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Synthesis of a Water-Soluble Ytterbium Porphyrin–Bovine Serum Albumin Conjugate

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Abstract—The ytterbium complex of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin was synthesized as an IR-fluorescent label and covalently bound to bovine serum albumin. The resulting conjugate fluoresces at 985 nm and is of interest for use in IR-fluorescent tumor diagnostic, immunoassay, and energy transfer studies.

Key words: conjugates, fluorescence, near IR region, serum albumin, ytterbium porphyrins

INTRODUCTION

Previously, we have obtained fluorescent probes on the basis of palladium metalloporphyrins with isocyanate group [1].[2] The probes on the basis of metalloporphyrins with some lanthanides that fluoresce in the near IR region of spectrum (900–1050 nm) are especially interesting [2]. Any irradiance of biogenic substances is practically absent, and, subsequently, the sensitivity of the probe detection is higher in this range [3]. Among the lanthanide ions whose metalloporphyrin derivatives exhibit an IR-fluorescence, the complexes with Yb^{3+}, which has the least ionic radius (0.642 Å), are the most stable.

The ability of porphyrins to accumulate in malignant tumors underlies the photodynamic therapy and the fluorescent diagnostics of cancer diseases [4]. In this case, the main problem is the enhancement of selectivity of the porphyrin accumulation in tumor cells. The selectivity of porphyrin delivery to cancer cells increases when metalloporphyrin–albumin conjugates are used [5]. The possibility of using the IR fluorescence of YbTCPP for the diagnostics of tumors and an extremely low phototoxicity of YbTCPP were demonstrated in [6, 7].

The use of highly ordered oriented systems, such as LB films, make realizable the creation of immunosensor systems with the IR-fluorescent detection principle [8]. We have previously demonstrated [9] a basic possibility of the development of an IR-fluorescent immunosensor on a model system.

The metalloporphyrin–albumin conjugate is of interest for studying the process of energy transfer in lipid membranes (including lipid LB films), and its use may partially clear the problem of the role of protein–lipid environment in the migration of exciting energy [10]. Therefore, the synthesis of ytterbium metalloporphyrins and the obtaining of their conjugates with proteins appear to be important problems of bioorganic chemistry.

RESULTS AND DISCUSSION

We prepared TCMPP (**I**) as the starting compound for the synthesis of YbTCPP (**IV**) by the method [11] and hydrolyzed it by warm caustic potash in aqueous pyridine. The reaction mixture was evaporated and acidified by hydrochloric acid. The precipitated green solid was TCPP dihydrochloride (**II**).

YbTCPP (**IV**) is usually synthesized according to the procedure of obtaining tetrasulfophenyllantanides described in [12]. This procedure results in the water-soluble form of YbTCPP, its tetrasodium salt ($YbTCPP^{4-} \cdot 4Na^{+}$) (**III**). The acid YbTCPP, soluble in anhydrous organic solvents, is necessary for activation of carboxyl groups.

We partially modified the procedure of the ytterbium metalloporphyrin synthesis. A mixture of TCPP (**II**) and ytterbium acetylacetonate was fused in imidazole for 40 min. The reaction mixture was dissolved in a small quantity of methanol and treated with diethyl ether. The unreacted TCPP and resulting YbTCPP were precipitated, whereas Yb acetylacetonate and imidazole remained in solution. The TCPP and YbTCPP (**IV**) pre-

