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pH, serum proteins and ionic strength influence the uptake of merocyanine 540 by WiDr cells and its interaction with membrane structures

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Abstract

It has been suggested that selective uptake of photosensitizers is due to significantly lower pH of the interstitial fluid in tumors compared to normal tissue. Therefore, the cellular uptake of merocyanine 540 (MC 540) was examined at two pH values: 6.8 ± 0.1 and 7.4 ± 0.1. There was no difference in spectral properties (absorption and fluorescence maxima positions, fluorescence intensity) of the drug in the presence of increasing amounts of either human blood plasma or FCS (0–2%) at the two pH values investigated. Nevertheless, significantly higher amounts of the drug were taken up by WiDr cells at pH 6.8 ± 0.1, both in the presence of 10% FCS and in the absence of FCS. The absorption spectra of MC 540 in the presence of egg phosphatidylcholine (PC) liposomes turned out to be NaCl concentration-dependent (0.00–0.30 mol l^{-1}). Membrane fluidity, as measured by fluorescence anisotropy of diphenylhexatriene (DPH), was unchanged within the experimental error in the NaCl concentration range 0.01–0.30 mol l^{-1}. The spectral changes indicated an enhancement of the incorporation of MC 540 into lipid membranes with increasing ionic strength. Such a salt concentration dependence suggests a possible involvement of the surface potential in the interaction of MC 540 with lipid membranes. The results might provide an explanation of the pH dependency of the cellular uptake of MC 540 observed in this study.

Keywords: Cellular uptake; MC 540; pH; Ionic strength

1. Introduction

Selective uptake of photosensitizers by tumors has been reported [1–3]. However, underlying mechanism has not been satisfactorily explained. A role of different environmental properties in the tumor versus the normal tissue has been suggested [4]. In particular, significantly lower pH of the interstitial fluid in tumors [5–7] might influence physico-chemical properties of photosensitizers and/or membranes and, thus, cellular uptake of drugs.

Selective cellular binding and ability to sensitize photodynamic killing of cells have been found in case of merocyanine 540 (MC 540) [8–10]. MC 540 is a lipophilic dye with high affinity for lipids. It is generally accepted that the negatively charged sulphate group of MC 540 prevents the dye to penetrate cellular membranes of intact cells [8]. As a result, the plasma membrane is believed to be the main site of its localization [8]. MC 540 binds selectively (i.e. in a significantly higher content than to normal cells) to leukemic and lymphoma cells (e.g. Refs. [11–13]) circulating in the blood stream. However, blood is very well buffered and substantial differences in blood pH are not likely to occur. Therefore, even if pH was a determinant for selective uptake of MC 540 by tumor cells, additional factors could be expected to be involved as well. MC 540 binds also to electrically

Abbreviations: DMPA, dimyristoyl L-α-phosphatidic acid; DPH, diphenylhexatriene; FCS, fetal calf serum; HBP, human blood plasma; MC 540, merocyanine 540; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SBPC, soybean phosphatidylcholine.

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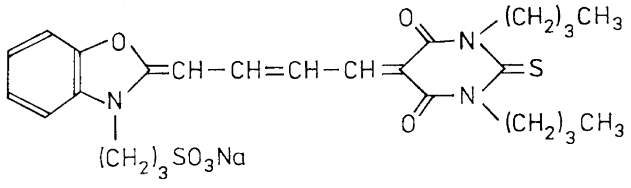
excitable cells [14]. It belongs to a group of probes being used for monitoring of the changes in transmembrane potential (e.g. Refs. [15,16]). Such changes are connected with variations in concentrations of different ions close to the cell membrane. Accordingly, membrane potentials, ion distributions in membrane proximity and their modifications due to possible special properties of cancer cells (e.g. surface charge) should be considered as factors affecting cellular uptake of MC 540 in addition to the factors investigated until now (fluidity, lipid packing, surface charge, phospholipid asymmetry).

The purpose of this study is to investigate the nature of the interaction of MC 540 with the membrane lipid bilayer of living cells and cell-like structures, and identify factors that might influence interactions of MC 540 with cellular membranes and result in selective cellular uptake. For instance, detailed studies on the effects of perturbation of the membrane structure of erythrocytes have been made by Lagerberg et al. [17,18]. We focused our attention on the effect of environmental factors on the interaction of MC 540 with solid tumor cells. Two pH values were chosen: pH 6.8, which is a mean pH value reported in tumors [19], and pH 7.4, as a reference pH, usually reported for normal tissue. The effects of both the pH in the incubation medium and the presence of serum proteins have been investigated in a WiDr cellular uptake study. pH modifications might affect different components of the tumor, such as tumor cells themselves or serum proteins in extracellular fluid. Therefore, the effects of the pH value on the interaction of MC 540 with serum proteins and with cells in the absence of serum proteins have been examined. Moreover, in order to check whether variations in ionic strength itself might influence interaction of MC 540 with lipid membrane, the effect of increasing salt concentration on incorporation of MC 540 into liposomes was examined. Model membranes were chosen for this purpose because different competitive phenomena might be involved in the case of cells. Such phenomena are both difficult to be maintained under a strict control and not easy to separate.

