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H-Aggregation of Azobenzene-Substituted Amphiphiles in Vesicular Membranes

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Photochemical switching has been studied of double-tailed phosphate amphiphiles containing azobenzene units in both tails in aqueous vesicular dispersions and in mixed vesicular systems with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Since the ease of switching depends on the strength of the bilayer packing, particular emphasis has been placed on the occurrence of H-aggregation in the hydrophobic core of the vesicles. UV-vis spectrometry was employed to monitor H-aggregation and showed how this process depends on the ionic strength and on the mode of preparation of the vesicles. Two types of H-aggregates were observed in mixed DOPC vesicles with 5 mol % of azobenzene phosphate: one with λ_{max} at around 300 nm and one with λ_{max} at 305–320 nm. Those with λ_{max} at 300 nm could not be trans-cis photoisomerized, whereas those with λ_{max} at 305–320 nm are more loosely packed and can be photochemically switched. The permeability of the vesicular bilayers, as probed with leakage experiments using calcein as a fluorescent probe, was examined as another measure for the strength of bilayer packing. Leakage occurred only for DOPC vesicles containing more than 20 mol % of azobenzene phosphate, irradiated with UV light to induce trans-cis photoisomerization. We contend that detailed information on bilayer packing will be of crucial importance for fine-tuning the lateral pressure in vesicular membranes with the ultimate aim to steer the opening and closing of mechanosensitive protein channels of large conductance.

Introduction

Important aspects of the chemistry of biological cell membranes can be successfully mimicked by using bilayer vesicles formed from synthetic amphiphiles.¹ An interesting class of synthetic surfactants involves photoresponsive amphiphiles, particularly those with azobenzene units in the tails. *trans*-Azobenzenes can be isomerized into their *cis* isomers by UV light with high yields (up to 80% *cis* isomer) and without decomposition.² The reverse isomerization can be realized either thermally or upon irradiation with visible light.

Azobenzene-derived surfactants have been used for the control of membrane permeability^{3,4} and other macroscopic properties⁵ and can be employed in aggregates such as bilayers/vesicles,^{1,3,6} monolayers/Langmuir-Blodgett (LB) films,⁷ and micelles.⁸

Interesting phenomena in such aggregates containing azobenzenes are J-aggregation and H-aggregation.⁹ A

blue shift of the absorption maximum of the azobenzene group can be assigned to parallel interaction modes of the chromophores, called H-aggregation. By contrast, a red-shifted absorption band has been attributed to aggregation of the chromophores in a head to tail fashion (J-aggregation).⁹ These phenomena are not restricted to azobenzene-functionalized amphiphiles. For example, stilbene-derivatized surfactants have been thoroughly investigated by Whitten et al.¹⁰ Other examples include dye-containing surfactants such as squaraine¹¹ and salicylideneaniline.¹² In related studies,¹³ small amounts of cationic micelle-forming surfactants were added to aqueous dye solutions. At concentrations below the critical micelle concentration (cmc), H-aggregation was observed. H-Aggregation of azobenzenes has been observed in monolayers, LB films,⁷ bilayers, membranes, and vesicles.^{1,3,6} Interestingly, H-aggregation does not occur in spherical micelles containing azobenzene surfactants.⁸ Apparently the packing is too loose and the chromophores cannot adopt the required configuration in the micelle.

For the formation of bilayers, membranes, or vesicles, three types of azobenzene surfactants have been used in the literature. The overall trend for single-tailed, azobenzene-containing surfactants is that they form molecular dispersions, hydrated microcrystals, micelles, or bilayers.¹⁴

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At shorter chain lengths, there is a preference for micelles, and at higher tail lengths, for bilayers. Double-chain amphiphiles with one azobenzene-functionalized tail often form vesicles.^{5,14} In the case of two azobenzene-functionalized chains, usually flat bilayer structures of extended or folded sheets are formed.^{5,15}

Azobenzene-derived amphiphiles, which do not form vesicles, are often mixed with vesicle-forming surfactants. Kunitake et al.¹⁴ suggested different modes of aggregates of azobenzene amphiphiles and their spectral characteristics. It was found that there are two types of blue shifts for mixtures of azobenzene-containing surfactants and nonfunctionalized double-tailed surfactants: one at 330–340 nm, which was accounted for by a dimeric chromophore, and one at ca. 300 nm, which was attributed to H-aggregation. H-Aggregation was associated with microdomain formation in a multicomponent bilayer. An interdigitated bilayer structure was found for single-tailed azobenzene-containing amphiphiles, which possessed a 300 nm absorption band.¹⁶ Later¹ it was stated that every blue shift relative to the absorption of the isolated chromophore results from H-aggregation. More recently, Whitten et al.¹⁵ concluded on the basis of induced circular dichroism (ICD) measurements and Benesi–Hildebrand approach mixing experiments that the absorbance at ca. 320 nm should be attributed to trimers of an extended glide layer. However, for one azobenzene-functionalized phospholipid an aggregation number of 42 was obtained with a maximal absorbance at 315 nm. This study¹⁵ involved azobenzene-containing phospholipids which were chiral, and it was stated that the chirality is responsible for the trimer formation.

The temperature dependence of H-aggregation in vesicles has been studied by Kunitake et al.^{14,17} The general outcome was that H-aggregation decreased upon increasing temperature, indicating that the orientation of the azobenzene chromophore is affected by the fluidity within the membrane.

