranes were prepared from zwitterionic lipid phosphatidylcholine (PC), anionic lipid phosphatidylglycerol (PG) and cholesterol (Chol). Hb-lipid binding was followed by the pyrene fluorescence quenching. Hb-induced decrease of pyrene monomer fluorescence was followed by the increase of relative intensities of vibronic bands. Presumably, Hb penetration into the bilayer increases the space between neighbouring lipids and promotes water penetration into the membrane core. Pyrene excimer emission quenching was interpreted in terms of resonance energy transfer. The greatest depth of Hb penetration into the lipid bilayer was observed in PC vesicles. In Chol-containing liposomes sterol condensing effect prevents deep protein penetration into the membrane. PG has an ability to stabilize lipid bilayers due to the ordered state of its lipid tails and H-bonding interactions between lipid molecules. This also can prevent Hb access to the inner membrane regions.

KEY WORDS: hemoglobin, model membranes, protein-lipid complexes, pyrene, fluorescence spectroscopy.

**ДОСЛІДЖЕННЯ ЗВ'ЯЗУВАННЯ ГЕМОГЛОБІНУ З ФОСФОЛІПІДНИМИ
МЕМБРАНАМИ ЗА ДОПОМОГОЮ ПІРЕНУ****O.K. Куценко, Г.П. Горбенко, В.М. Трусова***Харківський національний університет імені В.Н. Каразіна, пл. Свободи, 4, Харків, 61077*

В даній роботі комплексоутворення гемоглобіну (Hb) з ліпідними бішарами різного складу досліджувалось за допомогою флуоресцентного зонду пірену. Зв'язування Hb з ліпідами характеризувалось гасінням флуоресценції зонда, що супроводжувалось зростанням відносної інтенсивності вібронних смуг. Можливо, Hb, проникаючи у бішар, розсуває сусідні молекули ліпідів, сприяючи проникненню води у мембрану. Гасіння випромінювання ексимерів інтерпретувалось у термінах резонансного переносу енергії. Найбільша глибина проникнення білка спостерігалась у PC везикулах. У ліпосомах, які містили Chol, конденсуючий ефект стерола перешкоджав проникненню білка у мембрану. Структурні властивості PG сприяють стабілізації ліпідного бішару, що також може перешкоджати проникненню Hb у внутрішні регіони мембрани.

КЛЮЧОВІ СЛОВА: гемоглобін, модельні мембрани, білок-ліпідні комплекси, пірен, флуоресцентна спектроскопія.

**ИССЛЕДОВАНИЕ СВЯЗЫВАНИЯ ГЕМОГЛОБИНА С ФОСФОЛИПИДНЫМИ
МЕМБРАНАМИ С ПОМОЩЬЮ ПИРЕНА****O.K. Куценко, Г.П. Горбенко, В.М. Трусова***Харьковский национальный университет имени В.Н. Каразина, пл. Свободы, 4, Харьков, 61077*

В данной работе комплексообразование гемоглобина (Hb) с липидными бислоями различного состава исследовалось с помощью флуоресцентного зонда пирена. Связывание Hb с липидами характеризовалось тушением флуоресценции пирена, что сопровождалось увеличением относительной интенсивности вибронных полос пирена. Возможно, Hb, проникая в бислои, раздвигает соседние молекулы липидов, способствуя проникновению воды в мембрану. Тушение испускания эксимеров интерпретировалось в терминах резонансного переноса энергии. Наибольшая глубина проникновения белка наблюдалась в PC везикулах. В липосомах, содержащих Chol, конденсирующий эффект стерола препятствовал проникновению белка в мембрану. Структурные свойства PG способствуют стабилизации липидного бислоя, что также может препятствовать проникновению Hb во внутренние области мембраны.

КЛЮЧЕВЫЕ СЛОВА: гемоглобин, модельные мембраны, белок-липидные комплексы, пирен, флуоресцентная спектроскопия.

