The different kinetic behaviour of two potential photosensitizers for PDT

The different kinetic behaviour of two potential photosensitizers for PDT

Nadia Barbero,*[a] Sonja Visentin[b] and Guido Viscardi[a]

[a] *University of Torino, Department of Chemistry, NIS Centre of Excellence, Via P. Giuria 7, 10125 Torino, Italy. Fax: +39 011 670 7591; Tel: +39 011 670 7596; E-mail: nadia.barbero@unito.it*

[b] *University of Torino, Department of Molecular Biotechnology and Health Sciences, Dept. Innovation Center, via Quarello 15B, 10135 Torino, Italy.*

Abstract

Photodynamic therapy (PDT) efficiency is affected by the nature of the binding between the photosensitizer and serum albumin. The kinetic interaction between two potential PDT dyes with bovine serum albumin was studied providing not only the rate constants but also demonstrating that the kinetic of the interaction of the two dyes is very different.

Keywords

Photodynamic therapy, fluorescence kinetics, bovine serum albumin, squaraine, protoporphyrin

ABBREVIATIONS

PDT, Photodynamic therapy; PS, photosensitizers; ROS, reactive oxygen radicals; BSA, Bovine serum albumin; PPIX, protoporphyrin IX; SQ, squaraine dye

Introduction

Photodynamic therapy (PDT)[1] consists in the activation of molecular oxygen under irradiation by light in the presence of photodrugs (photosensitizers, PS) that have been previously selectively accumulated in the target tissues. In comparison with conventional treatment methods (i.e. surgery, chemotherapy, radiation), PDT is considered to be a clinical treatment with high safety, few side effects, reliable repeatability, and relatively low cost. The sensitizer, activated by light, reacts with the oxygen present in the tissue, forming highly toxic oxygen radicals (ROS). These species react with biological molecules such as proteins, amino acids, lipids, nucleotides and nucleic acids inducing tissue necrosis/apoptosis or autophagy. Therefore, PS is responsible of the efficiency of PDT and even if some important developments in PS have been achieved, some

problems still exist[2]. The way of binding of PS to serum albumin is thus crucial to clinical use of PDT.

Serum albumins are the most extensively studied proteins because of their availability, low cost, stability and unusual ligand binding properties[3]. Albumin is the most multifunctional transport protein and has an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood[4] due to the existence of a limited number of binding regions of very different specificity[5]. The nature and the efficiency of the binding between the PS and serum albumin plays an important role for PDT efficiency. Recently, it has been shown that if a molecule possesses affinity for serum albumin, it would probably exhibit efficient PDT applications[6]. Moreover, from a pharmacokinetic point of view, if the binding is very tight and the release is too slow, it will seriously restrict the action of the drug. For this reason, a complete and in-depth study is of the upmost importance to know the affinity and the way of interaction of any PS for albumin.[7]

Among the PS used so far, porphyrins derivatives have an excellent PDT efficacy[8] and some of them have been approved for clinical use[9]. Porphyrins are macrocycles formed by four pyrrole rings bridged by four sp^2 hybridized carbon atoms. The macrocycle is planar, fully conjugated and has a main conjugation pathway of 18 π electrons. Porphyrins have interesting photochemical characteristics and exhibit desirable properties for drug candidates in PDT. These properties include: high quantum yield of singlet oxygen production, absorption at longer wavelengths, preferential tumor location, minimal dark toxicity and stability[10]. These physico-chemical properties are affected by many factors and are important to understand the behaviour of porphyrins and their derivatives in organisms. Therefore, studies of their interaction with membranes, macromolecules and other isolated biological structures are relevant in view of the challenges faced in formulating these drugs into stable, effective and safe dosage forms. Porphyrins have some drawbacks such as relatively poor photostability, low solubility in water, serious skin photosensitization and specific (sometimes undesired) biodistribution[11]. For these reasons there is a need to develop next generation of PS in order to improve photophysical and pharmacokinetic properties; a variety of organic photosensitizers have been investigated like cyanines[12], phtalocyanines[13], dipyrromethenes[14] and squaraines.

Quite recently, the interest in squaraine compounds[15] has been renewed due to their potential application in a large number of fields such as solar-energy conversion[16], nonlinear optics[17], biological applications[18], and sensitizers for photodynamic therapy[19]. Squaraine dyes[20] are the dicondensation product of electronrich

molecules with squaric acid[21]. The main characteristic of this class of compounds is the sharp and intense absorption coupled with a strong fluorescence in solution.