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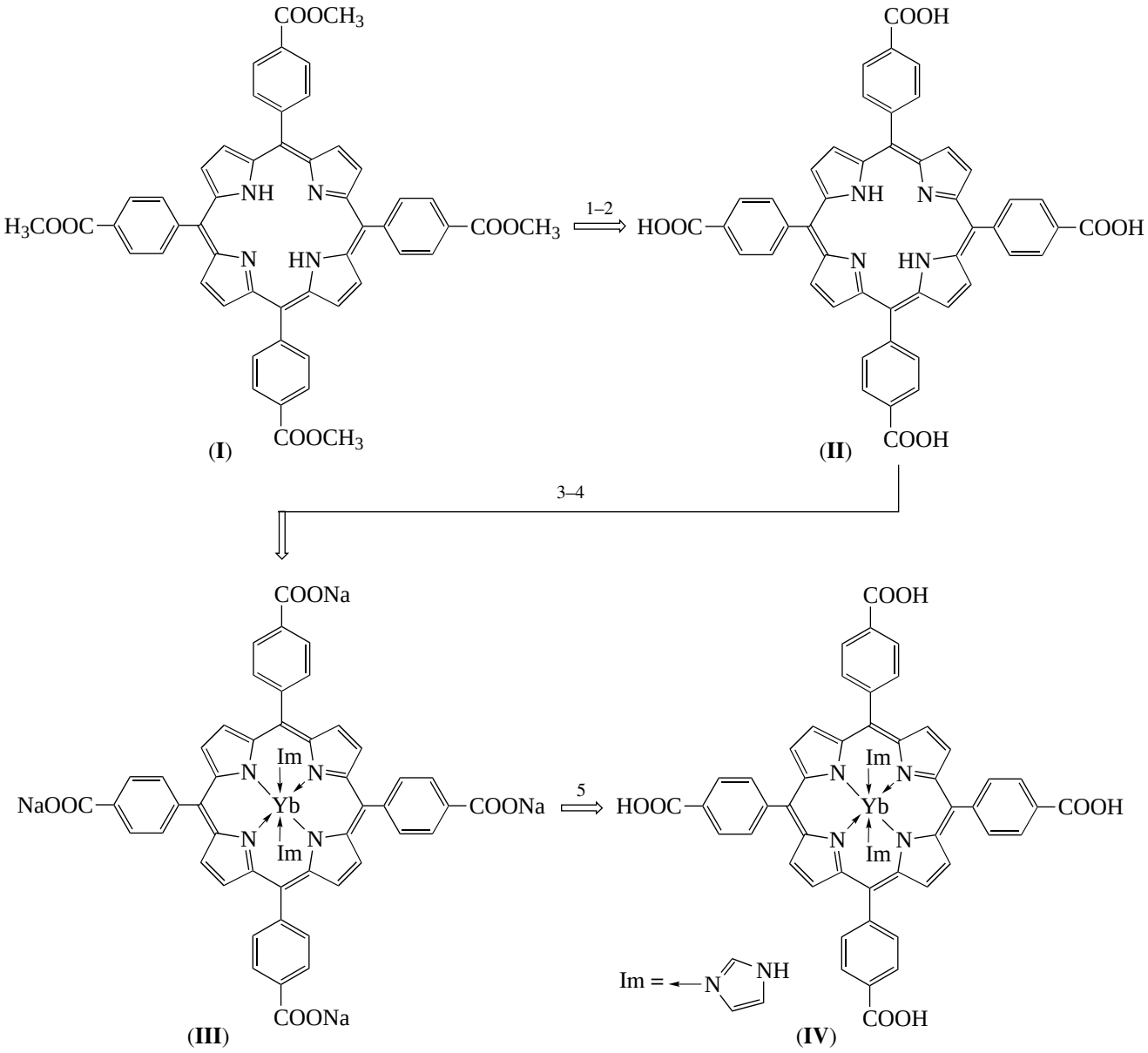
[2] Abbreviations: BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide; Im, imidazole; LB, Langmuir–Blodgett technology; NSI, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; TCMPP, 5,10,15,20-tetrakis(4-carbomethoxyphenyl)porphyrin; TCPP, 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin; and YbTCPP, TCPP ytterbium complex.



Scheme. Reagents and reaction conditions: (1) 2 M KOH, pyridine, 1.5 h; (2) 0.1 M HCl; (3) $Yb(acac)_3$, imidazole, 240°C, 40 min; (4) 0.5 M Na_2CO_3; (5) 0.1 N HCl

cipitate was dissolved in aqueous solution of sodium carbonate, applied onto a chromatographic column packed with Trisacryl, and eluted with water. The first colored zone contained TCPP sodium salt and the second, YbTCPP sodium salt. The aqueous solution of ($YbTCPP^{4-} \cdot 4Na^+$ (**III**)) was then slowly acidified with 0.1 N HCl, which led to the crystallization of YbTCPP acid (**IV**). This procedure should be carried out carefully, because an excessive acidification was observed to cause destruction of the metallocomplex. The precipitated YbTCPP acid is soluble in a number of organic solvents.