2. Materials and methods

2.1. Chemicals

Merocyanine 540 (Scheme 1) was obtained from Eastman Kodak (USA) and was used without further purification. Stock solutions of 2 or 1.3 mg ml^{-1} were prepared in water or in the mixture of water/ethanol (4:1), respectively, and were kept frozen until used. Fetal calf serum (FCS) purchased from Saveen (Norway) and fresh human blood plasma sampled in EDTA tubes were kept frozen until used. L-α-Phosphatidylcholine from frozen egg yolk (egg PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphate disodium salt (DMPA) and soybean L-α-phosphatidylcholine (SBPC) were purchased from Sigma, Fluka and Calbiochem, respectively. All chemicals used were of the highest purity commercially available.



Scheme 1. Merocyanine 540.

2.2. Binding of MC 540 to blood proteins

Phosphate-buffered solutions of different pH values [20] containing FCS or human blood plasma (concentrations are specified in Results) were used for sample preparation. In order to keep the ionic strength of all the samples constant, 0.14 mol l^{-1} NaCl was added to phosphate-buffered solutions.

Different amounts of FCS or human blood plasma were pre-incubated with phosphate-buffered solutions of the appropriate pH value for 1 h. The pH value of the sample containing FCS or human plasma without drug was checked before and after measurement, and was found to be constant within experimental error. Within 1 h after preparation significant changes in pH values of the samples were observed only at high concentrations of FCS (>5% FCS) at pH values <6.3 and >8.0. MC 540 from a stock solution was diluted in pre-incubated plasma to the final concentration 0.5 μg ml^{-1}.

The fluorescence emission and excitation spectra were recorded using a Perkin Elmer Luminescence Spectrometer LS50 B. According to the specifications of the instrument, the emission spectrum was uncorrected while the excitation spectrum was corrected up to about 610 nm. A quartz cuvette (1.0×0.4 cm^2) was used for the measurements. For excitation, 530 nm light was used. Fluorescence excitation spectra were recorded at $\lambda_{em} = 620$ nm. A cut-off filter was used to remove scattered light of $\lambda < 515$ nm. The fluorescence from the control (i.e. without MC 540) showed no fluorescence under these conditions. The absorption spectra were recorded using Specord M40 or Perkin Elmer Lambda 40 spectrophotometers. Quartz cuvettes (1.0×1.0 cm^2) were used for the measurements. A Metrohm AG CH-9101 Herisau pH-meter was used to measure pH values of the samples. Measurements were performed at room temperature immediately after the addition of the drug to the sample.

2.3. Cellular uptake

Human colon adenocarcinoma cells of the line WiDr were used for cellular uptake experiments. The cells were grown in monolayers at 37 °C in a humidified incubator with 5% CO_2 and subcultured twice a week in RPMI-1640

medium supplemented with 10% fetal calf serum, 1% penicillin and streptomycin, 0.2% insulin and 1% L-glutamine.

The carbon dioxide–bicarbonate buffer system was used to adjust pH of the medium (manuscript in preparation). Briefly, different combinations of the CO_2 partial pressure in the incubator atmosphere and the bicarbonate concentration in the medium were used to achieve different pH values (0.5 g l^{-1} bicarbonate and 19% CO_2 for pH 6.8, 2 g l^{-1} bicarbonate and 5% CO_2 for pH 7.4). Plastic tissue-culture flasks were inoculated with 5×10^5 cells. The cells were incubated for 48 h under the usual conditions (37 °C, 5% CO_2, RPMI 1640 medium with 10% FCS). Medium with (10%) or without FCS, with appropriate concentrations of bicarbonate to achieve desired pH, was used to wash the cells. The cells were then pre-incubated in the medium to achieve desired pH in the flask. MC 540 was added to the medium from the stock solution afterwards. The pH value of the medium in the flasks with cells was checked after pre-incubation and after the measurements. It was found to be unchanged within experimental error ($\pm$ 0.1) within the time period of the experiment. After incubation for different time periods (specified in Results), the medium was removed and the cells were washed three times with ice-cold phosphate-buffered saline (PBS). After addition of PBS (3 ml), the cells were removed from the bottom of the flasks by means of a soft cell scraper (KEBO Lab., Norway). Corrections for slightly different cell numbers in the samples were performed by light scattering measurements at 630 nm or absorbance measurements at 274 nm. Both the light scattering as well as the absorbance were linearly dependent on the number of cells. Neither the light scattering nor the absorbance was influenced by fluorescence or absorbance, respectively, from the photosensitizing drug.

Binding of MC 540 to WiDr cells, after the incubation at two different pH values, was assayed according to Lagerberg et al. [17] with a slight modification. One hundred microlitres of cell suspension in PBS was added to 2 ml of *n*-butanol and vortexed for 10 s. The amount of MC 540 in the butanol layer was determined from the fluorescence emission spectrum (excitation 540 nm, emission 580 nm) using a Perkin Elmer LS50 B Luminescence spectrofluorimeter. The absorption spectra were recorded in the region 200–700 nm, using a Perkin Elmer Lambda 40 spectrophotometer. The curve corresponding to the light scattering was subtracted from the original spectrum of cell-bound MC 540.