In the present work we studied the kinetic interaction between Bovine Serum Albumin (BSA) and protoporphyrin IX (PPIX) and we compared the results obtained with the interaction between BSA and a squaraine dye (SQ) (Figure 1). Both interactions have already been studied in the literature but only thermodynamic binding constants have been calculated[22,18]. The equilibrium association constants obtained for the interaction between BSA and PPIX are in the order of 10^5 M^{-1} and have been obtained by UV-Vis[22a] and cyclic voltammetry[23]. The same interaction has been studied by surface plasmon resonance[23] to evaluate the association and dissociation binding constants. Moreover, fluorescence spectroscopy[24] has been used to study the effect of pH on the interaction between protoporphyrins and BSA. From the thermodynamic point of view, the two dyes seem to interact in the same order of magnitude but their kinetic behaviour is completely different. The kinetics of these interactions may be important in determining the distribution and metabolic half-life of these potential photosensitizers. To our knowledge this is the first example of a kinetic study of the interaction between BSA and a squaraine dye.

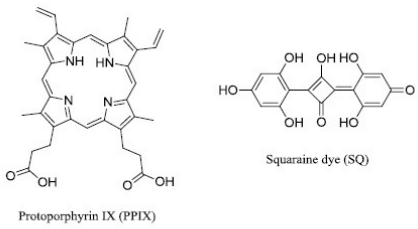


Figure 1. *Structures of protoporphyrin IX (PPIX) and squaraine dye (SQ).*

Experimental procedures

Materials

All reagents were of analytical reagent grade and double distilled water was used. BSA solutions were prepared based on its molecular weight of 66,000. The solution concentrations were chosen after UV-Vis analysis in order to check that no aggregates were present.[25]

Squaraine dye was synthesized by the condensation reaction between squaric acid and phloroglucinol in glacial acetic acid following the literature procedure[20] except for a slightly modification of the crystallization process which was performed in chloroform.

Fluorescence spectroscopy

Fluorescence measurements were recorded using a LS55 Perkin Elmer spectrofluorimeter equipped with a xenon lampsource, a 5 mm path lenght quarz cell and a thermostat bath. As a preliminary analysis, fluorescence spectra were recorded in the range of 300–500 nm upon excitation at 295 nm in order to investigate the binding of PPIX or SQ to BSA. Then, fluorescence experiments were performed in a time drive mode in order to check whether and when the solution had reached the stability. Samples were excited at 295 nm and monitored at 353 nm to check BSA fluorescence. Samples were excited at 409 nm and monitored at 626 nm to check BSA fluorescence. Slits widths were 2.5/5 nm. Fluorescence intensity was recorded every 60 seconds.

Stopped flow fluorescence

Fluorescence kinetics measurements were recorded using an Applied Photophysics SX20 stopped-flow spectrometer fitted with a 320 nm cut-off filter between the cell and fluorescence detector and equipped with a thermostat bath (25°C). Data acquisition, visualisation and analysis were provided by Pro-Data software from Applied Photophysics Ltd.

BSA concentration (2 μM in phosphate buffer pH 7.4) was kept constant and several shots of different SQ concentrations were performed over the range 10-120 μM in phosphate buffer. The reported concentrations are syringe concentrations, this means that the real concentrations in the cuvette are halved. Each experiment (whole concentration set) was repeated five times, each time using new mother solutions of BSA and SQ. For each dilution condition at least five scans were acquired and averaged. Each experimental point is therefore an average of 25 shots. Raw data were analyzed and plotted to a double exponential function by using Pro-Data Viewer 4.0.17 and from this data treatment the observed rate constants (k_{obs}) were obtained. The excitation wavelength was 295 nm and slits widths of the excitation monochromator were 1.5 mm.

