Binding ability of porphyrins in liposomes determined by optical spectroscopic methods

Theses of the doctoral dissertation

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Introduction

The phenomenon of photosensitization has been known for more than a hundred years. However, it has been rediscovered only recently because of its potential therapeutic uses. One of the most important clinical applications of porphyrinoid sensitizers is photodynamic therapy (PDT). PDT has been developed as a cell killing method therefore it has been applied mostly for tumor treatment. However some of the most successful PDT applications are related to nontumorous diseases. PDT involves a nontoxic dye – the photosensitizer (PS) – which is excited by visible light and arrives to a triplet state via nonradiant transitions. In this state, the PS interacts with cellular oxygen to form toxic reactive oxygen species. This subsequently leads to cell death. In addition, the characteristic fluorescence of PS is also used in diagnostics (photodynamic detection – PDD) to determine the location of neoplastic tissues. Photodynamic methods have also been considered for the inactivation of viruses and killing of other microorganisms.

The most important factor governing the outcome of the photodynamic effect is the way the PS interacts with cells in the target tissue. One of the main steps in the process is the generation of singlet oxygen. Due to its short lifetime and diffusion pathway, the primary reaction occurs mainly in the close vicinity of the PS molecules. Thus the key aspect of this interaction is the binding ability and the subcellular location of the PS. The favorable pattern of location depends on the nature of the sensitizer as well as on the complex environmental conditions.

Most of the sensitizers used in photodynamic methods are porphyrin derivatives. Because of their hydrophobic or amphiphilic character, they bind to various cellular lipid membranes. Hence the investigation of the binding of porphyrin derivatives to the membrane and their distribution between the membrane compartments is particularly important. On the basis of this information, one can predict the effectiveness of a photodynamic reaction and decide whether a sensitizer can be considered a good candidate for photodynamic applications.

Many conclusions presented in this field are based on data obtained from membrane model systems such as liposomes, which can mimic specific cellular environments. Numerous articles report on the location of porphyrin molecules and on the nature of interactions based on different fluorescence experimental techniques. The location along the hydrocarbon chains of the PSs in the bilayer has also been studied, and a direct correlation between location depth and photosensitizing activity was demonstrated.

Generally, the properties relevant to the interactions between the PS as chromophore and the membrane may be revealed from the optical spectra of the PS. In conventional spectra, besides temperature induced homogeneous broadening, slight fluctuations of the surrounding matrix (the membrane) lead to inhomogeneity in the chromophore environment and cause broadening of the spectral bands. Site-selective fluorescence spectroscopy (such as fluorescence line narrowing – FLN) is a high-resolution method capable of monitoring the environmental effects free of inhomogeneous broadening.

Research objectives

In my work I applied different optical spectroscopic techniques to examine several systems composed of porphyrins and liposomes as a photosensitizer–lipidmembrane model. I studied the binding ability and location of two mesoporphyrin (MP) derivatives, namely mesoporphyrin IX dimethyl ester (MPE) and mesoporphyrin IX dihydrochloride (MPCl), in monocomponent small unilamellar vesicles (SUVs). The SUVs were composed of three kinds of saturated phospholipids, namely, dimyristoyl-, dipalmitoyl-, and distearoyl-phosphatidylcholine (DMPC, DPPC, DSPC). On these bases my detailed research objectives were:

1. Prepare a stable, homogeneous, aggregation free sample that is also suitable for (low temperature) site-selective fluorescence measurements.
2. Determine the parameters of binding ability by conventional fluorescence spectroscopy in various MP–SUV models.
3. Demonstrate the existence of non-equivalent binding sites by conventional fluorescence spectroscopy.
4. Demonstrate that the fluorescence line narrowing technique (FLN) can be used to examine photosensitizer–lipidmembrane models.
5. Examine the applicability of the “quasi-FLN” methodology.
6. Demonstrate the existence of non-equivalent binding sites by the FLN method.
7. Propose a consistent interpretation at the molecular level about location of the MPs in the SUVs based on the inhomogeneous distribution functions (IDF).