MALDI mass spectrometry and ^1H NMR spectroscopy allowed us to find that two imidazole molecules are coordinated with ytterbium ion (Fig. 1). A similar situation was previously found for an ytterbium sulfoporphyrin [12].

The homogeneity of the resulting porphyrins was confirmed by TLC. The benzene–methanol–triethylamine systems were efficiently used for the separation of mixtures of porphyrins containing no metal ions. Addition of various quantities of methanol allowed the regulation of the eluent polarity.

Ytterbium complex (**IV**) demonstrates the zero mobility when being analyzed in the systems suitable

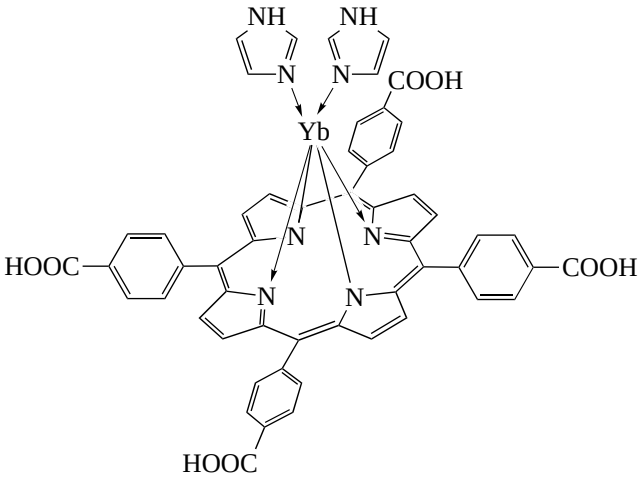


Fig. 1. Spatial structure of YbTCPP

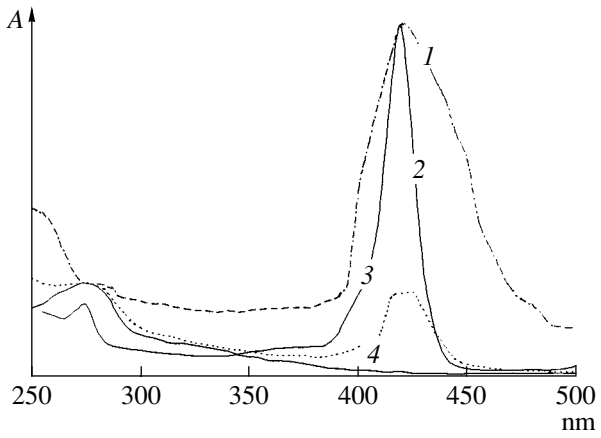


Fig. 2. Electronic spectra of (*1*) covalent conjugate BSA–YbTCPP, (*2*) YbTCPP, (*3*) noncovalent conjugate of BSA with YbTCPP, and (*4*) BSA in PBS, pH 7.4

for the porphyrins devoid of metal ions. This is probably connected with the presence of the third valence of ytterbium, which is partially free. An addition of 1% acetylacetone to the methanol–triethylamine system (60 : 1) increases the chromatographic mobility of the YbTCPP acid complex to R_f 0.19; TCPP has R_f 0.27 in this system.

Next, we conjugated YbTCPP with BSA. Free amino groups, mainly ε-amino groups of Lys residues, are located on the surface of BSA molecule. The BSA–YbTCPP was synthesized in two stages. At the first stage, the YbTCPP succinimide ester was prepared under anhydrous conditions. An interaction of acid (**IV**) with isobutyl chloroformate in pyridine resulted in the active mixed anhydride, which was then transformed into the less active *N*-succinimide ester. A TLC monitoring showed that, under the found reagent ratio (see the Experimental section), the YbTCPP with one active carboxyl group is preferentially formed. At the second stage, the succinimide ester was conjugated with the protein in water–organic medium at pH 9–9.3.

The composition of the resulting synthetic conjugate was determined after its gel-chromatographic purification. The number of porphyrin molecules per protein molecule, α, was determined by using spectrophotometric method [13]. In UV region, the conjugate absorption is summed up from the absorptions of BSA and (**IV**), whereas only YbTCPP absorbs visible light (Fig. 2).