A Metrohm AG CH-9101 Herisau pH-meter was used to measure the pH values of the samples. All the measurements were carried out at room temperature.

2.4. Liposomes

Multilamellar vesicles were prepared by vortexing the lipid (egg PC, egg PC/DMPA, SBPC; 20 mg ml^{-1}) in the presence of an appropriate salt solution (0.00 or 0.15 mol l^{-1} NaCl). In the case of egg PC/DMPA liposomes, lipids (egg PC and DMPA) were first dissolved in chloroform, solutions of lipids were mixed to give an appropriate molar ratio (9.3 mol% of negative lipid) and chloroform was removed afterwards. The lipid dispersion was subjected to five cycles of freezing and thawing to achieve equilibrium transmembrane solute distribution [21]. Large unilamellar vesicles were prepared by extruding multilamellar vesicles (19 times) through two (stacked) polycarbonate filters of 100 nm pore size using an extrusion device (LipoFast-Basic, Avestin, Ottawa, Canada). SBPC liposomes were prepared by sonication of multilamellar vesicles. Samples were prepared by 70-fold (egg PC) or 100-fold (egg PC/DMPA) dilution of the vesicle suspension into an appropriate salt

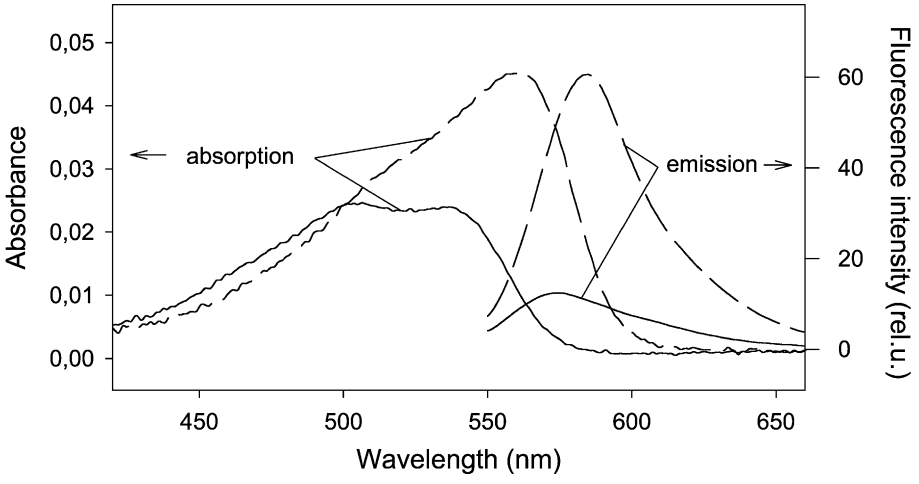


Fig. 1. Absorption and fluorescence emission spectra of MC 540 (0.5 μg ml^{-1}) in 0.14 mol l^{-1} NaCl with 0.3% (- - -) and without human blood plasma (—). λ_{exc} = 530 nm.

solution (0.00–0.30 mol l^{-1}). MC 540 was added afterwards. As judged from the light scattering curve, stability of the liposomes was not interrupted. The final concentration of the drug in the sample was 20 μg ml^{-1}. The absorption spectra of liposome-bound MC 540 were recorded using a Specord M40 spectrophotometer. The light scattering signal in the region of the MC 540 main absorption was less than 5% of the MC 540 absorption. Thus, no subtraction of the curve corresponding to light scattering was performed.

The fluorescent probe diphenylhexatriene (DPH) was added to the lipid dispersion of large unilamellar egg PC liposomes to a concentration 10^{-6} mol l^{-1}. After 90-min incubation in the presence of DPH in the darkness, the liposomes without MC 540 were diluted in a salt solution to the same concentration as used in other experiments with liposomes, and fluorescence anisotropy was estimated. For excitation of DPH, 360 nm light was used. All the measurements were performed at 24–25 °C.