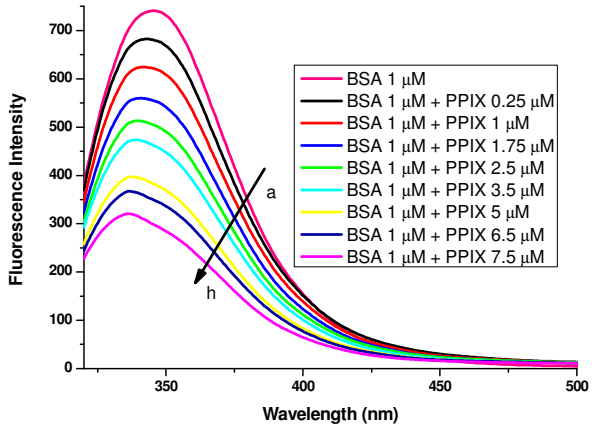
Results

BSA interaction with PPIX

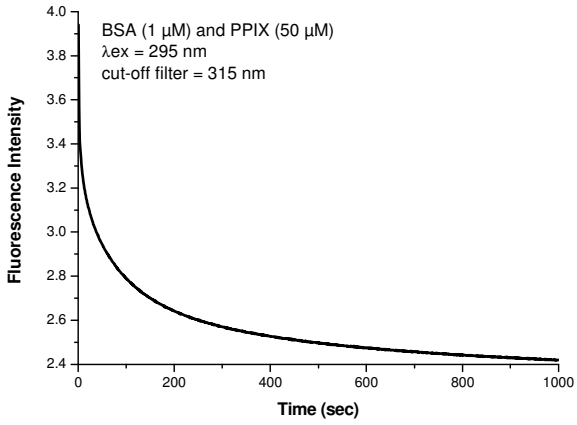
The interaction between BSA and PPIX was followed keeping the BSA concentration constant (1 μM) and

increasing PPIX concentration (from 0.25 µM up to 7.5 µM). The addition of PPIX led to an hypsochromic shift from 345 nm to 336 nm with a decrease in fluorescence intensity (Figure 2A). The same was reported previously and fluorescence quenching demonstrates that porphyrin binding sites are near the tryptophan residues of BSA.[23] But from the kinetic point of view, if we monitor this interaction with time we can notice that fluorescence intensity at 345 nm is continuously changing even after one hour. We report in Figure 2B and 2C the change of fluorescence intensity with time after mixing BSA (1 µM) and PPIX at different concentrations. We then tried to monitor the same interaction by following fluorescence intensity of the fluorophore therefore exciting at 626 nm but also in this case, as is evident in Figure 2D, the signal is continuously changing even after some hours. We tried to record the intensity change of fluorescence by mixing PPIX (2.5 µM) and BSA (5 µM) and we noticed that it never reached a plateau even after 5 hours (Figure 2D). The kinetic constant of the interaction was therefore impossible to evaluate.

a



b



c

d

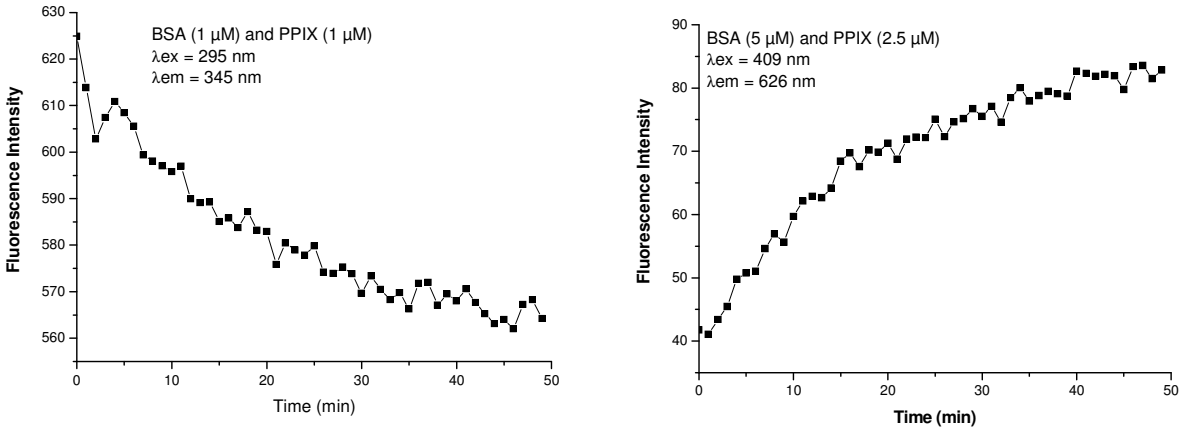


Figure 2. *A) Change in fluorescence emission of BSA (1 µM) with the addition of PPIX in phosphate buffer, after 30 minutes incubation. [PPIX] (a) 0, (b) 0.25 µM, (c) 1 µM, (d) 1.75 µM, (e) 2.5 µM, (f) 3.5 µM, (g) 5 µM, (h) 6.5 µM, (i) 7.5 µM. B) Fluorescence intensity changes vs. time recorded by stopped-flow apparatus (excitation at 295 nm, cut-off filter at 315 nm) for the interaction between BSA (1 µM) and PPIX (50 µM). C) Fluorescence intensity changes vs. time recorded at 345 nm upon excitation at 295 nm for the interaction between BSA (1 µM) and PPIX (1 µM). D) Fluorescence intensity changes vs. time recorded at 626 nm upon excitation at 409 nm for the interaction between BSA (5 µM) and PPIX (2.5 µM).*