Hemoglobin (Hb) is a major protein of red blood cells, responsible for the oxygen transport. Inner surface of erythrocyte membrane possesses two kinds of Hb binding sites. The high-affinity sites of first type contain band 3 protein [1] while low-affinity sites of second type include phospholipids [2]. Hb association with lipid bilayers has long been in a focus of research efforts. This problem deserves further investigation for several reasons. First, interactions with lipids may essentially modify the protein biological function. Second, Hb is a suitable model protein for elucidating the general mechanisms of protein-lipid interactions. At last, one of the most important facets of this problem is the development of blood alternate materials involving Hb encapsulated into the lipid bilayer vesicles [3,4]. These oxygen carrying systems are based on the idea of reconstituting some properties of red blood cells without the danger of their reactions with immune system or blood antigens. In this field a question of major importance is the achievement of stable and effective encapsulation. Variation in proportion of membrane lipid constituents is one of the ways to increase protein binding and to avoid lipid oxidation and protein degradation [5]. Therefore, investigation of Hb interaction with lipid bilayers of different composition represents an important step in the development of blood substitutes.

In this work the binding of Hb to lipid vesicles composed of zwitterionic lipid phosphatidylcholine and its mixtures with anionic lipid phosphatidylglycerol and cholesterol was investigated using fluorescent probe pyrene. Due to its peculiar spectral properties pyrene has found widespread application in exploring a variety of membrane processes [6,7]. The alterations in solvent polarity result in the changes in relative intensities of pyrene emission bands [8]. This effect is caused by the enhancement of symmetrically-forbidden bands (Ham effect). This feature of polycyclic aromatic hydrocarbons was first detected for benzene, however it turned out to be the most pronounced for pyrene. Ham effect may arise from the reduction of molecular symmetry in the field of surrounding solvent molecules or distortion of the π -electron cloud by the environmental perturbations [9]. The enhancement of vibronic bands with increasing solvent polarity is interpreted in terms of vibronic coupling originating from the dipole – induced solvent dipole interactions [10,11]. This property is essential for characterization of membrane environmental polarity. Another important feature of this probe is an ability of excited pyrene molecule to form complexes with non-excited one (called excimer) with broad and red-shifted (as compared to monomers) emission. Excimer formation is usually considered as a diffusion-controlled process [12,13]. Therefore, the efficiency of pyrene excimerization can be used for evaluation of the probe lateral mobility in lipid bilayers, which is determined by the membrane microviscosity. Location of pyrene monomers and excimers in different membrane regions [8,14,15] allows comprehensive characterization of the influence of protein-lipid binding on the membrane structure and physicochemical properties.

MATERIALS AND METHODS

Pyrene and bovine hemoglobin in met form were purchased from Sigma (Germany). Butylated hydroxytoluene (BHT) was from Merck (Germany). Egg yolk phosphatidylcholine (PC) and cholesterol (Chol) were purchased from Biolek (Kharkov, Ukraine). Phosphatidylglycerol (PG) was from Avanti Polar Lipids (Alabaster, AL). All chemicals were of analytical grade. Lipid vesicles were formed using the extrusion technique [16]. The thin lipid film was obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). Lipid suspension was extruded through a 100 nm pore size polycarbonate filter. Hb concentration was determined spectrophotometrically using the extinction coefficient $\varepsilon_{Hb}^{406} = 1.415 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Fluorescence measurements were performed with Perkin Elmer spectrofluorimeter. Emission spectra of pyrene were recorded with excitation wavelength 340 nm. Excitation and emission slit widths were set at 2.5 nm. Fluorescence intensity measured in the presence of Hb was corrected for reabsorption and inner filter effects using the following coefficients [17]:

$$k_{corr} = 10^{(A_{ex} + A_{em})/2} \quad (1)$$

where A_{ex} and A_{em} are Hb absorbancies at the excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