Materials and methods

MP-liposome sample preparation. Stock solutions of mesoporphyrin IX-dimethyl ester (MPE) and mesoporphyrin IX dihydrochloride (MPCl) were prepared in dimethyl-formamide (DMF) with a concentration of around 2 mM. Dimyristoyl-, dipalmitoyl-, and distearoyl-phosphatidylcho-line (DMPC, DPPC, DSPC) were dissolved in chloroform and then dried. Lipids were hydrated with phosphate buffered saline solution (PBS, pH 7.4) at a temperature just above the main (liquid − gel) transition temperature (T_m) of the corresponding lipid membrane (≈ 24, 42, and 55 °C for DMPC, DPPC, and DSPC, respectively). SUVs were prepared by sonication (US) or extrusion technique. Remnants of multilamellar vesicles and contaminants were removed by centrifugation. The final phospholipid concentration was approximately 15 mM. Porphyrin was added to liposome at room temperature ($RT \approx 22$ ° C).

Samples for binding measurements. A series of MP−liposome samples of constant mesoporphyrin [*MP*] (and DMF) but varying lipid concentrations [*L*] was prepared by mixing for half an hour. On the basis of the spectroscopic check, I found that binding equilibrium was established by the end of this time.

Samples for site-selective (FLN) measurements. Porphyrin from the stock solution was added to the liposome in excess and mixed for 45 min. Then, glycerol was added for cryoprotection and to ensure sample transparency at low temperatures (final concentration was 40% (v/v)). The final concentration of the samples was approximately 20 μ M and 7 mM for MP and phospholipids, respectively. Spectroscopic measurements were carried out immediately after sample preparation at cryogenic temperature (10 K). Following each step of the sample preparation, the size distribution of liposomes was measured by dynamic light scattering (DLS). Thus, the homogeneity and the possible disintegration or aggregation of the liposome samples were checked.

Dynamic Light Scattering (DLS). DLS measurements were performed with equipment consisting of a goniometer, a solid-state laser light source (457 nm), and a sensitive light detector. The evaluation software yielded the autocorrelation function of scattered light intensity, which was further analyzed by the maximum entropy method from where the different contributions of this function were determined. With r^{-2} used as a weighting factor, where r is the radius of the vesicle, the particle size distributions were determined.

Conventional fluorescence spectroscopy. Fluorescence emission spectra were measured with a luminometer which has a spectral resolution of about 0.5 nm. Samples were kept in a

temperature-controlled sample holder at 22 ° C, and they were excited at the maximum of the Soret-band (397 nm). Fluorescence emission spectra were recorded by scanning the 600−640 nm range in 0.5 nm steps.

FLN spectroscopy. The basic principles of this technique are the following. Fluorescence emission spectra are measured on samples which are cooled fast from room temperature to cryogenic temperatures. As a consequence of the cooling, the sites populated at room temperature freeze in the sample. This information, as a snapshot, is conserved and can be studied by spectroscopic methods. The meaning of the term “site” in this context is a molecular environment (almost without fluctuations) of the dye molecule as well as a detuned electronic transition energy (characterized by a certain wavenumber) of the dye molecule in this environment. With the use of narrow bandwidth on the excitation side, part of the molecules will be simultaneously but selectively excited. This subpopulation (in electronic transition energy) of the chromophores corresponds to such a specific frozen environment. Thus the sites are defined in this sense. Because of the selective excitation, the emission spectra consist of sharp emission lines (line narrowing) resulting from resonant excitations, superimposed on the background of broad bands which are also present, due to the emissions from nonresonant (phonon-coupled) excitations. The intensity of the sharp lines is proportional to the number *(N)* of selectively excited molecules at a given excitation frequency *(ν)*. With the *N(ν)* function we can create the so called inhomogeneous distribution function (IDF), which characterize the inhomogeneous environment.

