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Preliminary Note

pH Dependence of fluorescence and absorbance spectra of free sulphonated aluminium phthalocyanine and its conjugate with monoclonal antibodies

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Abstract

Water-soluble phthalocyanines and phthalocyanines linked to targeting monoclonal antibodies are considered to be one of the most promising photosensitizers in photodynamic therapy. Here the spectrum characteristics of sulphonated aluminium phthalocyanine and its protein conjugate in the pH range 1.8–12.0 in solution have been studied. The pK values are determined.

1. Introduction

One of the present directions in photodynamic therapy (PDT) development is a search for new photosensitizers which would increase the efficiency of PDT. Recent results have demonstrated the considerable potential of phthalocyanines [1]. The combining of photosensitizers with monoclonal antibodies specific to tumour cells could significantly increase the selectivity of PDT [2, 3].

The nature of the mechanism responsible for the selective biodistribution of phthalocyanines is not yet clear. Several possible factors have been suggested [4].

Cells are known to display strong heterogeneity in local pH value. Moreover, malignant tissues demonstrate lower pH than normal tissues [5]. The examination of various ionic species that predominate throughout the physiological pH range is of

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substantial interest because of their possible role in selective retention of neoplastic tissues.

Ferraudi *et al.* [6] have determined the absorption spectrum for sulphonated aluminium phthalocyanine (AlSPc) in a solution in which the pH could be varied in such a manner that is consistent with the existence of two acid-based equilibria with pK 3.5 and pK 9.5 that have been assigned to the deprotonation of axially coordinated water. In this paper we report the studies of water-soluble AlSPc and the dependences of its protein conjugate absorption and fluorescence spectral changes on pH in aqueous solution. pK values were determined to be 6.9 and 9.8. The influence of the chlorine ion on the pK value was observed.

2. Materials and methods

AlSPc was synthesized as follows. A solution of oxyaluminium phthalocyanine (3.0 g) in chlorosulphonic acid (concentrated) was mixed for 3 h at 138–140 °C. Then thionylchloride (8 ml) was added during 3 h at 85–87 °C and the mixture was stirred for 30 min at this temperature. The reaction mixture was cooled to 15 °C and poured out on ice. The suspension was filtrated. The precipitate was washed with cold water to neutral reaction and dried under vacuum. The elemental composition was found to be as follows: C, 41.02–41.24%; H, 2.82–2.35%; N, 11.00%; S, 11.88–11.70%; Cl, 7.73–7.76%; ashes, 3.07–2.96. This was compared with the calculated values: C, 42.06%; H, 1.66%; N, 12.26%; S, 14.03%; Cl, 7.76%. In accordance with the elemental composition the degree of sulphonation was found to be 3.5. The degree of chlorination is 1 and one Cl^- is coordinated to aluminium.

Monoclonal antibodies of the mouse against horse-radish peroxidase were obtained by a standard technique and were found to belong to the IgG1 subclass of immunoglobulins [6].

Chromatographic separation of the conjugate was performed using a column with Sephadex G-50 and Sepharose CL-6B (Pharmacia, Sweden). Chromatograms were recorded at 280 nm.

The conjugate synthesis was performed in the following way: 0.2 mg of phthalocyanine was dissolved in 0.025 ml of dimethylsulphoxide and was gradually added to 2 ml of IgG solution (1 mg ml^{-1}) in carbonate buffer (0.1 M; pH 9.2) with constant stirring. After incubation for 1 h at room temperature and during the night at 4 °C the reaction mixture was chromatographed in a combined column with Sephadex G-50 and Sepharose CL-6B in the buffer tris-HCl (0.03 M; pH 7.4) containing 0.15 M NaCl. Three fractions were obtained. The first corresponding to cross-linked protein and the third corresponding to unbound phthalocyanine were discarded. The fraction with the retention time corresponding to unbound immunoglobulin G was used in the experiment. The coupling degree of AlSPc to protein was approximately 2:1.