Therefore, α was calculated according to the equation:

$$\alpha = A^{(417)}_{\text{BSA–YbTCPP}} \times \varepsilon^{(280)}_{\text{BSA}} / A^{(280)}_{\text{BSA–YbTCPP}} - A^{(280)}_{\text{YbTCPP}} \times \varepsilon^{(417)}_{\text{YbTCPP}},$$

where $A^{(417)}_{\text{YbTCPP}}$, $A^{(417)}_{\text{BSA–YbTCPP}}$, and $A^{(280)}_{\text{BSA–YbTCPP}}$ are the absorptions of YbTCPP, BSA–YbTCPP, and BSA at the wavelength 280 or 417 nm; $\varepsilon^{(280)}_{\text{BSA}}$ and $\varepsilon^{(417)}_{\text{YbTCPP}}$ are the molar absorption coefficients of BSA (36 000 M^{-1} cm^{-1} at 280 nm) and YbTCPP (450 000 M^{-1} cm^{-1} at 417 nm) (see table). The α value reached 5.4–6.6 when the initial quantity of YbTCPP was equal or more than 100 mol/mol of BSA. At such a conjugate composition, no substantial disturbances in the structure or functions of protein are usually observed and no concentrational quenching of the porphyrin fluorescence occurs.

We carried out a control bare experiment to estimate the degree of sorption (noncovalent) binding of BSA with YbTCPP (**IV**). BSA was conjugated with YbTCPP acid bearing nonactivated carboxyl groups. The value of $\alpha \le 0.1$ was found for such noncovalent conjugates (Fig. 2), and, therefore, the proportion of nonspecifically bound porphyrin in the BSA–YbTCPP complex is small.

A study of the IR-fluorescent properties of YbTCPP and BSA–YbTCPP complex showed that the IR fluorescence of ytterbium porphyrins is due to the energy transition of inner 4*f* electrons of Yb^{3+} ions from the $^2F_{5/2}$ to the $^2F_{7/2}$ state [2]. The excitation energy is absorbed by the π-electron system of the ligand porphyrin and then migrates from its triplet level to the ytterbium $^2F_{5/2}$ resonance level. Therefore, the fluorescence in the near IR range is the result of the intramolecular energy transfer, and, hence, it should substantially depend on the complex environment.

A weak IR fluorescence is characteristic of aqueous solutions of YbTCPP: the calculated value of its quantum yield is 0.006. At the same time, increased fluorescence intensity with the maximum at 986 nm is observed in water–micellar solutions. The fluorescence intensity of the BSA–YbTCPP conjugate with 985-nm maximum is even greater (Fig. 3). An increase in quantum yields n_f (see table) observed for YbTCPP upon the transitions buffer ⟶ water–micellar system ⟶ fixa-

Spectral properties of YbTCPP and BSA–YbTCPP

Conditions	Compound	λ_{max}^{abs}, nm	ε, M^{-1} cm^{-1}	n_f
0.1 M Na_2CO_3, 0.9 M $NaHCO_3$, pH 9.2	YbTCPP	417	$4.5–10^5$	1.00
0.05% Triton X-100	YbTCPP	419	$3.9–10^5$	2.00
8.6 mM K_2HPO_4, 1.4 mM KH_2PO_4, 0.15 M NaCl, pH 7.35	BSA–YbTCPP	425	$4.0–10^5$	3.03

tion on protein may be explained by a certain decrease in the heat molecular mobility of complex (**IV**) and its preferential occurrence in monomeric form. The contribution of some amino acid residues of BSA into the energy of YbTCPP molecules cannot be also excluded. The table also lists the values of molar extinction coefficients of YbTCPP and BSA–YbTCPP at the wavelengths of their absorption maxima, λ_{max}^{abs}.

Thus, we demonstrated a possibility of synthesis of proteins with an IR-fluorescent label by the example of a BSA conjugate with a water-soluble Yb-porphyrin (YbTCPP). This conjugate exhibits fluorescence at 985 nm, the quantum yield of which substantially depends on its environment. An inclusion of the conjugate into biosensor films or into biological or artificial membranes would probably enhance intensity of its IR fluorescence. An appropriate choice of optical equipment would allow a prospective use of this conjugate.