The thermodynamic binding constants can be determined by fixing an incubation time of one hour and by using the Modified Stern-Volmer Equation (equation 1)[3c] from which it is possible to get the K_a from the antilog of the intercept and the n (i.e. the number of binding sites) value from the slope of the straight regression line (R^2=0.98) as showed in Fig. 3:

$$\log\frac{F_0 - F}{F} = \log K_a + n\log[Q] \qquad (1)$$

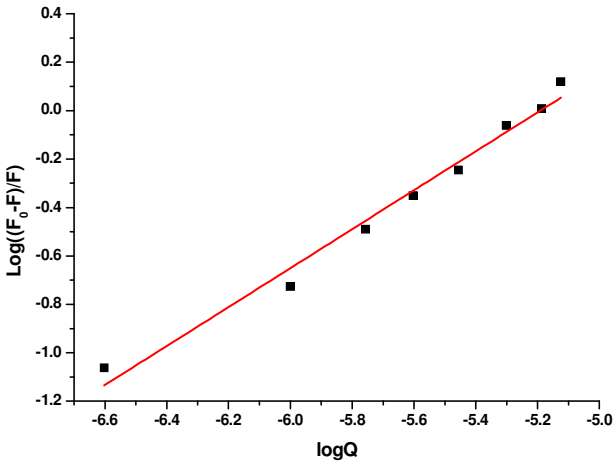


Figure 3. *Modified Stern–Volmer plot of the BSA-PPIX experiment.*

The values of K_a and n were $1.15 \cdot 10^4$ M^{-1} and 0.8, respectively, indicating that there is only one binding site for porphyrin on BSA. These results are in agreement with the data obtained by Xiao et al.[23], in particular with their data obtained by SPR technique. On the contrary, our results differ from their fluorescence quenching data, always reported in the same paper. This difference can be due to the fact that we pay

attention to reach the saturation. Therefore, our fluorescence results are a more realistic description of the interaction and indeed more comparable with their kinetic rather than thermodynamic studies. Actually, Sulkowski et al.[22a] had studied the time dependence of the spectral properties of PPIX and observed a change in the UV-Vis spectra of the PPIX alone in solution.

BSA interaction with SQ

On the contrary of what observed for PPIX, the interaction between SQ and BSA was immediate with a decrease in fluorescence intensity with an hypsochromic shift from 345 to 329 nm (Figure 4a). The greater solubility of SQ allows us to work at higher concentrations compared to that used for PPIX. As we monitored this interaction with time, we noticed that in this case the interaction was too fast to be followed by a common spectrofluorimeter.

