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Incorporation of hydrophobic chlorin photosensitizers into a liposome membrane

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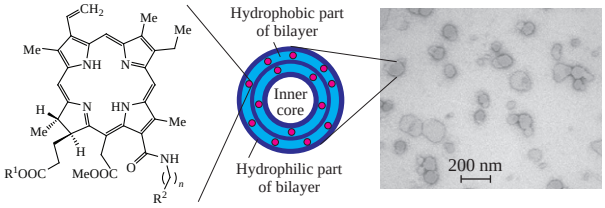
A reliable procedure for the incorporation of hydrophobic chlorin photosensitizers into a lipid bilayer was proposed to obtain water-soluble agents for the photodynamic therapy of cancer.

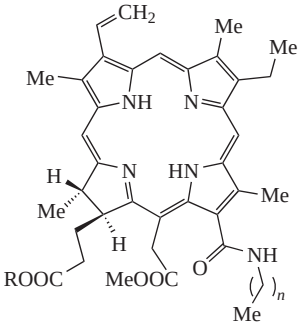


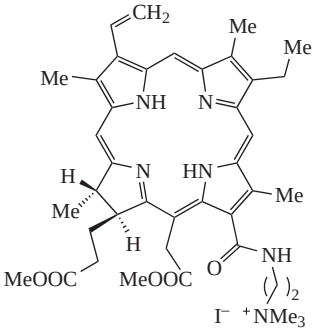
Photodynamic therapy (PDT) is a promising method for the treatment of different deseases.[1–4] However, a great number of photosensitizers are hydrophobic and, as a consequence, insoluble in biological fluids. To overcome solubility problems, the chemical modification of the structure or carriers such as polymers or nanoparticles can be used. In our opinion, the incorporation of water-insoluble compounds in lipid vesicles (liposomes) is the most effective method. Liposome systems are widely employed.[5] The main advantage of liposomes is the ability of transforming hydrophobic compounds into nanodisperse forms soluble in water systems. This allows one to use the well-known enhanced permeability and retention effect (EPR effect) for intravenous administration and thus to increase the efficacy and therapeutic index.[5–7]

The incorporation of drugs into the lipid bilayer has been studied.[8–10] The modification of water-soluble structures to hydrophobic ones for further incorporation into the hydrophobic part of a liposome was described.[11] Thus, we choose the hydrophobic amide derivatives of chlorin e_6 as key compounds for liposome preparation.

Here, we describe the preparation and characterization of liposomes loaded with six hydrophobic chlorin e_6 derivatives containing 6–12 carbon atoms in the alkyl chain (compounds **1**–**6**).

The synthesis of the amide derivatives of chlorin e_6 from pheophorbide *a* and its methyl ester was described in detail earlier.[12,13]





1 R = H, *n* = 5
2 R = H, *n* = 6
3 R = Me, *n* = 5
4 R = Me, *n* = 6
5 R = H, *n* = 11

6

In order to produce the liposomal forms of photosensitizers, we used soybean phosphatidylcholine (PC, Lipoid S100, $t_m \approx 27$ °C) as a lipid. The photosensitizers were encapsulated according to a thin-film hydration method.[10] Lipid was dissolved in $CHCl_3$ and mixed with a photosensitizer solution in $CHCl_3$ leading to a composition with PC : photosensitizer molar ratios from 5 : 1 to 50 : 1. The organic solvent was removed in an evaporator, and the resulting dry lipid films were hydrated with 2 ml of NaCl buffered saline (pH 7.4). Then, the solution was frozen and thawed three times to form a hydrated lipid suspension of multilamellar liposomes with incorporated photosensitizers. The suspension was sonicated in an ultrasonic bath at 26–29 °C. This step is optional, but it facilitates the subsequent extrusion process.

Liposome suspensions were thermostated at 29 °C and extruded 17–19 times through 0.2 μm-pore-diameter polycarbonate membrane filters (Isopore™; Millipore) using an extruder (Northern Lipids Inc.). In a case of the incomplete incorporation of photosensitizers after an extrusion process, we observed a green precipitate of photosensitizers on the membrane filter. Varying the conditions of liposome preparation, mainly the lipid : photosensitizer molar ratio, we succeeded in obtaining liposomes with the complete incorporation of photosensitizers.