Fluorescence emission spectra were measured with different luminometers (L1 and L2a, L2b). The spectral resolution of L1 luminometer is about 0.1 nm both in excitation and emission side. This spectrometer setup consists of a stabilized CW tunable ring dye laser pumped by an Ar ion laser. Because the IDF turned out to be broad, and the lasing range of the dye is limited relative to this scale I tried L2 luminometers. The spectral resolution of these equipments is approximately 0.5 nm in both side. Because of the wider excitation and emission bands we call this measurements “quasi-FLN” technique. The applicability of L2s was based on comparison of the IDFs obtained with the two luminometers (L1 and L2a). All fluorescence measurements were carried out at 10±1 K adjusted using a temperature-controlled closed-cycle helium cryostat. A series of fluorescence (line narrowing) emission spectra were recorded by scanning the wavelength over the inhomogeneous bands (600-640 nm) in steps matching the estimated spectral resolution (0.1 or 0.5 nm). The excitation wavelength was also varied in uniform steps (1 nm) in a defined range (555-585 nm). From the recordings, the inhomogeneous distribution function (IDF) was determined.

Results

The examination of the stability, homogeneity and aggregation. The frequency distribution of the liposomes among their hydrodynamic radius is lognormal. The mean liposome size was characterised by the mode of the distribution. The distribution of the used samples was homogeneous (only one peak present). The mean size of liposomes made via sonication was 12 nm, 18 nm, 22 nm for DMPC, DPPC, DSPC respectively. In the case of the extrusion method the sizes were nearly identical (24 nm). The deviation from the mode in the US technique was at most 1.5 nm and 2.5 nm in the extrusion technique. The full width at half height (fwhh) was 4.5–11.5 nm and 4–8 nm for US and extrusion respectively. The effect of porphyrin addition (solved in DMF) was negligible even after the conventional measurement (the mode and the fwhh increased at most 2 nm or at most 4.5 nm). For the low temperature measurements glycerol had to be added to the sample. It had no effect to the nature of the distribution – the mode and fwhh deviated at most 1 and 2 nm. The cryogenic measurement had no effect to the distribution.

I had checked the transparency of the samples containing different amount of glycerol in cryogenic temperature. I found at least 35% (v/v) of glycerol needed to keep the sample transparent. The effect of glycerol on the binding ability of MP to liposomes was examined by conventional fluorescence spectroscopy. The spectrum of the MP–SUV and the MP–SUV–glycerol samples (with the same MP concentration) was the same, but the spectrum of the MP–glycerol (without SUV) sample was different. This shows glycerol addition did not significantly affect the amount of MP associated with liposomes.

In the case of conventional fluorescence spectroscopy I used a porphyrin concentration that did not increase the amount of light scattering (measured by DLS) – compare with the pure PBS. I also checked the aggregation in the samples of FLN measurements. I measured FLN spectra on sample contains different amount of porphyrin (just below and above the saturation concentration). I found that there was no change in the resolved part of the spectra that corresponds to the associated MP. On the contrary there was a difference in the unresolved part of the spectra that corresponds to the free – and based on this – differently aggregated porphyrins. Thus remnants of free MP (or its aggregated forms) did not disturb the fluorescence measurements because of the significant spectral separation from the emission maximum of associated MP.

Determination of the parameters of binding ability and the demonstration of the existence of non-equivalent binding sites by conventional fluorescence spectroscopy. I based my calculations on the classical equilibrium mass action equation that can be rearranged to express the concentration of bound mesoporphyrin $[MP]_b$ as an explicit function of the two independent variables – the lipid $[L]$ and the total mesoporphyrin concentration $[MP]$:

$$[MP]_b = \frac{1}{2}\left\{n\cdot[L]+[MP]+K_d-\sqrt{(n\cdot[L]+[MP]+K_d)^2-4\cdot n\cdot[L]\cdot[MP]}\right\},$$

where n is the number of possible binding sites per lipid and K_d is the dissociation constant. I used a new evaluation method and a novel representation to estimate better $[MP]_b$ and show the existence of non equivalent binding sites in cases when it was not obvious at all. In this method I used the intensity function of the maximum concentration of lipid ($[L]$max) as a reference to the intensity function of a given concentration of lipid ($[L]$*). The basis of this representation showed in **figure 1**.