EXPERIMENTAL

Isobutyl chloroformate and imidazole were from Fluka (Switzerland); *N*-hydroxysuccinimide from Merck (Germany); acetylacetone and potassium monophosphate and diphosphate from Sigma (United States); Triton X-100 and cetyltrimethylammonium bromide (CTAB) from Ferak (Germany); methanol, ethanol, pyridine, benzene, triethylamine, diethyl ether, sodium chloride, sodium bicarbonate, sodium carbonate, hydrochloric acid, and potassium hydroxide of analytical or reagent purity grades were of domestic production (Reakhim, Russia). Pyridine was stored over molecular sieves (4 Å), and BSA (Sigma, United States), in lyophilized state at +4°C.

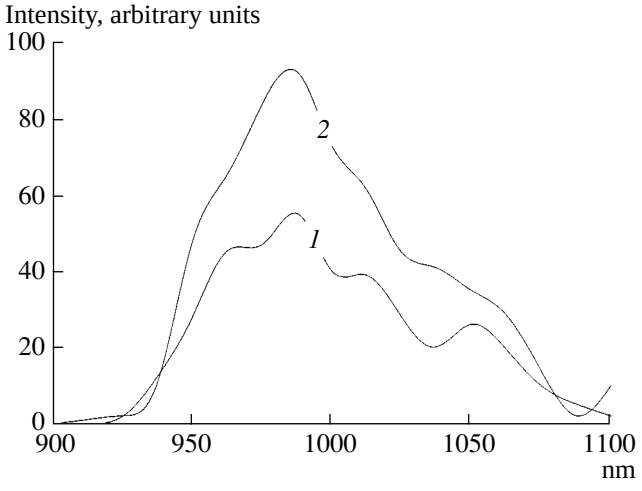


Fig. 3. Fluorescence spectra of (*1*) YbTCPP in 0.05% aqueous solution of Triton X-100 and (*2*) BSA–YbTCPP in tridistilled water at room temperature.

The reagent $Yb(acac)_3$ was obtained from $YbCl_3$, and TCMPP was synthesized according to [14].

Trisacryl GF-05 (LKB, Sweden) was used for the column chromatography of YbTCPP. The column size was 10 × 300 mm, and the flow rate 1–1.5 ml/min. Precoated Silufol UV-254 plates (20 × 70 mm, Kavalier, Czechia) were used for TLC in systems: (A) 15 : 15 : 1 benzene–methanol–triethylamine and (B) 60 : 1 methanol–triethylamine containing 1% acetylacetone. A gel-chromatographic purification of BSA–YbTCPP was carried out on a column (10 × 180 mm) packed with Sephadex G-25 (Pharmacia, Sweden). The eluate from the column was detected at 280 nm using an Uvicord (LKB, Sweden) instrument.

^{1}H NMR spectra were registered on a Bruker AMX400 spectrometer (Germany) at 295 K. The values of chemical shifts (δ, ppm) were calculated relative to the residual signals of HOD (δ 4.63) for solutions in D_2O, $CHCl_3$ (δ 7.26) for solutions in $CDCl_3$, and $(CH_3)_2SO$ (δ 3.57) for solutions in DMSO-d_6 [14]. The spin–spin-interacting signals were revealed by means of double resonance. Mass spectra were measured on a Kompact MALDI 4 (KRATOS Analytical, United States) instrument. Electronic spectra were obtained on a Shimadzu UV-VIS 3100 spectrophotometer (Japan). Melting points were determined on a Boetius hot plate (Germany). Fluorescence spectra were measured on a spectrofluorimeter Shimadzu RF 5000 (Japan). Quantum yields were calculated by the procedure in [15] using a solution of 5,10,15,20-tetraphenylporphyrin in toluene as a standard (Q = 0.12). CTAB and concentrated solutions of HCl and NaOH were used for the determination of molar extinction coefficients.