Note that cholesterol is a commonly used constituent of liposomal drug formulations due to its ability to modulate membrane permeability and biological stability.[14,15] Membrane permeability depends on the composition of the membrane, the transition temperature of the bulk phospholipid used and the amount of cholesterol incorporated. For example, cholesterol at concentrations above 30 mol% causes an increase in chain disorder at temperatures below the phase transition temperature of the bulk phospholipid component.[16,17] Since the structure of our photosensitizers incorporated into a lipid bilayer was rigid, it allowed us to avoid the use of cholesterol as a component affecting the liposome fluidity.

The size of the liposomes loaded with photosensitizers was determined using dynamic light scattering (DLS) and transmission electron microscopy (TEM). For DLS the samples were diluted with NaCl buffered saline (pH 7.4) and placed in a Delsa Nano C dynamic light scattering instrument (Beckman Coulter), which was equipped with a laser (653 nm). Note that substances that absorb in this spectral region make some difficulties

Table 1 Size distributions.

Photosensitizer	PC / photosensitizer molar ratio	Size/nm
1	10 : 1	223.9
	20 : 1	216.5
	40 : 1	232.9
2	5 : 1	110.3
	10 : 1	239.4
	20 : 1	214.2
	30 : 1	203.7
	40 : 1	236.6
3	30 : 1	148.5
4	30 : 1	136.4
5	20 : 1	103.6
	50 : 1	178.4
6	5 : 1	125.7
	10 : 1	140.1
	20 : 1	207.4

for obtaining fruitful results. Only samples with photosensitizer concentrations of 10^{-6} mol dm^{-3} or lower gave reliable results (see Table 1 and Figures 1 and 2). Micrographs were made using a JEM-100CX electron microscope (Jeol) and a Perfection 3200 Photo scanner (Epson).

The photosensitizer incorporation efficiency was evaluated by measuring the absorption of a liposomal sample after extrusion and free photosensitizer forms and calculating a ratio of peak intensities in the UV and visible regions of two spectra. The UV-VIS spectra were recorded on a JASCO UV/VIS 7800 spectrophotometer in a range of 350–750 nm in acetone. Before the UV-VIS spectroscopic measurements, the liposomes were disrupted by the addition of 80 volumes of acetone. In a case of the incomplete incorporation of photosensitizers, we observed a decrease in absorption intensity in the UV and visible regions of the spectra; the absorption intensity strongly depends on photosensitizer concentration. The dependence of the efficiency of incorporation of compound **2** on excess lipid used is shown in Figure 3. In our case, the incorporation efficiency is a ratio of spectrally measured amount of the incorporated photosensitizers in the samples after extrusion to that in the samples before extrusion / free photosensitizers.

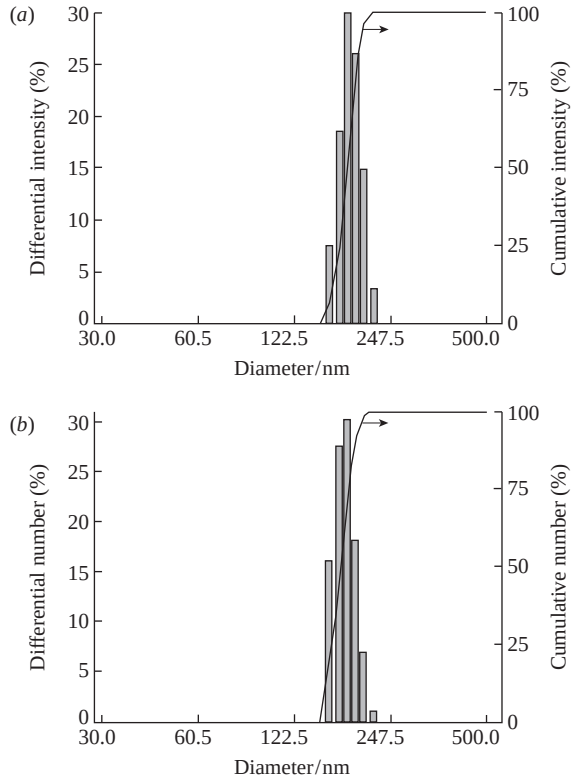


Figure 1 DLS results for liposomes with encapsulated photosensitizers **2** (a PC : photosensitizer molar ratio of 20 : 1): (*a*) intensity and (*b*) number distribution.

