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A pH-controllable protein container for the delivery of hydrophobic porphyrins

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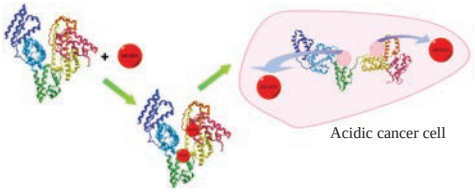
The possibility of creating a pH-controllable protein container for the delivery of hydrophobic porphyrins as photosensitizers in cancer cells has been demonstrated.



The capability of tetrapyrrole macroheterocyclic compounds (porphyrins, phthalocyanines and chlorines) to generate singlet oxygen or free radicals under irradiation is well known. The application of macroheterocyclic compounds to photodynamic therapy (PDT) is based on the indicated properties. Aluminum(III) sulfophthalocyanine (Photosens), 9-capronyloxytetrakis(methoxyethyl)porphycene, benzoporphyrin (Verteporfin), cationic porphyrins, chlorine e_6 and chlorine e_4, and tetrasulfophthalocyanine are used in current clinic practice.

Photosensitizers are under intensive investigation to raise the quantum yield of singlet oxygen and achieve an efficient therapeutic effect. Note that the local concentration and target delivery of photosensitizer become very important in biosystems. It is well known that the oxidation of a biological target is the initial stage of molecular oxygen light-induced reactions resulting in the irreversible destruction of cancer cells. There are three mechanisms of cell death: necrosis, apoptosis and autophagia. Necrosis is the most dangerous because nonprogrammable cell death leads to the release of hydrolases, lysosomal enzymes, *etc*., attacking neighboring healthy cells and provoking the inflammation of health tissues. Photosense and some cationic porphyrins invoke such an effect.[1–5] These photosensitizers are polycations or polyanions, and they accumulate in mitochondrions, lysosomes, Golgi apparatus, endoplasmic reticulum, cell membranes due to their hydrophobic/hydrophilic character and the presence of different reaction centers. Hydrophobic photosensitizers [Verteporfin[6,7] and 9-capronyloxytetrakis(methoxyethyl)porphycene[8,9]] are located in lysosomes and endoplasmic reticulum and invoke programmable cell death (apoptosis and autophagia). Bacellar *et al.*[10] concluded that (1) lipophilic photosensitzers are the most efficient; (2) due to the limited diffusion of photoexcited active forms in biomedia, photodestruction occurs in the places where photosensitizer is located; therefore, the oxidation of target biomolecules may lead to a higher positive effect than total and deep oxidation; and (3) apoptosis and autophagia are desirable mechanisms of cancer cell death.

The use of hydrophobic photosensitizers requires the development of delivery systems: nanospheres or molecular containers,[11,12] porphyrin–lipid complexes,[13] conjugates[14] or endogenous transport systems such as albumins.[15–17] Albumin is a multifunctional transport protein. Its main physiological functions are the maintenance of blood osmotic pressure, the reversible binding of different ligands (fatty acids, drugs, metabolites, *etc*.) and free radical scavenging. Albumin is a carrier of nitrogen oxide, which participates in important physiological processes and information transfer in nervous system (synapses). The transport function of albumin attracts the attention of researches in terms of biomimetics and application of the endogenous protein for delivery of lipophilic drugs. Traditionally, the binding of ligands to serum albumin is studied at physiological pH (7.0–7.4) when the protein occurs in native structure: the polypeptide chain forms a heart shaped asymmetric globule having dimensions of 80×80×30 Å[18] (normal or N-form[19]). Changes in pH lead to the structural alterations of albumin. For example, the basic form (B-form) occurs at pH > 8.0. The B-form is characterized by structure fluctuation,[20] loss of rigidity, increasing surface area of the protein molecule from 39000 to 47000 Å^2, decreasing helical content and increasing β-structures. It was supposed that the pH-sensitive conformational alteration of albumin can be used for the preparation of molecular containers for the delivery of hydrophobic porphyrins. For this purpose, it is necessary to study the complex formation of albumin in N- and B-forms with the model hydrophobic porphyrin hemin and to estimate the effect of pH on the stability of hemin complexes with albumin in B-form and the protein conformation.[†]

Hemin (Figure 1) is a hydrophobic compound, which can be dissolved in an aqueous medium only upon the ionization of peripheral carboxyl groups in the 6- and 7-positions of the

[†] Hemin from Sigma-Aldrich (⩾90%) was purified by HPLC. Bovine serum albumin (BSA), fraction V, for biochemistry, pH 7.0 (Acros Organics) and warfarin of analytical grade from Sigma-Aldrich were used as received. Tris-HCl buffers with pH 7.4, 8.0 and 8.5 were prepared according to a published procedure.[28] The spectroscopic measurements were performed using a Unico 2800 single-beam scanning spectrophotometer (United Products and Instruments) in a range of 200–800 nm. The fluorescence spectra were measured using an Avantes AvaSpec-2048 spectrophotometer. The light source was a xenon arc lamp with an LM-4 diffractional monochromator (Lumix). The characteristics of complex formation were estimated according to a Scatchard method[29] from fluorescence spectroscopic data.