The solutions were prepared in phosphate (0.01 M Na_2HPO_4, with different concentrations of NaCl (0 M, 0.15 M and 0.5 M)) and carbonate (0.1 M Na_2CO_4; 0.15 M NaCl) buffers. In the absence of NaCl, 0.2 M Na_2SO_4 was used to adjust for constant ionic strength. The titration was done with HCl or H_2SO_4.

The fluorescence and excitation spectra were recorded on a Perkin–Elmer LS-50 luminescence spectrofluorometer. The fluorescence spectrum was recorded at an excitation wavelength of 390 nm. The slits widths were 10.0 nm. A coloured glass filter was used to remove the scattered light at wavelengths below 530 nm. The absorption spectra were recorded using a Hitachi-557 spectrophotometer. The experiments were carried out at room temperature.

3. Data analysis

The observed titration curves were analysed in accordance with the equation (see Appendix)

$$\log\left(\frac{\lambda_{max}-\lambda}{\lambda-\lambda_{min}}\right)=n\ \mathrm{pH}-\mathrm{p}K$$

where λ_{max} and λ_{min} are the peak positions of different spectral forms, λ is the peak position obtained and n is the number of added protons.

An analogous formula was applied for the pK calculation in terms of fluorescence and absorption intensity [7].

4. Results and discussion

To evaluate the influence of aggregation on the observed AlSPc fluorescence and absorption data, spectra were measured in the concentration range 10^{-10}–10^{-5} M at pH 2, pH 7 and pH 11. The observed linear dependence of fluorescence and absorption intensity on AlSPc concentration was evidence of the absence of aggregation.

The AlSPc absorption and emission spectra at pH 5.5 and pH 11.0 are shown in Fig. 1. The AlSPc excitation spectrum did not differ from its absorption spectrum.

Figure 2 shows the effect of the pH variation on the fluorescence spectrum of free AlSPc in phosphate buffer. The increase in pH from 3.0 to 12.3 shifts the fluorescence peak position from 683 to 675 nm and increases the fluorescence intensity. The results do not depend on the direction of pH changes and the buffer applied. The titration curve has two pronounced points of inflection that indicate two chemical transformations in the AlSPc molecule.

As the pH exceeds 7, a new shoulder appears in the excitation spectrum of AlSPc and increases with increasing pH.

The fluorescence spectra changes of AlSPc conjugated to protein were similar to those for free AlSPc.

The AlSPc absorption spectral changes were consistent with those of fluorescence (Fig. 3). Curves 1 and 2 show the dependence of the absorption at $\lambda=350$ nm on
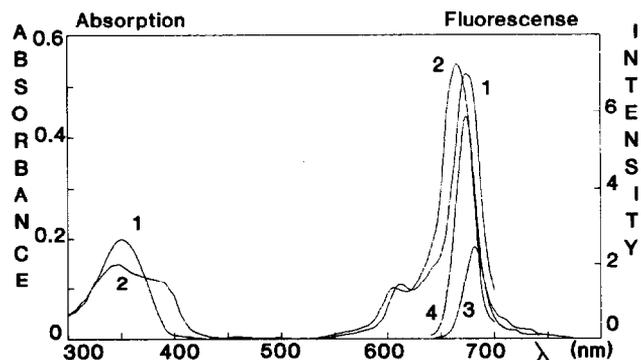