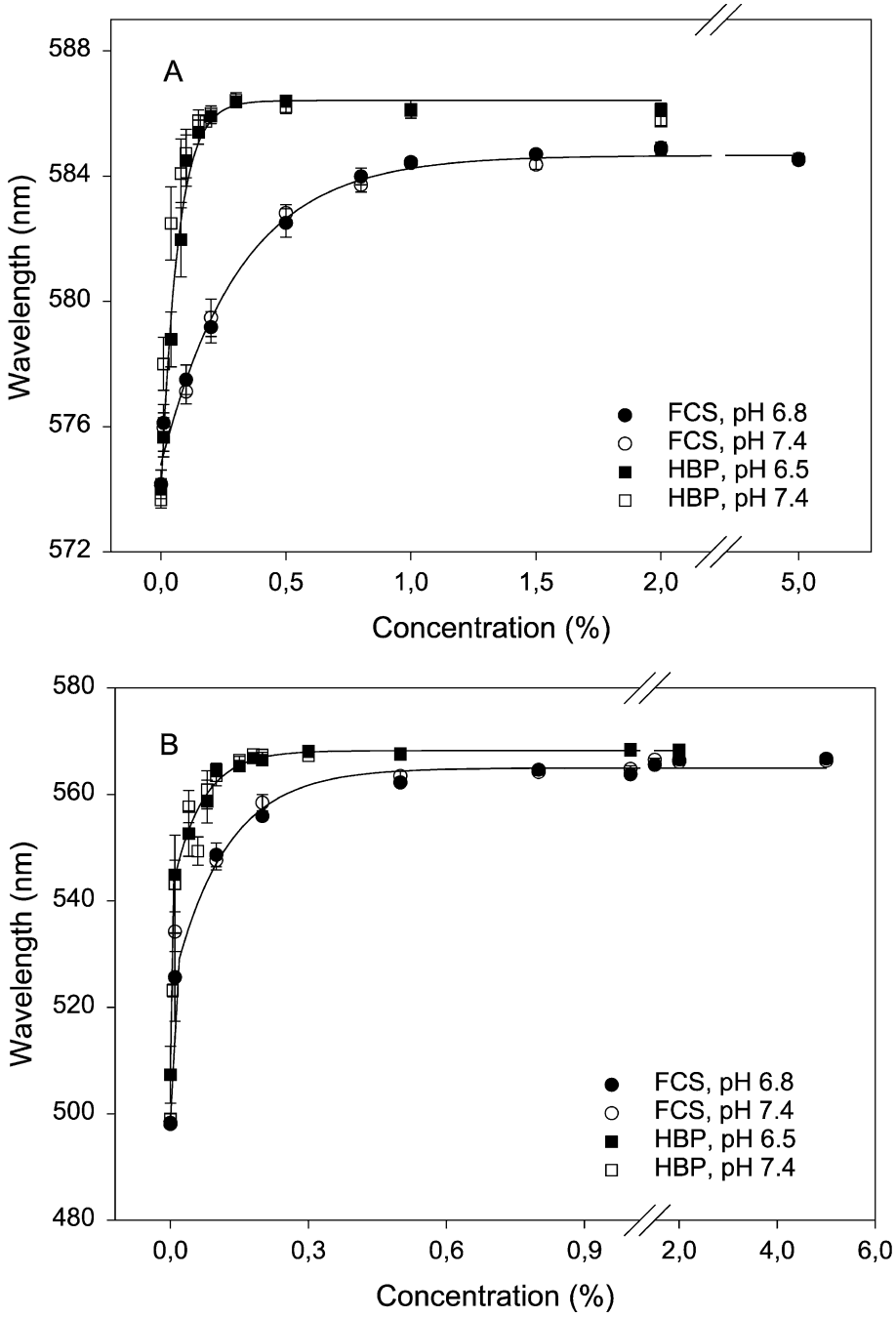


Fig. 2. FCS and human plasma concentration dependence of the fluorescence emission (A) and fluorescence excitation (B) peak position of MC 540 (0.5 μg ml^{-1}, λ_{exc} = 530 nm, λ_{em} = 620 nm). Data points were fitted to the exponential rise to maximum function (R^2>0.99 for all fits of the curves). Each data point represents mean ± S.E.M. from four (FCS) or seven (human plasma) measurements.

2.5. Statistical analysis

For curve fittings and statistical evaluation of data, Sigma plot and Sigma stat softwares were used. In most cases, the Student's *t*-test with 1% significance level was applied for statistical analysis. In cases when the normality test failed, Mann–Whitney rank sum test was used instead. Kolmogorov–Smirnov test with Lilliefors' correction and 5% significance level to test normality was automatically performed by Sigma stat software. The data are expressed as means ± standard errors of mean (S.E.M.).

3. Results

3.1. Interaction with blood plasma/serum

The absorption spectrum of MC 540 (0.5 μg ml^{-1}) in phosphate-buffered solutions in the presence of 0.14 mol l^{-1} NaCl without human blood plasma or FCS at pH 7.4 ± 0.1 showed peaks at 500.0 ± 0.8 and at 532.3 ± 0.7 nm (Fig. 1), respectively. The same peak positions, within experimental error, were observed at pH 6.8 ± 0.1. Increasing amounts of human blood plasma and FCS resulted in a bathochromic shift of the absorption bands (Fig. 1). The position of the long wavelength peak in the presence of 0.8% human blood plasma was localized at 561.5 ± 0.5 nm at pH 7.4 ± 0.1. Increasing the amounts of human blood plasma above this concentration did not result in any further shift of the absorption maxima. The same peak positions, within experimental error, were detected at pH 6.8 ± 0.1. Concomitantly, the increase of the molar extinction coefficient of the latter peak (relative to the former one) was observed (Fig. 1). Fluorescence emission and excitation maxima of MC 540 in phosphate-buffered solutions in the presence of 0.14 mol l^{-1} NaCl at pH 7.4 ± 0.1 were detected at 573.7 ± 0.4 and 499.0 ± 1.1 nm, respectively. A gradual bathochromic shift of both fluorescence maxima with an increase of human blood plasma concentration (Figs. 1 and 2) was accompanied by the increased fluorescence intensity (Fig. 3). Qualitatively the same tendency was observed after the addition of FCS, although higher amounts of FCS than those of human blood plasma were necessary (Figs. 2 and 3). There was no effect of a pH decrease from 7.4 ± 0.1 to 6.5 ± 0.1 (6.8 ± 0.1 in the case of FCS) on the above-mentioned spectral changes.