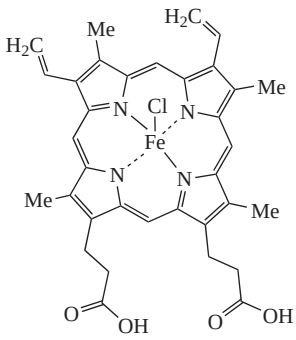


Figure 1 Structure of hemin.

macrocycle. The pK_a of many carboxylic acids is 4.6–4.8;[21] *i.e.*, hemin is soluble only in strongly basic media or mixed water–organic solvents.

Figures 2 and 3 show the results of the spectral titration of a bovine serum albumin (BSA) solution (1.2×10^{-5} M) by hemin solutions (1.4×10^{-6}–7.2×10^{-6} M) in aqueous DMF (0.19 M). At this concentration of DMF, considerable structural alterations of the protein are not observed.[22]

The BSA fluorescence is quenched with increasing hemin concentration (Figure 2). At an excitation wavelength of 295 nm, the fluorescence properties of BSA are due to two tryptophan residues, Trp-134 and Trp-213, located in domain IB and domain IIA, respectively (Figure 4). The fluorescence quenching of BSA may be caused by both a loss of native conformation and the interaction of the protein with the quencher. The hemin–BSA interaction leads to a red shift of the hemin Soret band by 15 nm (Figure 2). Probably, such a considerable shift is connected with a change of the pseudo-solvate environment of hemin at its embedding into the protein globule. Taking into account the data of electron absorption spectroscopy, we concluded that

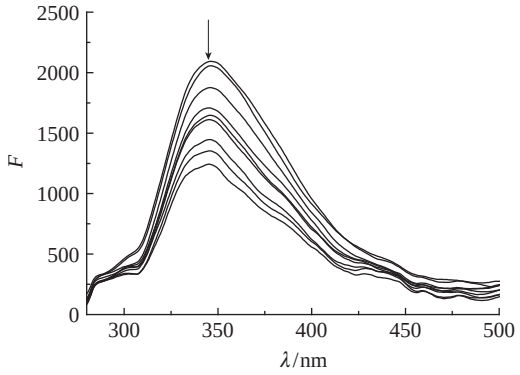


Figure 2 Fluorescence spectra of BSA upon the addition of hemin in solution with pH 7.4 (λ_{ex} = 295 nm).

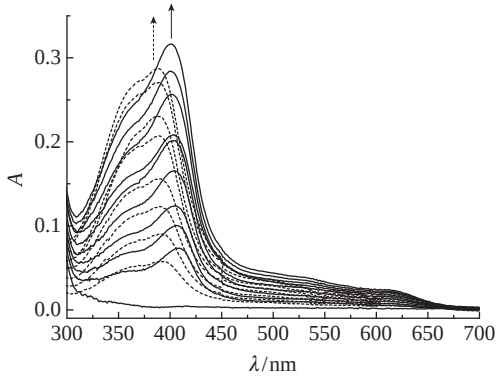


Figure 3 UV-VIS spectra of BSA upon titration with hemin in solution with pH 7.4 (solid lines) and appropriate solutions of hemin (dashed lines).

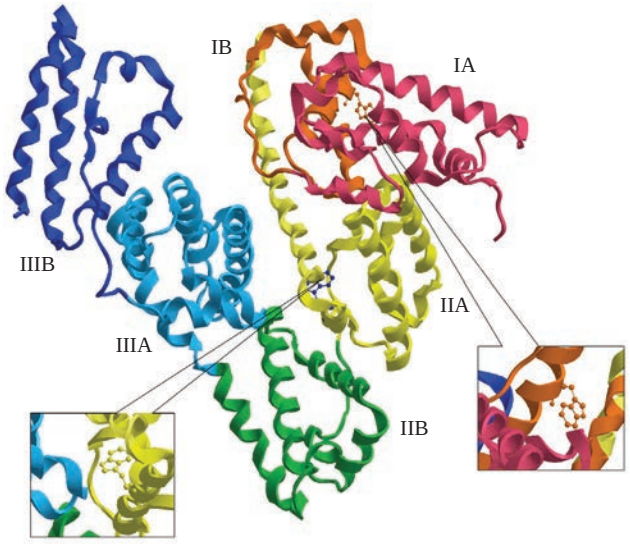


Figure 4 BSA structure, PDB code: 4F5S, tryptophan residues 213 and 134 on insets.

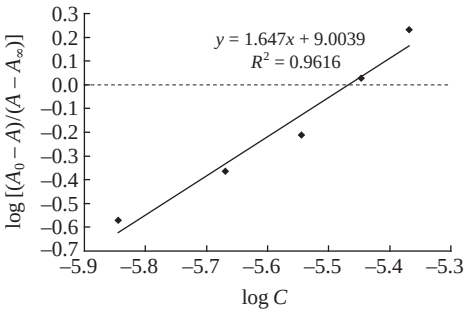


Figure 5 Scatchard plot for BSA upon titration with hemin in solution with pH 7.4.

the fluorescence quenching of BSA is due to its interaction with hemin. However, the full fluorescence quenching of the protein is not observed. It can be supposed that hemin is located only in one of the potential binding sites of BSA. The assumption is confirmed by a linear plot in the Scatchard coordinates (Figure 5) because hemin interaction with two sites of BSA should result in a deviation from linearity. The parameters characterizing a high affinity of hemin to BSA are presented in Table 1.