Fig. 1. The absorption (curve 1, pH 5.05; curve 2, pH 11.2) and fluorescence (curve 3, pH 5.05; curve 4, pH 11.2) AlSPc spectra in phosphate buffer (0.01 M NaH_2PO_4; 0.15 M NaCl; 10^{-6} M AlSPc).
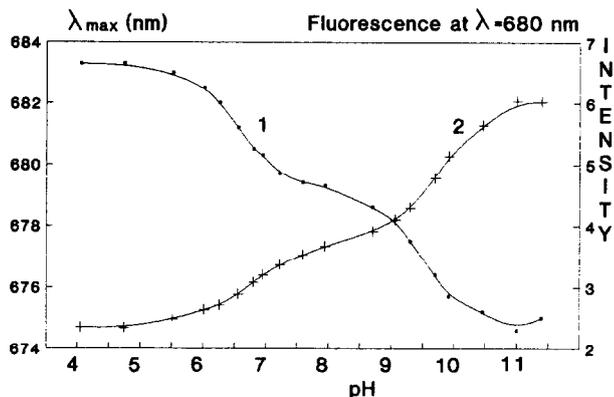
Fig. 2. Variation in the fluorescence spectrum of the free AlSPc in phosphate buffer (0.01 M NaH$_2$PO$_4$; 0.15 M NaCl; 10^{-7} M AlSPc): curve 1, peak position; curve 2, intensity at $\lambda = 680$ nm ($\lambda_{ex} = 390$ nm).

TABLE 1

The calculated pK values and number of added protons of free sulphonated aluminium phthalocyanine and its protein conjugate in solution

|	Buffer	n_1	pK_1	n_2	pK_2
AlSPc free	1	0.84 ± 0.27	6.66 ± 0.18	1.11 ± 0.13	9.90 ± 0.11
AlSPc conjugated	1	0.83 ± 0.20	6.76 ± 0.13	1.22 ± 0.25	9.78 ± 0.13
AlSPc free	2	0.91 ± 0.15	6.61 ± 0.17	0.98 ± 0.16	9.78 ± 0.12
AlSPc conjugated	2	1.03 ± 0.21	6.74 ± 0.12	0.96 ± 0.23	9.68 ± 0.19
AlSPc free	3	1.17 ± 0.3	6.44 ± 0.15	0.94 ± 0.11	9.72 ± 0.07
AlSPc free	4	1.14 ± 0.18	6.86 ± 0.14	0.96 ± 0.21	10.19 ± 0.05

Buffers 1, 2, fluorescence measurements; buffers 3, 4, absorption measurements.
Buffer 1, 0.01 M sodium phosphate buffer with 0.15 M NaCl; buffer 2, 0.1 M sodium carbonate buffer with 0.15 M NaCl; buffer 3, 0.01 M sodium phosphate buffer with 0.5 M NaCl; buffer 4, 0.01 M sodium phosphate buffer with 0.2 M Na$_2$SO$_4$.

pH. In the pH range 1.9–5.7 some spectral changes were observed which depended on the direction of pH changes and became more pronounced as the chlorine ion concentration increased. Curves 3 and 4 correspond to the absorption at $\lambda = 390$ nm. Two transitions are observed. The pK values and the number of added protons for each equilibrium determined from the absorption and fluorescence spectra changes are presented in Table 1.

In so far as the increase in chlorine concentration by six orders of magnitude did not dramatically influence the acid dissociation at neutral and basic pH, it is natural to assign these transitions to the deprotonation of axially coordinated water. This is also in agreement with the observed independence of the titration curve shape on the buffer nature.