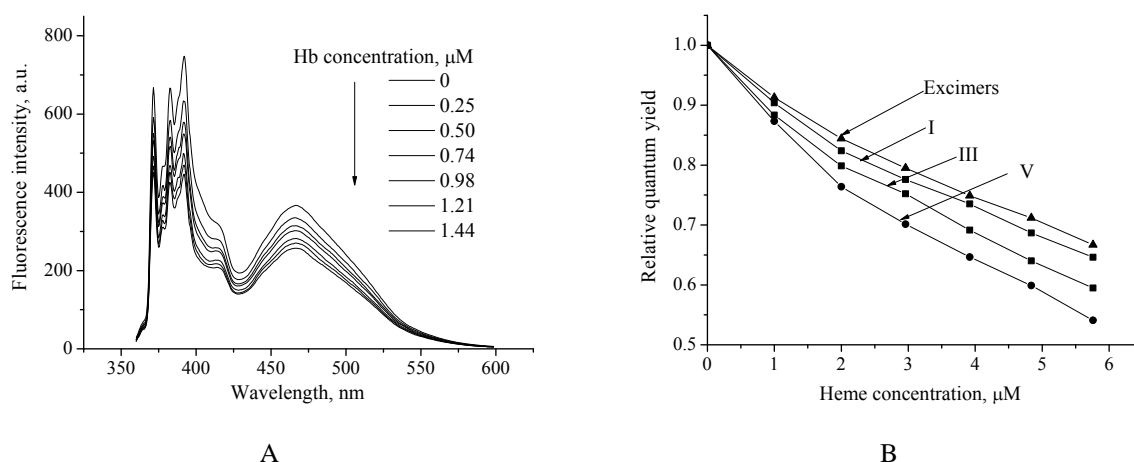


Fig. 1. Pyrene emission spectra in PC:PG 10% model membranes at varying Hb concentration (A) and relative intensity of I, III and V vibronic bands and excimer fluorescence in PG:Chol 30% model membranes (B). Lipid concentration was 46 μM.

Hb binding to lipid membranes is a complex process. Depending on the experimental conditions, it can involve protein-lipid surface complexation, Hb penetration into the membrane core, protein unfolding, oxidation and denaturation, formation of lipid free radicals and bilayer structural changes [18-23]. Initial complexation is driven by the electrostatic forces. As was demonstrated in lipid monolayer studies, Hb association with phosphatidylcholine and phosphatidylserine membranes decreased with pH decrease relative to physiological pH [24]. Electrostatic nature of this process manifests itself in the ability of some bacterial hemoglobins selectively bind to the membranes containing anionic lipids [22]. However, pH effect on Hb-lipid binding manifests itself only at the initial stage of binding process [18]. Subsequent protein penetration into the inner membrane regions is driven by hydrophobic forces [18]. This binding stage depends on membrane structure and composition [25]. Hb-lipid binding increases in the presence of anionic lipids and Chol [5,25,26]. Bilayer packing density and mobility of lipid molecules may also affect the association process [18,22,27]. Penetration of the protein into membrane interior is accompanied by the changes in membrane structure. It can initiate lipid disordering [22], augmentation of polarity and viscosity, increase of membrane permeability [23,26,28]. Likewise, Hb may initiate lipid peroxidation [19]. In neutral and Chol-containing bilayers this effect is expressed weakly while the presence of anionic lipids promotes the formation of free radicals [26].

Hb binding to lipid bilayers was followed by the decrease of pyrene fluorescence (Fig. 1A). Taking into account that the probe spectral changes in Hb-lipid system can be distorted by Hb-induced lipid oxidation, we conducted two series of experiments, with and without of antioxidant BHT. It appeared that the presence of BHT did not influence the observed effects.

Therefore we concluded that the intensity of oxidative processes in the systems under study is rather weak. Free radical processes were not detected even in liposomes containing 20 mol% of anionic lipid PG. It can be a consequence of specific properties of this lipid. PG has an ability to stabilize lipid bilayers [29]. Besides, its lipid tails are in more ordered state than those in PC bilayers. Hydrogen bonds between lipid molecules compensate electrostatic repulsion [29, 30]. It seems that PG effect on lipid bilayer structure prevents the interaction between heme iron and lipids which initiates the formation of free radicals.