Published data on hemin localization in the protein globule are very contradictory. Some authors believe that such large ligands as hemin and other porphyrins are bound in subdomain IIA.[23–25] On the other hand, there are X-ray diffraction data testifying about the location of hemin in subdomain IB.[26] Obviously, hemin localization depends on the presence of fatty acid or other ligands, ions and pH. Therefore, to ascertain hemin localization in the protein globule, competitive titration in the presence of warfarin,

Table 1 Parameters of hemin binding to BSA calculated using a Scatchard method.

pH	From UV-VIS spectral data			From fluorescence data		
	Binding constant	Number of binding sites	R^2	Binding constant	Number of binding sites	R^2
7.4	$K = 2.93\times10^5$	$n = 1.6$	0.96	$K = 2.28\times10^5$	$n = 1.5$	0.99
8.0	$K_1 = 3.00\times10^5$	$n_1 = 1.7$	0.99	$K_1 = 3.78\times10^5$	$n_1 = 2.6$	0.93
	$K_2 = 2.69\times10^5$	$n_2 = 3.5$	0.93	$K_2 = 3.08\times10^5$	$n_2 = 8.4$	0.93
8.5	$K_1 = 3.14\times10^5$	$n_1 = 1.4$	0.99	$K_1 = 3.84\times10^5$	$n_1 = 1.4$	0.99
	$K_2 = 2.97\times10^5$	$n_2 = 3.9$	0.98	$K_2 = 2.54\times10^5$	$n_2 = 7.5$	0.96

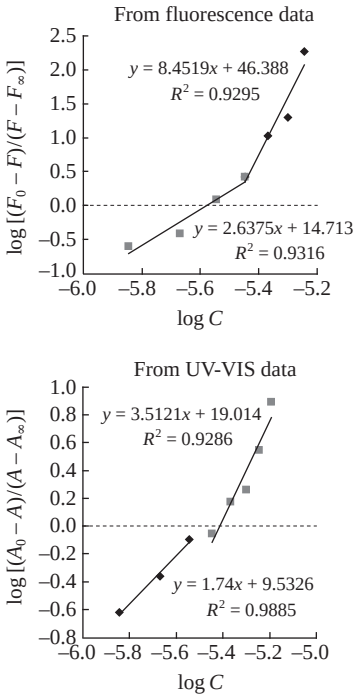


Figure 6 Scatchard plots for BSA upon titration with hemin in a buffer solution with pH 8.0.

which is known to bind in subdomain IIA of BSA, was carried out. The results showed that, at pH 7.4, hemin binds to the protein in the single site located in subdomain IB. The binding parameters are $K = 2.28 \times 10^5$ dm^3 mol^{-1} and $n = 1.5$.

Based on the results of the spectrophotometric and fluorescence titration of BSA solutions by hemin solution at pH 8.0 and 8.5, we observed the same spectral changes: a red shift of the hemin Soret band and protein fluorescence quenching, which increased with pH.

The spectral data obtained according to a Scatchard procedure (Table 1) showed that hemin binding to BSA is a pH-dependent process. In an alkaline medium, the second binding site of the protein becomes available for hemin due to a more friable protein globule. This leads to the increased fluorescence quenching of BSA and two linear sections on Scatchard plot (Figure 6).

The possibility of hemin release from the protein globule on changing pH was estimated using spectroscopic measurements. Figure 7 shows the spectrum of a hemin–BSA complex in an alkaline medium with a maximum ligand content of the globule. The solution was titrated with hydrochloric acid to neutral pH.

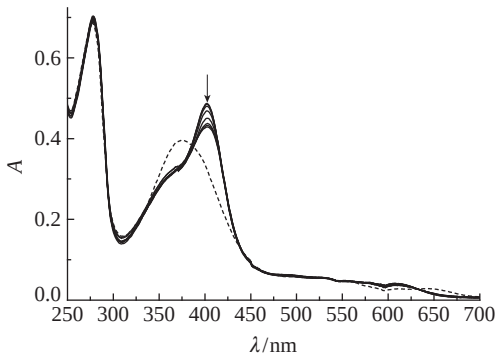


Figure 7 BSA with hemin upon titration with HCl in buffers at pH from 8.5 to 6.4. Dashed line shows an abrupt release of hemin from the protein container at pH 6.4.

The titration led to a decrease in the intensity of the Soret band. In weakly acidic media, the stepwise change corresponding to the released (free) hemin was detected. This fact demonstrates the possibility of hemin release from a BSA globule in weakly acidic solutions. The obtained result is very important for photosensitiser accumulation in cancer cells having a more acidic extracellular pH than normal cells.[27]

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