Poor reproducibility and hysteresis of the AlSPc spectrum changes that were obtained at $\lambda = 350$ nm and low pH seem to originate from the slow hydrolysis of chlorine to AlSPc. The increase in the amplitude of the changes with increasing chlorine concentration is consistent with this assumption.
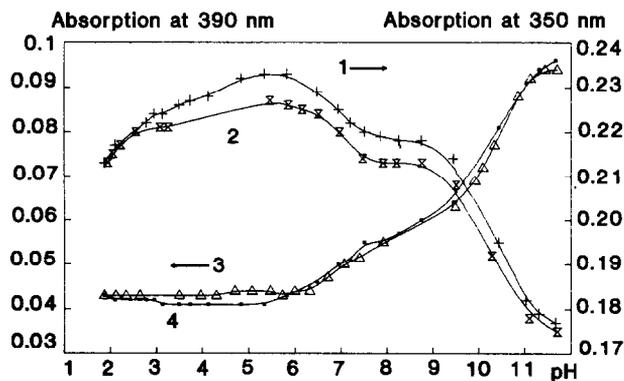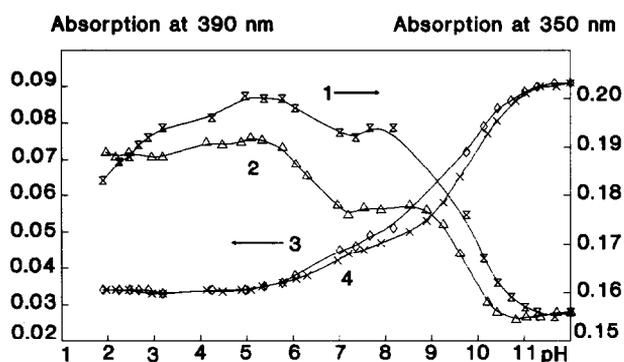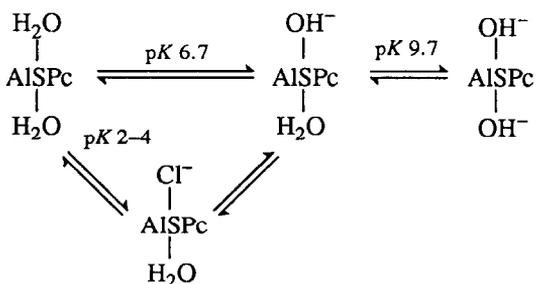
Fig. 3. AlSPc (10^{-6} M) in phosphate buffer (0.01 M NaH_2PO_4) ((a) with 0.2 M Na_2SO_4; (b) with 0.15 M NaCl) absorption spectra changes: curves 1, absorption at $\lambda = 350$ nm by pH change from 1.9 to 11.3; curves 2, absorption at $\lambda = 350$ nm by pH change from 11.3 to 1.9; curves 3, absorption at $\lambda = 390$ nm by pH change from 1.9 to 11.3; curves 4, absorption at $\lambda = 390$ nm by pH change from 1.9 to 11.3.

So in accordance with the observed data we suppose that for AlSPc in aqueous solution the following equilibria are valid:

$$
\begin{array}{c}
\underset{\underset{H_2O}{|}}{\overset{\overset{H_2O}{|}}{AlSPc}}
\;\underset{pK\,6.7}{\rightleftharpoons}\;
\underset{\underset{H_2O}{|}}{\overset{\overset{OH^-}{|}}{AlSPc}}
\;\underset{pK\,9.7}{\rightleftharpoons}\;
\underset{\underset{OH^-}{|}}{\overset{\overset{OH^-}{|}}{AlSPc}}
\end{array}
$$

pK 2–4

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\underset{\underset{H_2O}{|}}{\overset{\overset{Cl^-}{|}}{AlSPc}}
$$

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References

1 J. Spikes, Phthalocyanines as photosensitizers in biological systems and for the photodynamic therapy of tumor, *Photochem. Photobiol., 43* (1986) 691–699.
2 A. R. Ozeroff, G. Ara, D. Ohuoha, J. Aprille, J. C. Bommer, M. L. Yarmush, J. Foley and L. Cincotta, Strategies for selective cancer photochemotherapy: antibody-targeted and selective carcinoma cell photolysis, *Photochem. Photobiol., 46* (1987) 83–86.
3 D. Mew, V. Zum, C.-K. Wat, G. H. N. Towers, C.-H. C. Sun, R. J. Walter, W. Wright, M. W. Berns and J. G. Levy, Ability of specific monoclonal antibodies and conventional antisera conjugated to hematoporphyrin to label and kill selected cell lines subsequent to light activation, *Cancer Res., 45* (1985) 4380–4386.
4 R. Pottier and J. C. Kennedy, The possible role of ionic species in selective biodistribution of photochemotherapeutic agents toward neoplastic tissue, *J. Photochem. Photobiol. B: Biol., 8* (1990) 1–16.
5 A. J. Thistliethewaiye, B. Leeper, D. J. Moylar III and R. D. Nerlinger, pH distribution in human tumors, *Int. J. Radiat. Oncol. Biol. Phys., 11* (1985) 1647–1652.
6 G. Ferraudi, G. A. Arguello, H. Ali and J. E. van Lier, Types I and II sensitized photooxidation of aminoacid by phthalocyanines: a flash photochemical study, *Photochem. Photobiol., 47* (1988) 657–660.
7 A. P. Savitsky, E. V. Vorobyova, I. V. Beresin and N. N. Ugarova, Acid-base properties of protoporphyrin-IX its dimethyl ester and hem solubilized on surfactant micelles. Spectrophotometric and fluorometric titration, *J. Colloid Interface Sci., 84* (1981) 175–181.