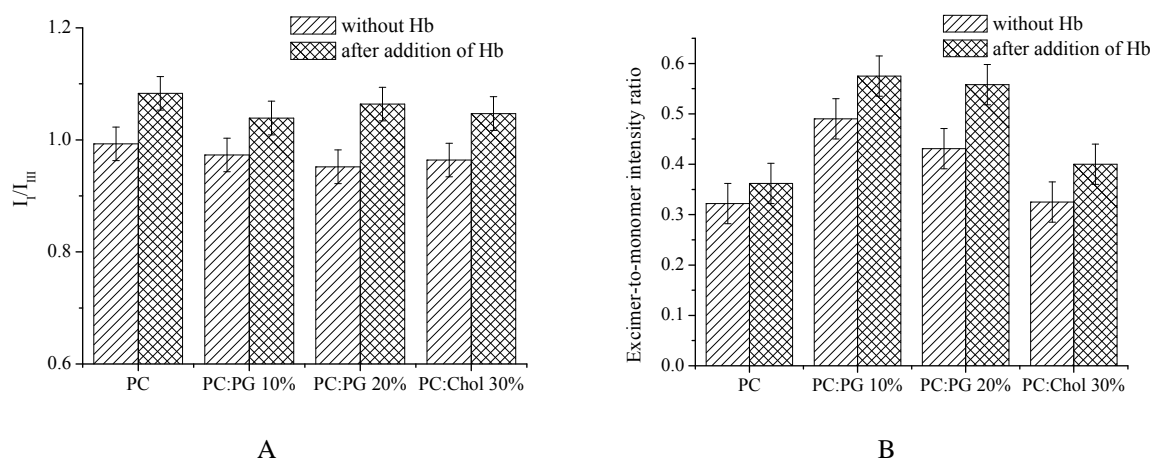


Fig. 2. Relative intensity of vibronic bands I_I/I_{III} (A) and excimer-to-monomer intensity ratio (B) in model membranes. Lipid concentration was 46 μM , pyrene concentration was 1.2 μM , Hb concentration was 1.4 μM .

Table 1 Forster radii for monomer and excimer pyrene species, nm

System	Monomers	Excimers
PC	4.2	3.4
PC:PG 10% PG	4.5	3.7
PC:PG 20% PG	4.4	3.6
PC:Chol 30% Chol	4.5	3.5

The fine structure of pyrene monomer emission is featured by five vibrational bands numbered I-V from the lowest wavelength. Their relative intensities are highly sensitive to the environmental polarity, with intensity ratio I_I/I_{III} being the most sensitive parameter. Its value increases with the increase of polarity, in water it is reported to be 1.96, while in n-hexane – 0.6 [31]. In our case I_I/I_{III} values were approximately 1. This is in good agreement with the data reported elsewhere for similar systems (0.9 – 1.2 in PC bilayers [12, 15]). The recovered estimates correspond to I_I/I_{III} values in relatively polar solvents [11]. Since pyrene is a hydrophobic probe, it might be expected that its monomers would locate in the most hydrophobic membrane region in the middle of lipid bilayer. However, they were found to reside in the acyl chain region near the lipid headgroups with their long axis oriented parallel to the bilayer normal [8,14]. Pyrene dimension along its long axis is 0.8 nm, therefore, it is localized near 4-13 carbons. The presence of hydrophobic pyrene molecules in a relatively polar environment can be explained as follows. First, Hoff [14] hypothesized that pyrene monomer location is favorable due to entropic reason. In the middle of the bilayer lipid tails are in high disorder, so that pyrene molecules residing there reduce lipid mobility and decrease entropy. Thus, it is more favourable for probe molecules to reside in a more ordered membrane region near lipid headgroups. Second, as was reported for benzene [32] charge distribution in aromatic compounds can lead to appearance of partially charged groups which can interact with charged lipid headgroups. Therewith, aromatic rings can act as hydrogen bond acceptor [33] and bind to PG molecules which have a pronounced ability to form hydrogen bonds [30]. This may also favour pyrene location near the lipid headgroups.

The differences in I_I/I_{III} values between various lipid model membranes before Hb addition did not exceed 4%, indicating that polarity of pyrene microenvironment in all membranes is nearly identical. Hb addition to lipid bilayers was followed by the decrease of pyrene fluorescence and slight increase of I_I/I_{III} value by 7-11% (Fig. 2A). It should be emphasized at this point that the use of pyrene for study of heme-containing proteins has some complications. The overlap between pyrene emission and heme absorbance spectra leads to resonance energy transfer (RET) from the probe to the heme [17]. Therefore, RET may distort the variations in pyrene spectral properties due to environmental changes and vice versa. Thus, decrease of pyrene fluorescence may be a consequence of RET from pyrene donors to heme group acceptors. However, in the case of RET emission of all vibronic bands must decrease proportionally to each other, since, as was shown by Kaprovich [10], pyrene quenching does not induce the changes in pyrene monomer relative intensity. However, this is not the case (Fig. 1B). Therefore, we assumed that the alterations in I_I/I_{III} values reflect the changes in the polarity of pyrene microenvironment. Hb increases lipid bilayer polarity in a concentration dependent manner. The changes of I_I/I_{III} are similar to those reported by Xi et al. [23] for Hb interaction with lecithin liposomes. It was supposed that protein penetration into the membrane core increases the space between neighbouring lipid molecules and promotes formation of gaps. Such changes in lipid bilayer structure favour water penetration into the membrane core. Though, the changes in I_I/I_{III} value are rather weak implying small perturbations of the polar membrane region. This can be explained either by surface binding of Hb and slight perturbation of membrane structure or by compaction of lipid molecules upon protein binding which prevents water penetration into bilayer interior. It is important to note that lipid peroxidation enhances membrane permeability and causes increase of water content in the lipid bilayer [34]. Thus, small increase of pyrene monomer intensity ratio confirms previous finding about the absence of significant lipid oxidation upon Hb-membrane complexation.