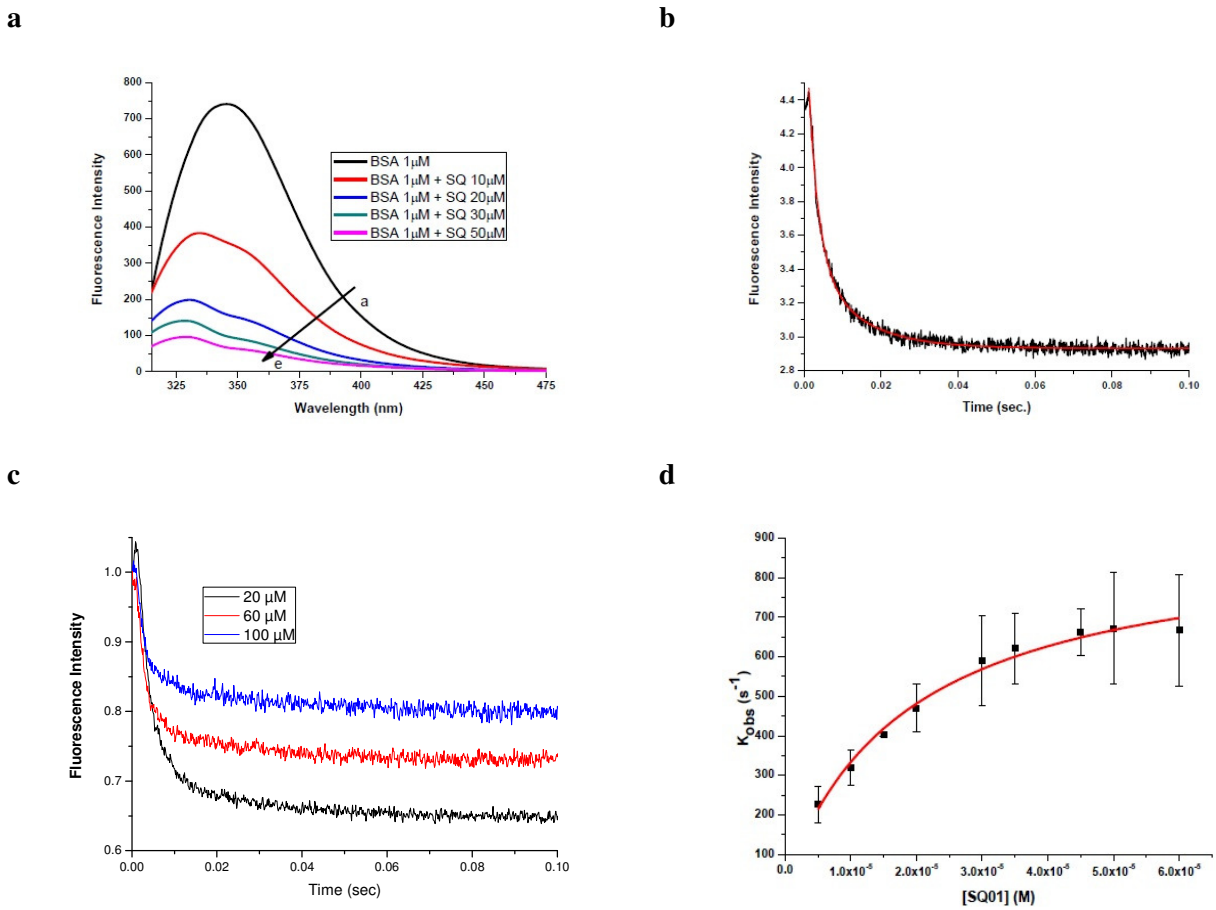


Figure 4. *A) Change in fluorescence emission of BSA (1 μM) with the addition of SQ in phosphate buffer. [SQ] (a) 0, (b) 10 μM, (c) 20 μM, (d) 30 μM, (e) 50 μM. B) The stopped-flow fluorescence intensity record of the binding of 1 μM BSA to 10 μM SQ. The solid thin red line is the best fit to the data providing k_{obs} values. C) The stopped-flow fluorescence intensity record of the binding of 1 μM BSA to 20, 60 and 100 μM SQ. D) The dependence of the k_{obs} determined by the fluorescence intensity of the binding of BSA to increasing SQ concentration. Data points are the mean of five independent experiments, standard deviation is also reported. In one point error bar is not visible as it is concealed by the data point.*

For this reason we examined the kinetic interaction of the binding between SQ and BSA by stopped flow fluorescence. The binding was investigated under *pseudo*-first order condition[26] ([SQ] >> [BSA]) by monitoring the fluorescence changes after the formation of the complex. Figure 4b shows that on mixing 1 μM BSA with 10 μM SQ (cell concentration) there is a decrease of the signal which reaches a plateau after

few ms. Raw data were analyzed and plotted to a double exponential function.

The interaction was studied by keeping BSA concentration fixed at 1 μM and changing the SQ concentration (Figure 4c). The dependence of the observed rate constant (k_{obs}) of SQ binding to BSA (SQ concentration over a range of 5-60 μM) is shown in Figure 4d.

Although the dependence is linear at lower concentrations, there is evidence of hyperbolic behaviour at higher concentrations. This suggests that there are two steps in the binding interaction.[27] A graph of $1/k_{obs}$ against 1/[SQ] is linear within experimental accuracy (see Fig. 5) and is compatible with a two-step binding mechanism.