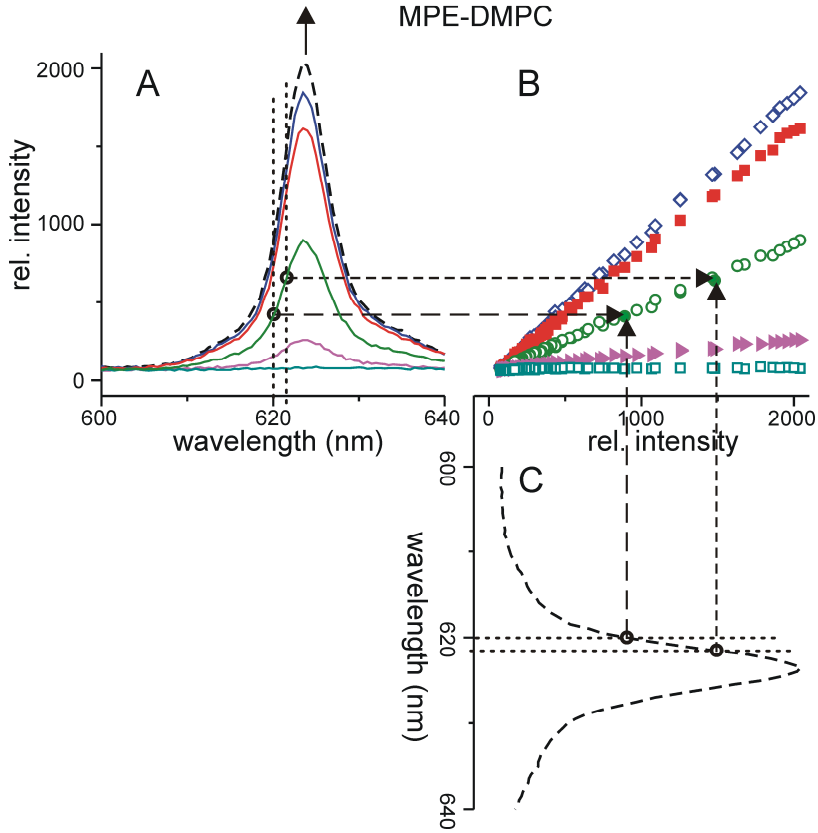

Figure 1. Basic principles of a new representation of evaluation process

(A) a series of conventional fluorescence emission spectra of the MPE–DMPC at constant MP and various lipid concentrations (marked with different colors. (C) the same kind of spectrum as (A), but at maximum concentration of lipid ($[L]$max), after a 90° clockwise rotation. (B) a series of $I_{[L]\max}(\lambda) \rightarrow I_{[L]^*}(\lambda)$ functions of relative intensities, at various lipid concentrations according to the simultaneously changing wavelength.(The broken lines with arrow tips represent the mapping at 620 nm and 621.5 nm respectively.)

Figure 2 shows three of these functions for all the MPE–SUV samples at a specific lipid concentration [*L*]*. In the case of DSPC, the data points form a loop instead of a linear function with random errors. I could explain it with two types of binding sites that are not completely equivalent with respect to the association process. A similar but weaker effect is also present in the case of DPPC but practically non existent for DMPC. These results cannot be read directly from the binding curves, but only from this novel representation. But there is no such a parameter in this novel evaluation that could characterise these different sites. Thus, I used the slope of the linear corresponds to the loops to estimate the $[MP]_{b.}$