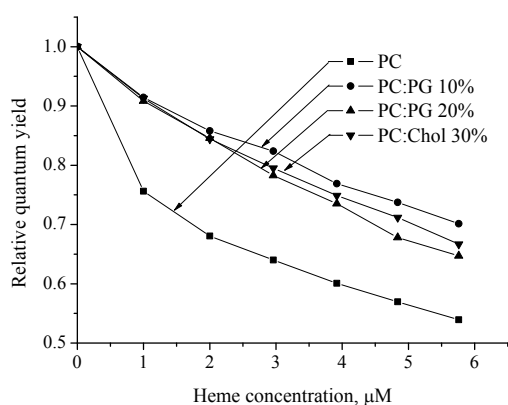


Fig. 3. Relative quantum yield of pyrene excimers. Lipid concentration was 46 μM .

Next, we analyzed the possibility of the interpretation of experimental results in terms of resonance energy transfer. As can be seen from Table 1, Forster radii for monomers are larger than that for excimers. Besides, monomers are located near lipid headgroups, while excimers are immersed into the center of lipid bilayer [8, 14, 15]. Allowing for this fact, it can be supposed that in the case of Hb surface binding the quenching of monomer fluorescence by RET must be stronger than that of excimer emission. As a result, Hb association with lipid bilayers must be accompanied by the increase of excimer-to-monomer emission ratio. Indeed, this was observed in the systems under study (Fig. 2B). On the other hand, E/M ratio can be influenced by the increase or decrease of the extent of excimer formation. If Hb-induced alterations in lipid bilayer structure promote excimer formation, the extent of RET is overrated, while in opposite case this value is understated. Using EPR technique, Gornicki [28] demonstrated that Hb-membrane interaction is followed by the increase of membrane microviscosity which is caused by the oxidative processes. The changes in lipid packing resulted in the decrease of pyrene excimer formation and excimer-to-monomer intensity ratio [8,13,35]. Thus, one should bear in mind that RET data cannot be used for quantitative analysis of the energy

transfer process itself. However, they permit the facile qualitative and semi-quantitative evaluation of the protein-lipid binding.

As seen from Fig. 3, Hb binding to PC model membranes was followed by the most efficient pyrene excimer quenching, while in the other systems RET curves were approximately identical. As was shown by Ushakova et al. the depth of Hb penetration into lipid bilayer increases with the increase of lipid tail mobility [27]. Since PG acyl tails are in more ordered state than PC tails, lipid ordering is likely to prevent Hb insertion into the membrane interior [29]. In the case of PC/Chol bilayer, lesser depth of the protein penetration may be also induced by the changes in lipid packing. Cholesterol may cause tighter packing of fatty acyl chains, thereby creating a steric barrier to the access of Hb to the inner membrane regions [18].

CONCLUSIONS

The results obtained provide some insights into the process of hemoglobin association with lipid membranes which may be useful for the development of blood substitutes. More specifically, we found that:

1. Protein interaction with neutral PC and PC/Chol and anionic PG-containing lipid bilayers is not followed by the formation of free radicals. This may be a consequence of specific packing properties of lipid molecules.
2. Complexation of Hb with lipid bilayers gives rise to the membrane perturbations and increase of lipid bilayer polarity. However, the magnitude of these changes is insignificant.
3. The depth of Hb penetration into lipid bilayer attains the greatest value in PC membranes. Steroid inclusion in the bilayer prevents penetration of Hb into the membrane core. PG stabilizing effect also restricts protein insertion into the bilayer interior.
4. Pyrene steady-state fluorescence study can be used for investigation of interactions between membranes and heme-containing proteins. However, some limitations of this approach must be taken into account.

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