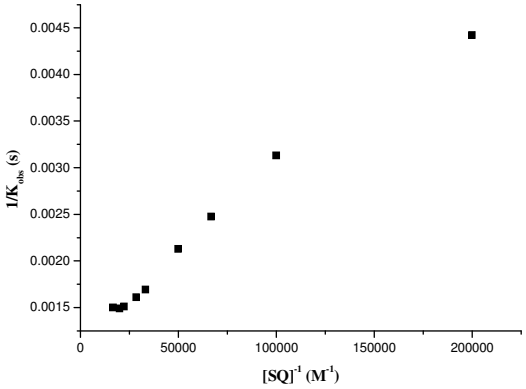


Figure 5. *Secondary plot of the data reported in figure 4d.*

The binding interaction between the squaraine (SQ) and the protein (P) can be described by a minimal, two-step mechanism (Equation 2) where a fast, diffusion controlled pre-equilibrium step is followed by a second slower step, which leads to the formation of a stable complex. The stopped-flow apparatus can follow and study only the second step, since step I is completed within the mixing time.

$$\text{SQ} + \text{P} \underset{\text{I}}{\rightleftharpoons} (\text{SQ-P})^* \underset{\text{II}}{\rightleftharpoons} \text{SQ-P} \qquad (2)$$

When the squaraine/protein ratio is under pseudo first-order condition the dependence of the observed relaxation rate constants on protein concentration fulfilled equation 3.[28] This allows the determination of the equilibrium association binding constant of step I (K_1) and the rate constants for step II (k_2 and k_{-2}).

$$k_{obs} = k_2 \frac{K_1[SQ]}{K_1[SQ]+1} + k_{-2} \qquad (3)$$

The experimental data (K_{obs} vs. squaraine concentration) were fitted to equation 3 finding: K_1 ($5.3 \cdot 10^4$ M^{-1})

which represents the association binding constants of step 1 and k_{+2} and k_{-2} which are the rate constants of the second step whose values are 880 $M^{-1}s^{-1}$ and 30 s^{-1} respectively. From these values the dissociation binding constant of step 1 (K_{-1} = $1.9 \cdot 10^{-5}$ M) and association and dissociation binding constants of step 2 (K_{+2} = 29.33 M^{-1} e K_{-2} = $3.4 \cdot 10^{-2}$ M) can be determined. It is not easy to compare these data with the binding constants obtained previously[18a] because in that paper they reported the binding constant of the overall process while, in this paper, we were able to better describe the way of interaction of BSA with SQ01. In both cases there is evidence of a two-site interaction (i.e. SQ01 interacts on both BSA sites). The power of this approach is that we can better describe the interaction showing its occurrence in a two steps mechanism.

The evaluation of the binding between the photosensitizers to their macromolecular targets is crucial for pharmacological activity. Copeland et al.[29] asserted that sometimes the *in vivo* efficacy of a ligand can be related to the equilibrium dissociation constant measured *in vitro*. In fact, one of the most important aspects for *in vivo* drug efficacy is not only the apparent affinity of the drug for its target, but rather the period in which the receptor is occupied by the ligand (i.e. residence time). Also in the case of plasma protein-drug complexes the duration of the interaction may play an important role in the equilibrium process between unbound and bound drug concentrations.

Conclusions

The nature and of drug–albumin interaction significantly influences the pharmacokinetics of the drug and the binding parameters influence absorption, distribution, metabolism, and excretion properties of typical drugs.

Here we showed that the kinetic behaviour of PPIX and SQ is completely different: SQ has a very fast kinetic interaction with BSA and a greater solubility. These aspects make the class of squaraines very interesting as potential drugs in PDT. Further studies are in progress to understand the squaraines structure-relationship influence on the kinetic interaction with serum albumins. The correct determination of the binding parameters of ligand–receptor interaction is important for fundamental investigations and practical aspects for the development of potential drugs like squaraines in PDT.

Acknowledgements

The authors thank University of Torino (Ricerca Locale ex-60%, Bando 2012). S.V. acknowledges financial support by NANOMED project (PRIN 2010-2011, 2010FPTBSH_003) from Ministero dell'Istruzione,

dell'Università e della Ricerca. N.B. thanks MIUR for partial financial support of their Research grants.

References

1 (a) Lang K, Mosinger J, Wagnerová DM. Photophysical properties of porphyrinoid sensitizers non-covalently bound to host molecules; models for photodynamic therapy. Coord Chem Rev; 2004;248: 321–50. (b) Moan J, Peng Q. An outline of the hundred-year history of PDT. Anticancer Res; 2003;23: 3591–600.