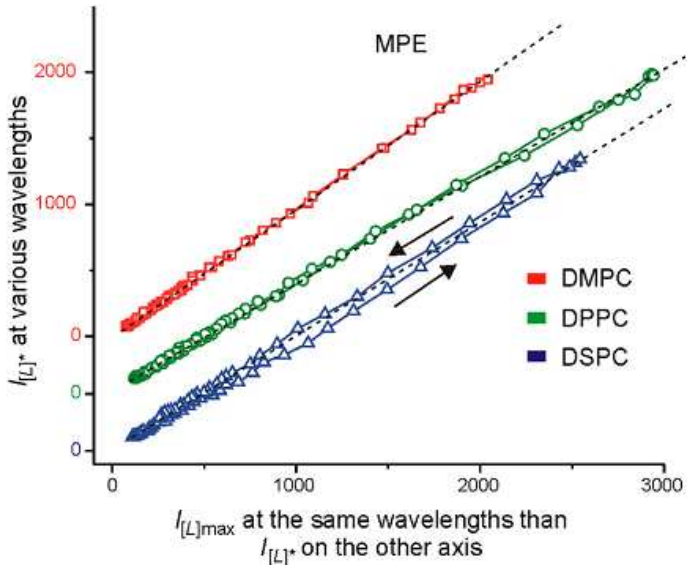

Figure 2. $I_{[L]max}(\lambda) \rightarrow I_{[L]^*}(\lambda)$ functions of MPE in various SUV samples
The novel representation at specific lipid concentrations ($[L]^* \approx 6.5$ μM), with fitted broken lines: DMPC (red), DPPC (green) and DSPC (blue) (for the better visibility they are shifted along the vertical axis).

The calculated parameters of binding ability are shown in **table 1**.

Table 1. Parameters of binding ability

n (number of all the possible binding sites per lipid molecule), Kd (dissociation constant), n/Kd = Kb (binding constant)

	DMPC [14]		DPPC [16]		DSPC [18]	
	MPE	MPCl	MPE	MPCl	MPE	MPCl
n	0.0083	0.0037	0.0044	0.0026	0.0079	0.0038
K_d (nM)	30 ±10	52 ±16	28 ±7	53 ±13	33 ±9	15 ±4
K_b (M^{-1})	2.8×10^5	7.1×10^4	1.6×10^5	4.9×10^4	2.4×10^5	2.5×10^5

FLN and "quasi-FLN" methods. The basis of the evaluation of the IDF is to determine the number of molecules (*N*) with a given transition energy that corresponds to the given environment. Here *N* is proporcional to the emission intensity. I observed the intensity change of the emission peak series corresponds to the change in excitation frequency. The different peak series correspond to the different absorption transition probabilities which are taken into consideration by a constant parameter. The inverse of these parameters was used as a weighting factor for normalization. The normalized series together give us the IDF.

I compared the determined IDFs of MPE–DPPC samples obtained by L1 and L2a luminometers**. Figure 3** shows that the difference between the IDFs was negligible. (Thus, in the further measurements I used the L2 luminometers.)

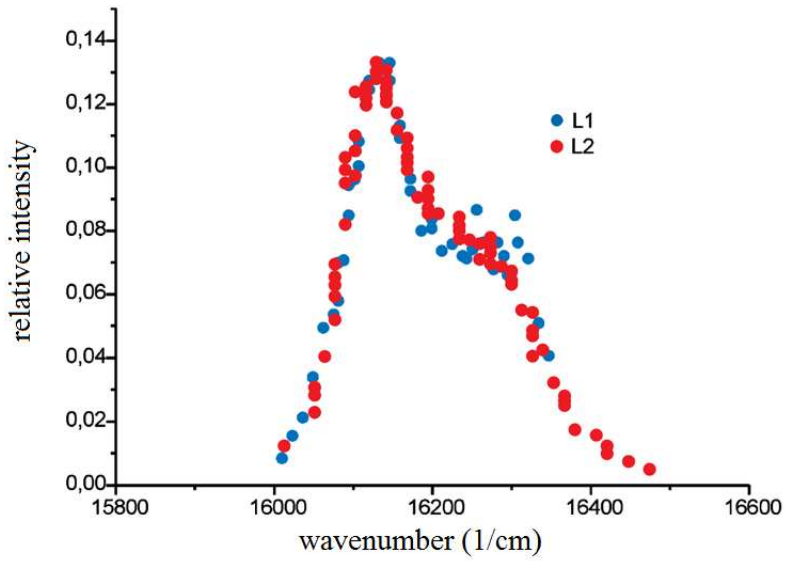

Figure 3. Two IDFs for comparison determined from data obtained by different luminometers (L1 and L2)

IDF mesured by L1 (blue) and L2 (red) luminometers.