Appendix A

Under pH titration in solution in the absence of aggregation the fluorescent form transfers into another form:

$$A^- + nH^+ = AH_n$$

$$k = \frac{C_1[H^+]^n}{C_2} \tag{A1}$$

where C_1 and C_2 are the concentrations of A and AH_n respectively and n is the number of added protons per molecule:

$$\frac{k}{[H^+]^n} = \frac{C_1}{C_2} \tag{A2}$$

If the fluorescence bands of the fluorophores have the Lorenz shape, the total fluorescence curve has the shape

$$\phi = \frac{\Gamma^2 C_1 q_1}{(w-w_1)^2 + \Gamma^2} + \frac{\Gamma^2 C_2 q_2}{(w-w_2)^2 + \Gamma^2} \tag{A3}$$

where $w_1 = 1/\lambda_1$ and $w_2 = 1/\lambda_2$ are the fluorescence peak positions of the fluorophores, q_1 and q_2 are the quantum yields and Γ is the full width at half-maximum (FWHM) of each fluorophore.

To obtain the peak position of the total fluorescence we find

$$\frac{\delta\phi}{\delta w} = -\frac{\Gamma^2 C_1 2(w-w_1)q_1}{[(w-w_1)^2 + \Gamma^2]^2} - \frac{\Gamma^2 C_2 2(w-w_2)q_2}{[(w-w_2)^2 + \Gamma^2]^2} = 0 \tag{A4}$$

Then

$$\frac{q_1 C_1}{q_2 C_2} = -\frac{w - w_1}{w - w_2}\left(\frac{(w - w_1)^2/\Gamma^2 + 1}{(w - w_2)^2/\Gamma^2 + 1}\right)^2 \tag{A5}$$

If the fluorescence peak positions of the fluorophores differ by less than the FWHM, $|w_1 - w_2| < \Gamma$ and $w_1 < w < w_2$, we have

$$\frac{q_1 C_1}{q_2 C_2} = -\frac{w - w_2}{w - w_1} \tag{A6}$$

and, in terms of λ,

$$\frac{q_1 C_1}{q_2 C_2} = -\frac{1/\lambda - 1/\lambda_2}{1/\lambda - 1/\lambda_1} = \frac{\lambda - \lambda_2 \lambda_1}{\lambda_1 - \lambda \lambda_2} \tag{A7}$$

Since $\lambda_1/\lambda_2 \approx 1$, we can write

$$\frac{q_1 C_1}{q_2 C_2} = -\frac{\lambda - \lambda_2}{\lambda_1 - \lambda} \tag{A8}$$

For the determination of pK of the transition (A1) and taking into account (A2) we obtain

$$pK - n\,pH = \log\left(\frac{\lambda_2 - \lambda}{\lambda - \lambda_1}\right) + \log\left(\frac{q_1}{q_2}\right) \tag{A9}$$

The standard deviation of pK determination is 0.1–0.2 which corresponds to the difference in quantum yields of 25–50%. In our case this difference did not exceed 25%. So we applied the formula

$$pK - n\,pH = \log\left(\frac{\lambda_2 - \lambda}{\lambda - \lambda_1}\right) \tag{A10}$$

In the case of the absorption measurements the calculations are analogous.