The positions of the absorption and fluorescence maxima of MC 540 in the presence of 0.3% human blood plasma or 1% FCS were independent of the pH in the range 5.8 ± 0.1 to 8.3 ± 0.1, within experimental error, as measured shortly after sample preparation.

3.2. Cellular uptake

Cellular uptake of MC 540 was examined at two pH values, 7.4 ± 0.1 and 6.8 ± 0.1, both in the presence and in the absence of FCS. The absorption spectra of the cell suspension in PBS following incubation in the absence of FCS were recorded after removal of incubation medium by

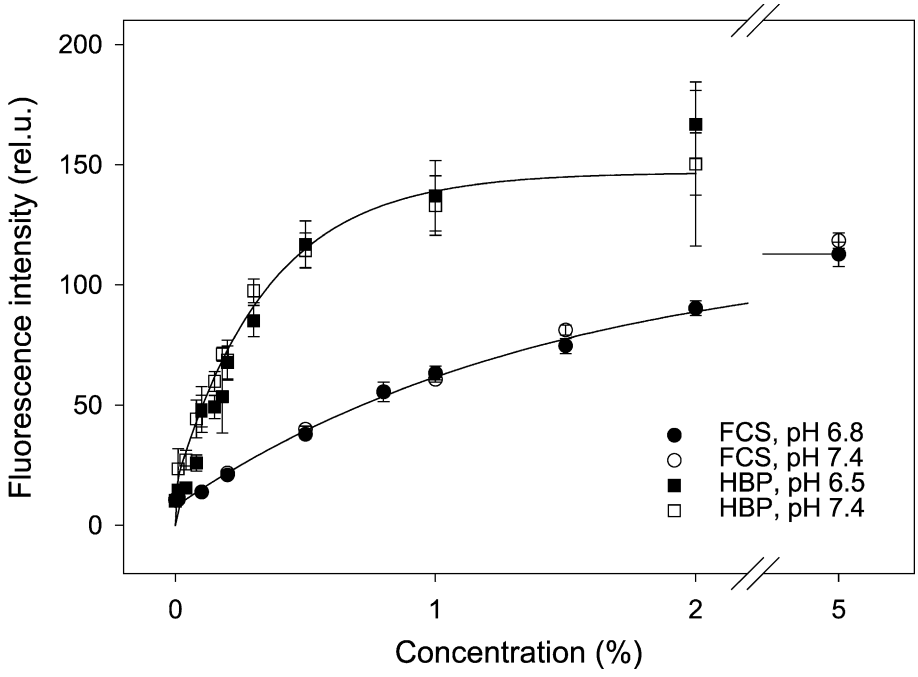


Fig. 3. FCS and human plasma concentration dependence of fluorescence intensity of MC 540 (0.5 μg ml^{-1}, λ_{exc} = 530 nm, fluorescence emission recorded at 585 and 586 nm, respectively). Data points were fitted to the exponential rise to maximum function (R^2 = 0.9889 and 0.9899 for FCS and human plasma, respectively). Each data point represents mean ± S.E.M. from 6 to 10 measurements.