The Gaussian curves fitted to the IDFs represent the different environments, the different "sites", namely the different porphyrin bindig sites. I observed that almost all of the samples have a composite IDF providing evidence for the existence of more than one type of MP sites. I decomposed the IDFs into Gaussian bands shown in **Figure 4.**

The different parameters of the Gaussian curves give us different information. The center of the curve is characteristic of the subpopulation of molecules, while the fwhh (full width of the band at half height) parameter measures the heterogeneity of its environment. The relative area under the band is proportional to the total amount of molecules associated with a certain site. Based on the center values of the Gaussian bands I found three distinguishable binding sites, namely “site I”, “site II” and “site III”. Based on the parameters of the Gaussian curves and the transition temperature of the corresponding membrane (that characterise the order of the membrane structure) I proposed a consistent interpretation at the molecular level about location of MPs in the SUVs.

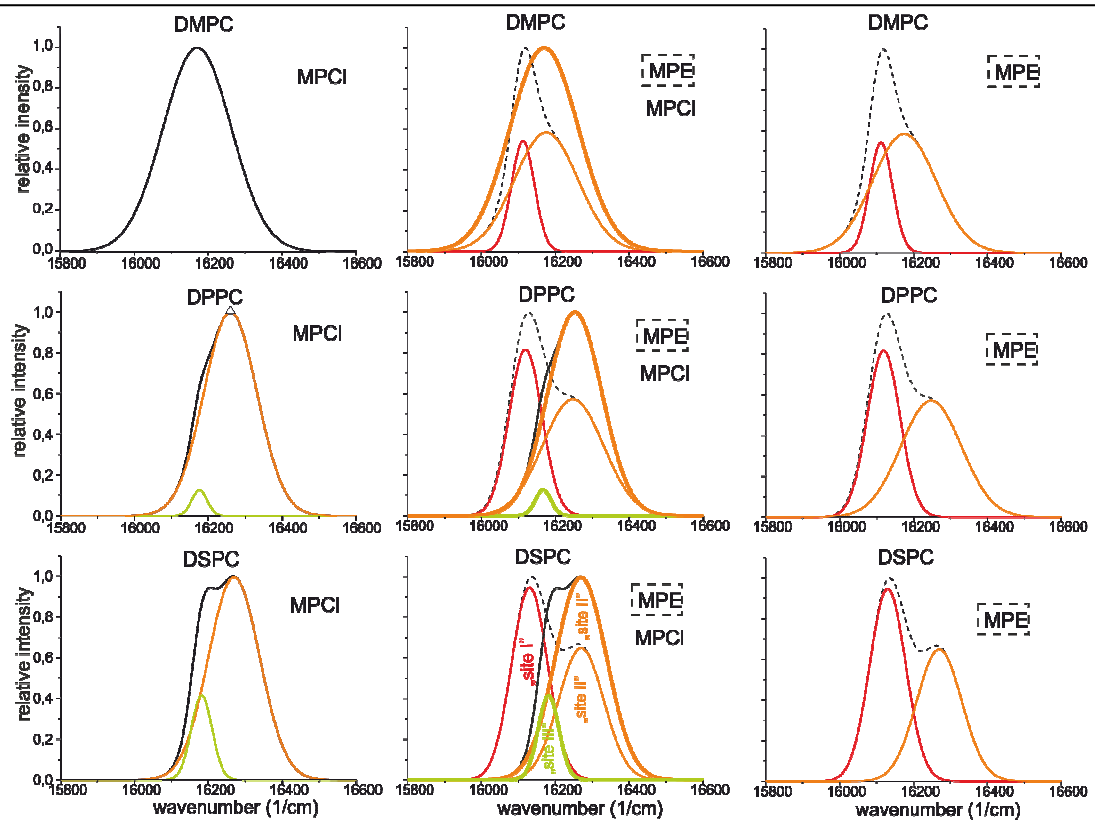

Figure 4. Inhomogeneous distribution functions (IDFs) (with their Gaussian decompositions) of the two MP incorporated in different SUVs

On the left side the MPCl, ont he right side the MPE, and on the center of the figure both MP’s IDF are shown with their Gaussian curves. The continous lines represent the MPCl samples. The dotted ones represent the MPE samples. The black curves shows the IDFs. The fitted Gaussian bands colered by the sites: the green is the “site I”, the red is the “site II” and the blue is the “site III”. According to the Gaussian decompositions, in the bottom I marked the bands by “site I-III”.