2 Bechet D, Couleaud P, Frochot C, Viriot M-L, Guillemin F, Barberi-Heyob M. Nanoparticles as vehicles for delivery of photodynamic therapy agents. Trends Biotechnol; 2008;26: 612–621.

3 (a) Peters T. Serum albumin. Adv Prot Chem; 1985;37: 161–245. (b) Carter DC, Ho JX. Structure of serum albumin. Adv Prot Chem; 1994;45: 153–203. (c) Barbero N, Barni E, Barolo C, Quagliotto P, Viscardi G, Napione L, Pavan S, Bussolino F. A study of the interaction between fluorescein sodium salt and bovine serum albumin by steady-state fluorescence. Dyes Pigments; 2009. 80;307–313.

4 Kosa T, Maruyama T, Otagiri M. Species differences of serum albumins: I. Drug binding sites. Pharm Res; 1997;14: 1607–12.

5 Kragh-Hansen U. Molecular aspects of ligand binding to serum albumin. Pharm Rev; 1981;33: 17–53.

6 (a) Szacilowski K, Macyk W, Drzewiecka-Matuszek A, Brindell M, Stochel G. Bioinorganic photochemistry: frontiers and mechanisms. Chem. Rev.; 2005;105: 2647-2694. (b) Pandey RK, Constantine S, Tsuchida T, Zheng G, Medforth CJ, Aoudia M, Kozyrev AN, Rodgers MAJ, Kato H, Smith KM, Dougherty TJ. Synthesis, photophysical properties, in vivo photosensitizing efficacy, and human serum albumin binding properties of some novel bacteriochlorins. J. Med. Chem.; 1997;40: 2770-2779.

7 Tsuchida T, Zheng G, Pandey RK, Potter WR, Bellnier DA, Henderson BW, Kato H, Dougherty TJ. Correlation between site II-specific human serum albumin (HSA) binding affinity and murine in vivo photosensitizing efficacy of some Photofrin components. Photochem. Photobiol.; 1997;66: 224-228.

8 Sternberg ED, Dolphin D, Brückner C. Porphyrin-based photosensitizers for use in photodynamic therapy. Tetrahedron; 1998;54: 4151–4202.

9 Henderson BW, Dougherty TJ. How does photodynamic therapy work? Photochem. Photobiol.; 1992;55: 145–157.

10 Rinco O, Brenton J, Douglas A, Maxwell A, Henderson M, Indrelie K, Wessels J, Widin J. The effect of porphyrin structure on binding to human serum albumin by fluorescence spectroscopy. J. Photochem. Photobiol. A-Chem.; 2009;208: 91–96.

11 Yano S, Hirohara S, Obata M, Hagiya Y, Ogura S, Ikeda A, Kataoka H, Tanaka M, Joh T. Current states and future views in photodynamic therapy. J. Photochem. Photobiol. C: Photochem. Rev.; 2011;12: 46-67.

12 Delaey E, van Laar F, De Vos D, Kamuhabwa A, Jaobs P, De Witte PJ. A comparative study of the photosensitizing characteristics of some cyanine dyes. Photochem. Photobiol. B-Biol.; 2000;55: 27–36.

13 Wöhrle D, Shopova M, Muller S, Milev AD, Mantareva VN, Krastev KK. Effect of delivery system on the pharmacokinetic and phototherapeutic properties of bis(methyloxyethyleneoxy)silicon-phthalocyanine in tumor-bearing mice. J. Photochem. Photobiol. B-Biol.; 1999;50: 124–128.

14 Gorman A, Killoran J, O'Shea C, Kenna T, Gallagher WM, O'Shea DF. In vitro demonstration of the heavy-atom effect for photodynamic therapy. J. Am. Chem. Soc.; 2004;126: 10619–10631.

15 Liang K, Law KY. Synthesis, Characterization, Photophysics, and Photosensitization Studies of Squaraine−Bipyridinium Diads. J. Phys. Chem. B; 1997;101: 540–546.

16 (a) Park J, Barolo C, Sauvage F, Barbero N, Benzi C, Quagliotto P, Coluccia S, Di Censo D, Grätzel M, Nazeeruddin MdK, Viscardi G. Symmetric vs. asymmetric squaraines as photosensitisers in mesoscopic injection solar cells: a structure-property relationship study. Chem. Commun.; 2012;48: 2782–2784; (b) Magistris C, Martiniani S, Barbero N, Park J, Benzi C, Anderson A, Law C, Barolo C, O'Regan B. Near-infrared absorbing squaraine dye with extended π conjugation for dye-sensitized solar cells. Renew. Energ.; 2013;60: 672–678.