5,10,15,20-Tetrakis(4-carboxyphenyl)porphyrin (II). Five ml of 2 M KOH were added to a solution of TCMPP (50 mg, 0.046 mmol) in pyridine (5 ml), and the mixture was refluxed for 1.5 h at intensive stirring and evaporated on a rotary evaporator at 80°C and 15 mmHg. The residue was diluted with 10 ml of distilled water and acidified with 0.1 N HCl to pH 2 at stirring. The color of the solution was changed from red to

green, and a green solid (**II**) was precipitated. The precipitate was filtered, washed with water (100 ml), and dried over phosphorus pentoxide; yield 33.5 mg (92%); R_f 0.23 (A); mp >300°C; electronic spectrum [5 : 1 benzene–methanol, λ_{max}, nm (intensity ratio)]: 420, 513, 548, 592, 645, (1.0 : 0.05 : 0.028 : 0.019 : 0.013); ^{1}H NMR (δ, ppm): 8.82 (8 H, s, β-H), 8.34–8.39 (16 H, s, PhH), 2.50 (4 H, s, COOH); MALDI MS: found *M* 790.84; calc. for $C_{48}H_{30}N_4O_8$ *M* 790.77.

Ytterbium complex of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (IV). A mixture of (**II**) (50 mg, 10.5 μmol), ytterbium acetylacetonate (100 mg, 0.21 mmol), and imidazole (700 mg) was carefully triturated on a watch crystal, transferred to a bulb equipped with a reflux condenser, and heated on an air bath at 240°C in a nitrogen flow for 40 min. Then the mixture was cooled, and imidazole excess was removed on a rotary evaporator (100°C, 15 mm Hg). The residue was triturated with methanol to get a thick suspension, treated with diethyl ether (20 ml), and kept for 30 min to permit the flaky precipitate to settle. The supernatant was decanted and discarded, and the procedure was repeated thrice. The residue was air-dried, dissolved in 0.5 M Na_2CO_3, and applied onto a column packed with Trisacryl GF-05, which was eluted with distilled water. Complex (**III**) was eluted in the second colored zone. The solution of (**III**) was filtered and treated dropwise with 0.1 M HCl until the formation of fine crystals of (**IV**) that worked to bottom. The mixture was centrifuged, supernatant was decanted, and the residue was twice washed with 0.001 M HCl and distilled water and air-dried to a constant mass. The yield of porphyrin (**IV**) was 45.0 mg (64.7%); R_f 0.19 (B); electronic spectrum [0.5 M Na_2CO_3, λ_{max}, nm ($\varepsilon \times 10^{-3}$) M^{-1} cm^{-1}]: 417 (450), 510 (2.7), 545 (17.3), 585 (3.7); ^{1}H NMR (δ, ppm): 8.61 (8 H, s, β-H), 8.48 (4 H, d, *o*-PhH), 8.32 (4 H, s, *m*-PhH), 8.05 (4 H, s, *o*-PhH), 7.30 (4 H, s, *m*-PhH), 4.80 (4 H, m, 4,5-ImH), 4.62 (2 H, m, 2-ImH), 2.73 (4 H, s, COOH), 0.43 (1 H, t, Im-NH); MALDI MS: found *M* 1098.6; calc. for $C_{54}H_{36}N_8O_8$ *M* 1097.95.

Conjugate of ytterbium complex of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin with bovine serum albumin. A solution of isobutyl chloroformate (5 μl, 35 μmol) in chloroform (40 μl) was added at intensive stirring and –10°C to a solution of (**IV**) (15.8 mg, 14.3 μmol) in pyridine (2 ml). The reaction mixture was additionally stirred for 1 h at –10°C and for 2 h at 0°C, then treated with *N*-hydroxysuccinimide (7 mg, 60.8 μmol), stirred overnight at room temperature, and centrifuged at 14 000 rpm for 10 min. The supernatant that contained a solution of *N*-succinimide ester of YbTCPP in pyridine (200 μl) was added to a stirred solution of BSA (2 mg, 0.03 μmol) in 0.1 M carbonate–bicarbonate buffer (1 ml, pH 9.1) at room temperature. The mixture was stirred at room temperature overnight, centrifuged at 14000 rpm for 10 min, and supernatant that contained the BSA–YbTCPP conjugate was separated (~1 ml). It was applied onto a Sephadex G-25 column (10 × 180 mm) preliminarily equilibrated with PBS buffer (8.6 mM K_2HPO_4, 1.4 mM KH_2PO_4, 0.15 M NaCl, pH 7.35), and the column was eluted with the same buffer at a flow rate of 0.2 ml/min. The fraction with retention time 25–35 min was used for further studies. The fractions with lower retention times (protein aggregates) were discarded.

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