Conclusions and new scientific findings

In my work I applied different optical spectroscopic techniques to examine several systems composed of porphyrins and liposomes as a photosensitizer–lipidmembrane model. In comparison with some other techniques, the FLN method has the advantage that the detected signal comes from the molecules studied and not from externally added labels. In this way all the real sites of these molecules contribute to the measured optical signal. Based on the measurements and the evaluations I made the following statements:

1. The prepared liposomes were homogeneous and stable. The homogeneity and stability was kept by the addition of MP (solved in DMF) and glycerol, and also on the time scale of the experiments. The samples with glycerol kept their mesoporphyrin content. I showed that the fluorescence signal was given by monomer forms of MPs. Thus the samples were suitable for fluorescence spectroscopic measurements.

2. Based on the mass action equation for all MP-SUV systems the number of all the possible binding sites per lipid molecule, the dissociation constant, and the binding constant were determined. I found in general that the binding ability of MPE is considerably greater than that of MPCl.

3. Based on a new evaluation method and a novel representation ($I_{[L]\max}(\lambda) \rightarrow I_{[L]}(\lambda)$ functions) I could show the existence of non equivalent binding sites in cases where it was not obvious at all. But the quantitative characterisation of the different sites is not yet.

4. I showed that the fluorescence emission spectra could be resolved and the IDFs could be determined. I showed that only one MP form is presented in the samples, so the IDFs characterise the inhomogeneous environment. This way I demonstrated the applicability of FLN techniques to examine photosensitizer–lipidmembrane models.

5. Based on the measured IDFs I demonstrated that the “quasi-FLN” technique is also applicable to examine photosensitizer–lipidmembrane models.

6. Based on the determined IDFs and their fitted Gaussian curves I pointed to the presence of different locations of MPs.

7. On the basis of the fit results of the decomposition of the IDFs into Gaussian curves, I proposed a consistent interpretation of our results at the molecular level. Namely, one of the locations (for MPE) is between the two lipid layers, another one (for MPE and MPCl) is located deeply between the hydrocarbon chains, and a third one (for MPCl) is along the outer part of the hydrocarbon chains partially inserted between the head groups of lipid molecules. I illustrated this notion schematically in **figure 5**.

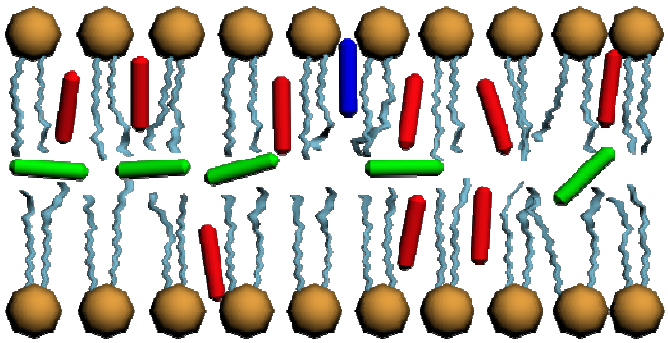

Figure 5. The schematic figure of the location of MPs in the membrane

The MP population of “site I” marked with green, for “site II” I used red, and “site III” represented by blue cylinders.

List of author's publications

Publication belongs to the dissertation:

Herenyi, L., **Veres, D.**, Békási, S., Voszka, I., Módos, K., Csík, G., Kaposi, A. D., Fidy, J. Location of mesoporphyrin in liposomes determined by site-selective fluorescence spectroscopy. J Phys Chem B. 2009, 113:7716-24.

Veres, D., Bőcskei-Antal, B., Voszka, I., Módos, K., Csík, G., Kaposi, A. D., Fidy, J. Herenyi, L. Comparison of binding ability and location of two mesoporphyrin derivatives in liposomes explored with conventional and site-selective fluorescence spectroscopy. J Phys Chem B. 2012, 116: 9644-52.