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The pH-dependent distribution of the photosensitizer chlorin e6 among plasma proteins and membranes: A physico-chemical approach

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Abstract

Decrease in interstitial pH of the tumor stroma and over-expression of low density lipoprotein (LDL) receptors by several types of neoplastic cells have been suggested to be important determinants of selective retention of photosensitizers by proliferative tissues. The interactions of chlorin e6 (Ce6), a photosensitizer bearing three carboxylic groups, with plasma proteins and DOPC unilamellar vesicles are investigated by fluorescence spectroscopy. The binding constant to liposomes, with reference to the DOPC concentration, is 6×10^3 M^{-1} at pH 7.4. Binding of Ce6 to LDL involves about ten high affinity sites close to the apoprotein and some solubilization in the lipid compartment. The overall association constant is 5.7×10^7 M^{-1} at pH 7.4. Human serum albumin (HSA) is the major carrier (association constant 1.8×10^8 M^{-1} at pH 7.4). Whereas the affinity of Ce6 for LDL and liposomes increases at lower pH, it decreases for albumin. Between pH 7.4 and 6.5, the relative affinities of Ce6 for LDL versus HSA, and for membranes versus HSA, are multiplied by 4.6 and 3.5, respectively. These effects are likely driven by the ionization equilibria of the photosensitizer carboxylic chains. Then, the cellular uptake of chlorin e6 may be facilitated by its pH-mediated redistribution within the tumor stroma.

Keywords: Photosensitizer; pH; Albumin; Lipoprotein; Model membranes

1. Introduction

The therapeutic use of photosensitizing drugs is based on light-induced generation of reactive species that damage surrounding biological structures [1]. The selective accumulation of photosensitizers in proliferating tissues and the possibility to define the limits of the irradiated zone are two main factors insuring the specificity of photodynamic action [2]. The space diffusion of the reactive species, namely oxyradicals and singlet oxygen, is limited by their extremely short life time [3]. Thus the extent of the photoinduced damage is restricted to the structures labeled by the photosensitizer. Consequently, the uptake and/or retention of photosensitizers by targeted cells or tissues are crucial determinants of their efficiency. Several explanations have been proposed to clarify the selective uptake of porphyrin-type photosensitizers by neoplastic tissues. Firstly, extracellular accumulation of lactic acid results in the acidification of tumor interstitial matrix [4,5]. Consistent data obtained on cultured cells [6,7], animals [8] and membrane models [9] present strong evidence for a major role of the pH gradient thus created in the selective tumoral uptake of photosensitizers bearing carboxylic chains.

Another important determinant of the cellular incorporation of photosensitizers is their binding to low density lipoproteins (LDL). This association influences both the overall cellular uptake and the internalization pathway of the drug. The role of lipoproteins as blood carriers of photosensitizers has been proposed by several authors [10–12]. LDL are considered as a targeting and delivery system of lipophilic or amphiphilic photosensitizers [13]. Moreover, increased cholesterol requirements of proliferating tissues result in the over-expression of LDL receptors on the cell surface [14,15]. Thus, the cellular incorporation of lipoprotein bound photosensitizers via LDL-specific endocytosis has been suggested to be one of the main mechanisms of their preferential accumulation by tumors. Low-

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density lipoproteins are nearly spherical, highly plastic particles with diameters of between 210 and 250 Å. The LDL lipid core containing cholesteryl esters and triglycerides is surrounded by a monolayer of cholesterol and phospholipids. The large apoprotein B100 (500 kDa), associated to the phospholipid envelope, contributes to the overall structure of the particle and ensures its recognition by cellular receptors [16]. The number of photosensitizers bound to LDL and their localization within these particles are important determinants of this transportation mode.

In addition, the bioavailability of photosensitizers is governed by the competitive binding to albumin, the major protein in plasma [17]. The distribution of certain photosensitizers with various degrees of lipophilicity and numbers of charges among plasma proteins has been studied by means of ultracentrifugation [18–21]. A general finding was that the fraction of the dyes bound to LDL increased, and the fraction bound to HSA decreased with decreasing polarity of the dyes. However, the relative binding to these proteins was also dependent on the position of charges around the macrocycle [18]. It must be noted that the permeability of neovessels may allow leakage of albumin-bound photosensitizers into the tumor stroma, which would also lead to some selective retention [22].

In this study, we consider chlorin e6 (Ce6) that bears three carboxylic groups (see Fig. 1). This molecule was chosen as it enabled to the verification of the effect of the number of carboxylic groups when compared to dicarboxylic porphyrins. It is also relevant to therapy as a second-generation photosensitizer [23]. Quantitative data on the interactions of Ce6 with various potential serum carriers, as well as with cell-mimicking membrane systems, are derived in this paper. The effect of pH is particularly emphasized.