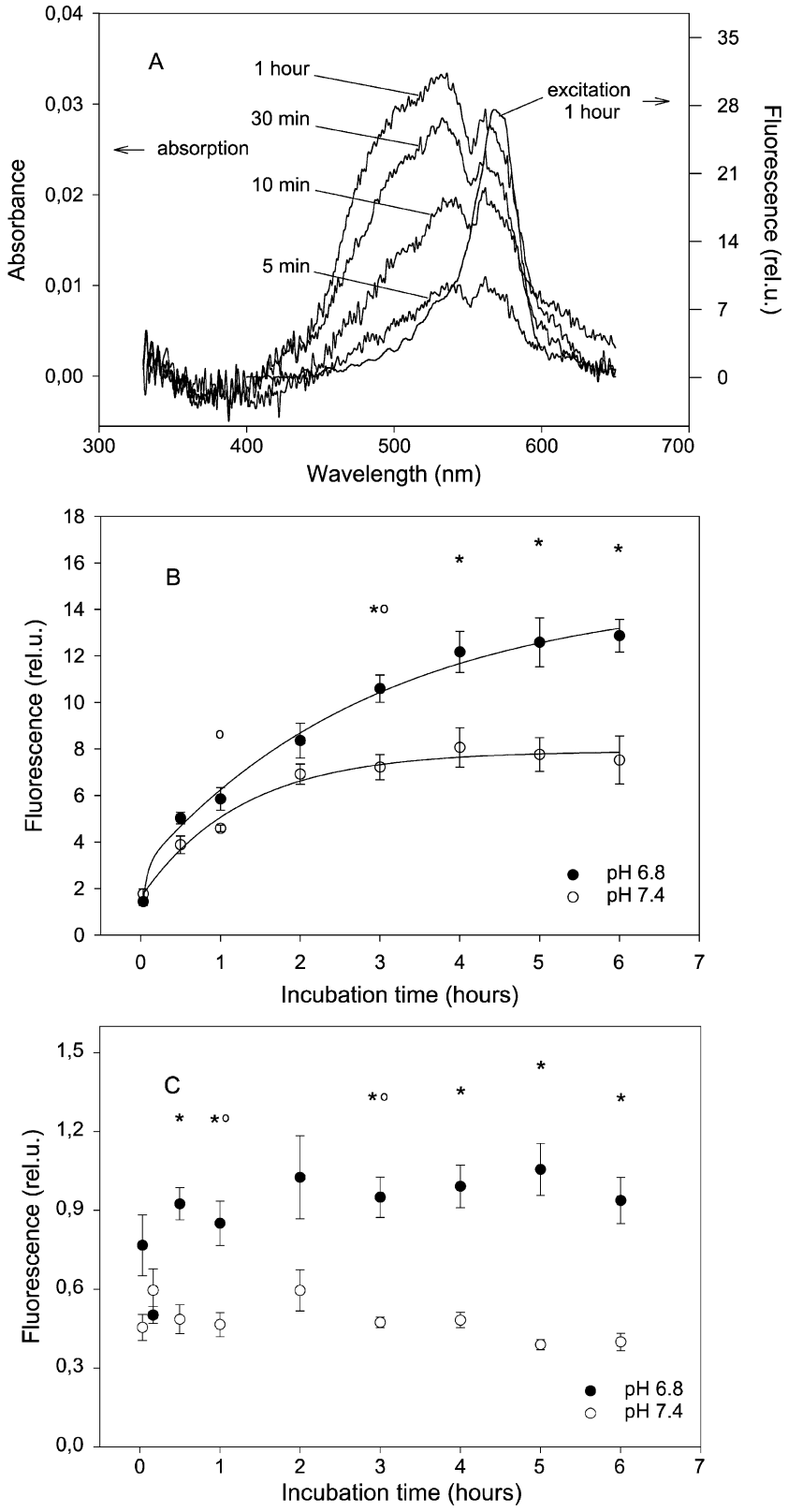


Fig. 4. (A) Absorption spectra of MC 540 bound to WiDr cells, incubated in the medium without FCS, in PBS after removal of the incubation medium and washing the cells with PBS. Incubation times are indicated in the figure. Cellular uptake of MC 540 as a function of incubation time at the indicated pH values in the medium without (B) and with (C) 10% FCS. MC 540 from the stock solution was added to the incubation medium to the final concentration of 13.2 μg ml^{-1}. Data points were fitted to the exponential rise to maximum function ($R^2 = 0.9820$ and 0.9937 for pH 7.4 and 6.8, respectively). Each data point represents mean ± S.E.M. from six independent measurements. The asterisks represent statistically significant difference (1% significance level). The Student's *t*-test was applied for statistical analysis. In cases when normality test failed (indicated by circle), Mann–Whitney rank sum test was used instead.

washing the cells with PBS (see Materials and methods). They confirmed a time-dependent increase of absorbance at wavelengths corresponding to monomers [22,23] and non-fluorescent species, as judged from the comparison with the corresponding excitation spectrum of the same sample (Fig. 4A). The sensitivity of the instrument does not allow an application of spectrophotometric analysis of the drug uptake, especially after the incubation of the cells in the presence of FCS. Therefore, the cellular uptake of MC 540 was evaluated after the extraction of cell-bound dye with *n*-butanol. The presence of FCS resulted in significantly lower amounts of MC 540 taken up by WiDr cells. Interestingly, the cellular uptake turned out to be pH-dependent (Fig. 4B,C) after the incubation of the cells with as well as without FCS.

3.3. Interaction with liposomes

Incorporation of MC 540 into liposomes was examined in the presence of NaCl. Absorption spectra of liposome-bound MC 540 were taken in the region 200–700 nm. No absorption peaks indicating lipid oxidation damage were detected. The lipid/drug ratio was chosen in such a way that the absorption peaks corresponding to MC 540 species in water phase were predominant in the sample without NaCl. Increasing the ionic strength in the suspension containing 100 nm egg PC large unilamellar vesicles led to changes in the absorption spectra of MC 540 in the presence of liposomes (Fig. 5). The absorption shoulder at 500.0 ± 0.8 nm, corresponding to MC 540 dimers in water phase [22,23], diminished with an increase of NaCl concentration. Concomitantly, the absorbance at 565.0 ± 0.5 nm, corresponding to MC 540 monomers incorporated in the lipid bilayer [22,24,25], increased. The same was true 2 min, as well as 2 h after the addition of MC 540 to the dispersion of liposomes. Qualitatively similar results (the lipid/MC 540 ratio was chosen so that the effect of increasing NaCl concentration could be seen) were obtained with egg PC/DMPA extrusion liposomes and sonicated SBPC liposomes. There was a salt concentration-dependent decrease of the absorbance ratio A_{530}/A_{565} irrespective of liposomes used (egg PC extrusion, egg PC/DMPA extrusion, sonicated SBPC). Alternatively, liposomes were prepared in 0.15 mol l^{-1} NaCl. Dilution of such dispersions unfortunately leads to contamination of the sample with ions, which might influence the results, especially at low ionic strength. However, a comparison of the absorption spectra of MC 540 in such liposomes (prepared in 0.15 mol l^{-1} NaCl) diluted in 0.15 mol l^{-1} NaCl showed no difference in the spectral shape as compared to MC 540 in liposomes prepared in water and diluted in 0.15 mol l^{-1} NaCl. No absorption peak corresponding to unbound MC 540 dimers or monomers in water phase was detected. The fluidity, as measured by fluorescence anisotropy of DPH in liposomes without MC 540, was unchanged, within the experimental error, in NaCl concentration range 0.01–0.30 mol l^{-1} (0.227 ± 0.009 and 0.231 ± 0.010 at 0.01 and 0.30 mol l^{-1} NaCl, respectively).