17 (a) Ashwell GJ, Ewington J, Moczko K. Orientation-induced molecular rectification and nonlinear optical properties of a squaraine derivative. J. Mater. Chem.; 2005;15: 1154–1159. (b) Beverina L, Salice P. Squaraine Compounds: Tailored Design and Synthesis towards a Variety of Material Science Applications. Eur. J. Org. Chem.; 2010;1207–1225.

18 (a) Jisha VS, Arun KT, Hariharan M, Ramaiah D. Site–Selective Binding and Dual Mode Recognition of Serum Albumin by a Squaraine Dye. J. Am. Chem. Soc.; 2006;128: 6024–6025. (b) McEwen JJ, Wallace KJ. Squaraine dyes in molecular recognition and self-assembly. Chem. Commun.; 2009;6339–6351.

19 Avirah RR, Jayaram DT, Adarsh A, Ramaiah D. Squaraine dyes in PDT: from basic design to in vivo demonstration. Org. Biomol. Chem.; 2012;10: 911–920.

20 Treibs A, Jacob K. Cyclotrimethine Dyes Derived from Squaric Acid Angew. Chem. Int. Ed. Engl.; 1965;4: 694.

21 Park JD, Cohen S, Lacher JR. Hydrolysis Reactions of Halogenated Cyclobutene Ethers: Synthesis of Diketocyclobutenediol. J. Am. Chem. Soc.; 1962;84: 2919–2922.

22 (a) Sulkowski L, Sulkowska A, Rownicka J, Bojko B, Sieron A, Pentak D, Sulkowski WW. The effect of serum albumin on binding of protoporphyrin IX to phospholipid membrane. Mol. Cryst. Liq. Cryst.; 2006;448: 73–81. (b) Jisha VS, Arun KT, Hariharan M, Ramaiah D. Site Selective interactions: Squarenedyes- Serum Albumin Complexes with Enhanced Flourescence and Triplet yields. J. Phys. Chem. B; 2010;114: 5912–5919.

23 Xiao C–Q, Jiang F–L, Zhou B, Lia R, Liu Y. Interaction between a cationic porphyrin and bovine serum albumin studied by surface plasmon resonance, fluorescence spectroscopy and cyclic voltammetry. Photochem. Photobiol. Sci.; 2011:10: 1110–1117.

24 Borissevitch IE, Tominaga TT, Imasato H, Tabak M. Fluorescence and optical absorption study of interaction of two water soluble porphyrins with bovine serum albumin. The role of albumin and porphyrin aggregation. J. Luminescence; 1996;69: 65–76.

25 (a) S. Das, T. L. Thanulingam, K. G. Thomas, P. V. Kamat, M. V. George, Photochemistry of squaraine dyes. 5. Aggregation of bis(2,4-dihydroxyphenyl)squaraine and bis(2,4,6-trihydroxyphenyl)squaraine and their photodissociation in acetonitrile solutions. J. Phys. Chem.; 1993;97: 13620-13624; (b) L. Monsù Scolaro, M. Castriciano, A. Romeo, S. Patanè, E. Cefalì, M. Allegrini. Aggregation Behavior of Protoporphyrin IX in Aqueous Solutions: Clear Evidence of Vesicle Formation. J. Phys. Chem. B; 2002;106: 2453-2459.

26 Barbero N, Napione L, Visentin S, Alvaro M, Veglio A, Bussolino F, Viscardi G. A transient kinetic study between signaling proteins: the case of the MEK–ERK interaction. Chemical Science; 2011;2: 1804-1809.

27 J. Wilting, J.M.H. Kremer, A. P. Ijzerman, S.G. Schulman, The kinetics of the binding of warfarin to human serum albumin as studied by stopped-flow spectrophotometry. Biochimica Biophysica Acta; 1982;706: 96-104.

28 I. Fitos, J. Visy, J. Kardos, Stereoselective kinetics of warfarin binding to human serum albumin: Effect of an allosteric interaction. Chirality; 2002;14: 442-448.

29 R.A. Copeland, D.L. Pompliano, T.D. Meek, Drug-target residence time and its implications for lead optimization. Nature Reviews Drug Discovery, 2006;5: 730-739.