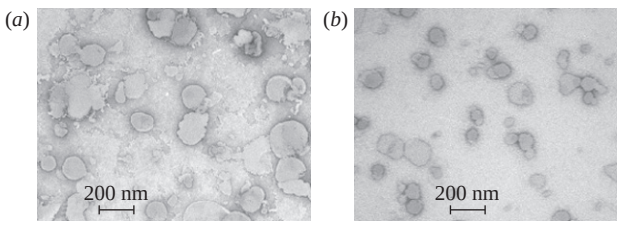


Figure 2 TEM images of liposomes with encapsulated photosensitizers: (*a*) PC : photosensitizer **1** (10 : 1), and (*b*) PC : photosensitizer **2** (5 : 1).

Unincorporated photosensitizers were not detected in the systems with compounds **1**, **2** and **6** even at a lipid : photosensitizer molar ratio of 10 : 1. Meanwhile, we observed unincorporated photosensitizers (as precipitates on filter membranes) for compounds **3**–**5** using a lipid : photosensitizer molar ratio of 20 : 1. It means that optimal conditions for liposome preparation depend on the length of a hydrophobic hydrocarbon moiety in photosensitizers.

In addition, we studied the changes occurred during the storage of samples. For the evaluation of the form and size stability of liposomal photosensitizers, we used DLS and TEM techniques. All of the test samples showed the high stability of liposomal complexes of chlorin e_6 amide derivatives (namely, equal in size and shape for samples before/after freezing) at –5 °C for 10 months and at 2–3 °C for 2 months without changes (after ultrasonic treatment). When the compounds were situated in the hydrophobic layer (as in our case), the liposomal form of a drug can be stable without a cryoprotectant.[18] Apparently, during freezing of the liposomal dispersion the drug compound remains in the bilayer in contrast to water-soluble substances.

In summary, we prepared the liposomal forms of the hydrophobic photosensitizers and studied the physical properties (UV-VIS spectra, size, incorporation efficiency and stability) of chlorin e_6-loaded liposomes. We found experimentally that the incorporation efficiency and the amount of lipid used depend on the photosensitizer structure, namely, its hydrophobicy. The photosensitizers with COOMe groups (compounds **3**, **4**) instead of COOH (compounds **1**, **2**) in the 17(3) position of a macrocycle required a greater molar excess of lipid per photosensitizer molecule for full incorporation into a lipid bilayer. At the same time, the cationic photosensitizer with the ester group in the 17(3) position (compound **6**) bearing a positive charge in the 13(3) position required a minimum excess of lipid for full incorporation into liposome.

To estimate the biological activity of our liposomal forms of photosensitizes, we carried out an *in vitro* experiment on cancer cells (P-388). The experimental procedure was the same as that reported earlier for the free form of photosensitizers.[13] The photoactivity of the liposomal forms of compound **2** was at the same level as that of the free form of photosensitizer **2** (the amount of damaged cells after irradiation). In this case, an

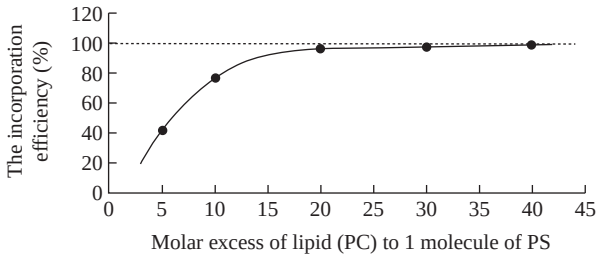


Figure 3 The dependence of incorporation efficiency of photosensitizer **2** on excess lipid used.

in vivo experiment, which allows us to evaluate the selectivity and the EPR effect, plays the main role in the estimation of PDT efficiency.

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