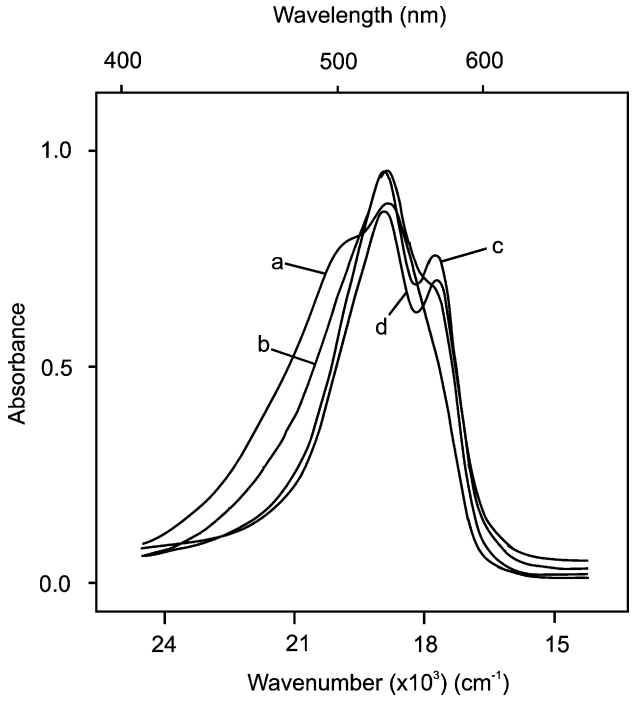


Fig. 5. Absorption spectra of MC 540 in the presence of egg PC extrusion liposomes—an effect of NaCl concentration. Liposome dispersion (20 mg ml^{-1}) was after extrusion through 100 nm polycarbonate filters diluted 70 times in (a) 0.00, (b) 0.01, (c) 0.15 and (d) 0.30 mol l^{-1} NaCl, and MC 540 was added afterwards. Spectra were recorded after 2-min incubation with MC 540.

4. Discussion

The present study shows that in order to explain selective localization of drugs in tumors, it is necessary to consider not only tumor cells but their environment as well. When investigating a contribution of pH in drug uptake by tumors, all the components of the system should be considered. The pH value and its changes might influence photosensitizer itself, the binding of the drug to blood proteins as well as interaction of the drug with cells. A detailed study dealing with spectral analysis of MC 540 water solutions at different pH values has been reported previously. It indicated that the protonation of SO_3^- group in the molecule of MC 540, resulting in neutralization of the charge of the drug, occurs at very low pH values [26,27], far outside the pH range examined in the present study. However, spectral changes comparable to those occurring at low pH values ($pH < 1.7$) have been observed in the presence of different cations even at physiological pH values [27].

Binding of MC 540 to blood serum and plasma components was judged from a gradual bathochromic shift of all the visible spectra and an enhancement of fluorescence

and absorption with increasing human plasma and FCS concentration in the sample (Figs. 1–3). Such changes have been attributed to the incorporation of MC 540 into a more hydrophobic environment as compared to water [14,22]. The dependence of fluorescence intensity on the human plasma concentration did not match the course of the curve based on wavelength position shifts. Since the fluorescence excitation spectra of MC 540 in the presence of the human plasma differed significantly from the corresponding absorption spectra (Fig. 4A), formation of non-fluorescent or very weakly fluorescent species might explain the observed difference. It is known that high polarizability of the chromophore of MC 540 in water containing environments allows concentration-dependent formations of dimers and higher aggregates with very weak fluorescence [23,28,29].

There was an obvious difference in the drug binding to FCS as compared to human plasma (Figs. 2 and 3). Such a difference is perhaps a consequence of different amounts of HDL and LDL contents within human plasma as compared to FCS [30] and/or species dependency of binding constants [31,32]. The difference should be taken into account in comparisons between in vivo and in vitro experiments. Selective uptake and retention of photosensitizers by tumor tissue in vivo is not always reproduced in experiments with malignant cells in vitro.

Consistently with the titration results, there was no significant difference in the binding of MC 540 to human plasma or FCS at the two pH values investigated (as measured shortly after drug addition). Neither was there any shift in the fluorescence spectra of MC 540 bound to human plasma/FCS in pH range 6–8. Nevertheless, variations in the interaction of MC 540 with different components and redistribution of the drug at longer time periods cannot be excluded.