2. Material and Methods

2.1. Chemicals

Chlorin e6 (Fig. 1) was purchased from Porphyrin Products, Logan (UT, USA). A stock solution (1 mM) was prepared in 20 mM $Na_2H\ PO_4$. The experimental Ce6 solutions were diluted in phosphate buffer saline, PBS (20 mM KH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH indicated for each experiment) and handled in the dark.

Human serum albumin (HSA) was purchased from Sigma (St. Louis, MO, USA). Experimental solutions were prepared in PBS at the desired pH and were used immediately.

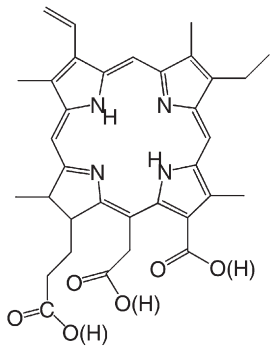

Fig. 1. Structure of chlorin e6.

Human low-density lipoproteins (LDL) were purchased from Calbiochem (San Diego, CA, USA). They were conditioned at a concentration of 9.52 mg/ml (protein content) in 150 mM NaCl pH 7.4 aqueous solution with 0.01% EDTA.

Dioleyol-*sn*-phosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform (Merck, Darmstadt, Germany) was of spectroscopic grade quality. Triton X-100 was purchased from Sigma (St. Louis, MO, USA).

2.2. Liposome preparation

DOPC was dissolved in chloroform and the solution was taken to dryness. The lipid film obtained was rehydrated in PBS and vortexed for several minutes. The liposome suspension was extruded 10 times through a stack of two polycarbonate membrane filters (Poretics, Livermore, CA, USA) with a pore size of 50 nm using an extruder device (Lipex, Biomembranes, Vancouver, Canada).

2.3. Fluorescence measurements

Fluorescence measurements were performed with an Aminco Bowman Series 2 spectrofluorimeter. The samples were contained in a 1 cm quartz cell and were stirred during the acquisition.

2.3.1. Partition experiments: Incorporation of Ce6 in DOPC vesicles

For experiments at equilibrium, the DOPC liposome solutions were prepared at different concentrations. 10 μL of 10 μM Ce6 solution were added to 2 ml of vesicle preparation and the fluorescence spectra were recorded. In order to correct the spectra for small differences in Ce6 concentration arising from experimental inaccuracy, 20 μL of Triton-X100 were added after measurement leading to disruption of vesicles and solubilization of all Ce6 in the Triton micelles. The spectra were normalized accordingly.

The global binding constant, K_B, was derived from changes in the fluorescence signal at a wavelength corresponding to the maximum of fluorescence emission of Ce6 incorporated into the membrane. We used the previously derived relationship [24]:

$$F = F_0 + \frac{(F\infty - F_0) \times K_B \times [\mathrm{DOPC}]}{1 + K_B \times [\mathrm{DOPC}]} \quad (1)$$

where F_0, F_∞ and F are the fluorescence intensities corresponding to zero, total and intermediate incorporation of chlorin into vesicles, respectively. DOPC being in large excess, the saturation of the bilayer is far to be reached and it can be assumed that Ce6 binding does not affect the properties of the model membrane. Then, regardless of the number of Ce6 molecules incorporated into a vesicle, [DOPC] was assumed to be equivalent to the total DOPC concentration added.

2.3.2. Binding to HSA and LDL

Contrary to former partition experiments, the interactions of Ce6 with HSA and LDL involved a limited number of sites. Moreover, due to the high affinity of chlorin to HSA and LDL, the concentration of Ce6 and that of the macromolecules were of the same order of magnitude in our experimental conditions. Consequently, the concentration of free sites on HSA or LDL was calculated by subtracting the amount of bound chlorin to the total number of binding sites per molecule. For this purpose, the concentrations of free and bound Ce6 were calculated by a spectral decomposition program running with the MatLab® (MathWorks, Natick, MA) software according to the equation:

$$(\mathrm{SP}) = \begin{pmatrix} Comp1 \\ Comp2 \end{pmatrix} \times (f_{\mathrm{PBS}}, f_B) \quad (2)$$

where SP is the experimental spectrum, Comp1 and Comp2 are the spectra of Ce6 in PBS and bound to the macromolecule, respectively. Comp2 was obtained using an excess of the macromolecule to insure total binding. The relative concentrations of free and bound chlorin are given by the factors f_{PBS} and f_B.

The equilibrium of Ce6 binding to the plasma proteins can be written as:

$$[\mathrm{Chl}]_F + [\mathrm{P}]_F \underset{}{\overset{K_B}{\rightleftharpoons}} [\mathrm{Chl}]_B$$