As a result, the presence of the serum was expected to influence the interaction of MC 540 with cells. Indeed, significantly higher amounts of the drug were taken up by WiDr cells in the absence of FCS (compare Fig. 4B and C). Likewise, staining of external membranes of normal intact cells, nonexcitable cells and normal healthy leukocytes by MC 540 was abolished by the addition of serum [12,14]. Leukemic cells, however, fluoresced even in the presence of 2% autologous plasma [11,14]. It was presumed that this difference resulted from specific properties of leukocyte plasma membranes [14]. Because a pH effect on cellular drug uptake was observed in the presence as well as in the absence of FCS, and protonation of MC 540 is expected to occur at much lower pH values, it is reasonable to assume that higher cellular uptake of MC 540 at lower pH value is related to cellular properties rather than to protonation of the drug.

MC 540 has been used to monitor changes in transmembrane potential given by the concentrations of mobile ions on both sides of the cellular membrane. Therefore, a tentative explanation might concern a modification of the transmembrane potential as a response to a different pH in the extracellular space. The changes in the pH value of the incubation medium were found to result in modifications of the transmembrane potential[1] in erythrocytes and microglial cells [18,33]. Lagerberg et al. [18] showed that the pH dependence of MC 540 uptake by leukemic cells and erythrocytes is connected to transmembrane potential[1] modification. Smith et al. [29] found that MC 540 was specifically sensitive to the transmembrane potential portion of the electrochemical gradient in submitochondrial particles. However, whether transmembrane potential modifications induced by pH occur in the case of WiDr cells remains to be checked.

The local membrane electric field can be influenced not only by transmembrane, but surface and dipole potentials as well. Although in theory, different types of potential might be distinguishable, a separation of particular responses to them is not straightforward [34,35]. In order to check whether surface potential, related to surface charge, could be involved in cellular uptake of MC 540 and to avoid artefacts due to competitive phenomena in cellular system, experiments with liposomes were undertaken. An application of protons and divalent cations was excluded in order to avoid artefacts [36–42]. Increasing NaCl concentration in the sample resulted in an increased incorporation of the drug into liposomes of different physico-chemical characteristics. This was judged from the formation of the absorption peak usually attributed to monomeric species of MC 540 in lipid environment (e.g. Refs. [22,23]) on the expense of the peak assigned to MC 540 dimers in water environment (e.g. Refs. [23,24]) (Fig. 5). The spectral properties of MC 540 in the absence of liposomes are same, within experimental error, in water and in 0.01 or 0.14 M NaCl [27]; still in the presence of liposomes, obviously different spectral shapes were observed. Qualitatively the same effect of NaCl was seen with different types of liposomes—egg PC extrusion, egg PC/DMPA extrusion and SBPC sonicated. Liposomes were different in size and thus in the radius of curvature and lipid packing [43], surface charge and also in osmotic characteristics. Comparison of the spectra of MC 540 in liposomes prepared and diluted in water with the spectra in liposomes prepared and diluted in 0.15 mol l^{-1} NaCl, as well as qualitatively same results in osmotically insensitive [44] sonicated liposomes, rules out significant contribution of osmotic phenomena. This comparison also shows that the effect of salts is still present even in the absence of concentration gradient of Na^+ ions across the lipid bilayer. Other factors to be considered are membrane fluidity and lipid packing. Fluidity (as measured by fluorescence anisotropy of DPH) in egg PC liposomes was unchanged within the experimental error in the NaCl concentration range 0.01–0.30 mol l^{-1} (it might be different among individual

[1] The authors [18,29,33] have used the term "membrane potential" instead of transmembrane potential.

types of liposomes), and all the measurements were performed above the T_t (temperature of phase transition) of the lipids used. Nevertheless, an effect of ionic strength on lipid packing in the proximity of the lipid headgroups and/or on orientation of phosphatidylcholine headgroup dipoles [45] might also be involved. Still, the similarity of results with both large unilamellar and sonicated liposomes, which certainly differ in the lipid packing in the proximity of the lipid headgroups, indicates at least partial contribution of surface potential in the interaction of MC 540 with biological membranes. A modification of interfacial potential of liposomes by Na^+ ions is a known effect [46]. As no quantitative analysis is given, the results do not rule out importance of other well documented factors in the interaction of MC 540 with lipid bilayers. The results only point to another factor — surface potential, which should also be taken into account. Contribution of surface potential alterations in responses of MC 540 and related fluorescent probes has already been mentioned [29, 35]. Thus, higher cellular uptake of MC 540 by WiDr cells might be a consequence of screening of negative surface charge by increasing H^+ concentration (i.e. lowering pH).

Based on our results, we conclude that the presence of serum, pH, ionic strength and perhaps membrane potential are important factors that might influence the uptake of MC 540 by cells. Variations of pH within the physiological range are not expected to influence MC 540/membrane interactions directly—through modification of chemical structure of the drug or protonization of membrane-bound charge in the main region of MC 540/membrane interaction (which is the hydrophobic lipid region and not membrane protein one [47]). Nevertheless, they might lead to changes in screening of negative surface charges and perhaps in membrane polarization. Such effects will certainly influence the response of potential sensitive probes such as MC 540.

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