The permeability of azobenzene-containing vesicles has been studied by 5(6)-carboxyfluorescein,¹⁵ K⁺,¹⁸ and calcein¹⁹ release experiments. It was found^{15,19} that lysis occurred upon irradiation with UV light when the azobenzene phospholipids were in a gel-phase matrix. In a matrix of a liquid-crystalline lipid, leakage upon irradiation did not take place in the 0–20 mol % concentration range of azobenzene phospholipid. In these studies and others,²⁰ not much attention was paid to the relation between H-aggregation and the membrane permeability upon irradiation.

In the present paper, we present a more detailed study of H-aggregation of amphiphiles carrying two azobenzene-functionalized chains in aqueous vesicular dispersions. The results of our study provide more detailed insights into the impact of the preparation of the vesicles on the H-aggregation and into the effect of H-aggregation on membrane permeability.

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Trans–cis isomerization of azobenzenes is expected to have a significant influence on the interchain packing of membranes and on the lateral pressure. The ultimate goal of the present study is to apply these properties to influence the opening and closing of mechanosensitive protein channels of large conductance (MscL).²¹

Experimental Details

General Remarks. Water was distilled in an all-quartz distillation unit. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids. Sodium chloride p.a. and sodium acetate (p.a.) were obtained from Merck. Triton-X-100 was obtained from Fluka. Calcein and EDTA (tetra sodium salt hydrate) came from Sigma. HEPES (free acid, utrol grade) was obtained from Calbiochem.

General Techniques. UV–vis absorption spectra were recorded on a Hewlett-Packard HP 8453 FT spectrophotometer. The solutions were diluted to an azobenzene concentration of 25 μ M. The pH values were measured with a Consort C835 multiparameter analyzer. Irradiations were performed with an 180 W Oriol Hg lamp adapted with a suitable Mercury line filter for 313, 365, and 436 nm irradiations (typically bandwidth, 10 nm). ¹H, ³¹P, and ¹³C NMR spectra were recorded at 25 °C on a Varian Gemini-200 spectrometer operating at 200 MHz for ¹H, at 80.96 MHz for ³¹P, and at 50.29 MHz for ¹³C channels.

Differential Scanning Calorimetry (DSC). DSC scans were taken on a VP-DSC apparatus (Microcal, Northampton, MA) with a scan rate of 1 °C min⁻¹. The total amphiphile concentration was 2 mM. The reference cell was filled with water. For the determination of the solubility temperatures, DSC measurements were performed using a Perkin-Elmer DSC-7 apparatus operated with stainless steel pans. The reference cell contained an empty pan. Heating and cooling scans were run with scan rates of 5 °C min⁻¹.

Transmission Electron Microscopy. Negatively stained electron micrographs were obtained using a JEOL JEM 1200 EX electron microscope operating at 80 kV. Samples were prepared on carbon-coated collodion grids and stained with uranyl acetate (UAc) or phosphotungstic acid (PTA).

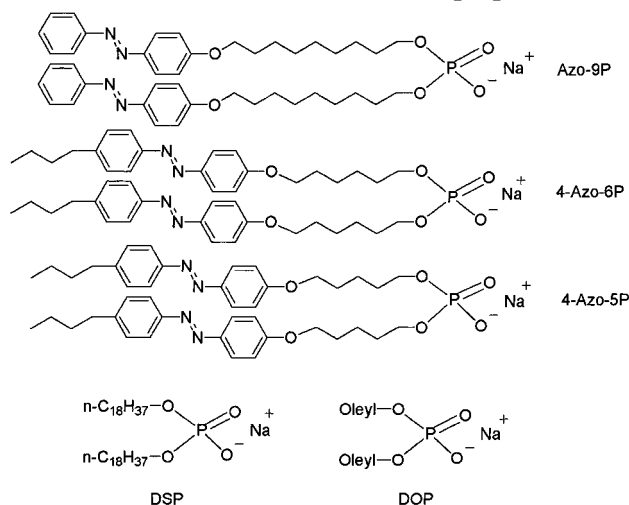
Vesicle Preparation. The appropriate amounts of the amphiphile stock solutions were mixed in methanol. These solutions were rotary-evaporated in tubes to yield thin lipid films, which were dried in a vacuum for at least 90 min. The appropriate amount of water was added to the vial, and the sample was vortexed for 30 s. Depending on the lipid composition, different methods were used to obtain vesicles. When sonication was applied, two different sonication methods were used. When tip sonication was applied, a Branson B15 sonication immersion tip (41 W, pulsed) was used. The second method employed a bath sonicator (Laboratory Supplies, model G112SPIT, 600 V, 80 KC, 0.5 A, 5–15 min), which was filled with water. For the calcein leakage experiments, a calcein solution of 20 mM (or 100 mM) was added to the amphiphile film (5 mM HEPES, 5 mM NaAc, 1 mM EDTA, pH 7.4, total lipid concentration of 5 mM). Vesicles were prepared as above. Nonencapsulated calcein was removed via gel filtration on a Sephadex G75 column (eluent: 5 mM HEPES, 5 mM NaAc, 1 mM EDTA, 29 mM NaCl (or 145 mM for the 100 mM calcein experiments), pH 7.4). The vesicle fraction was collected, and the fluorescence was monitored on an SLM-aminco SPF-500C spectrofluorometer. An excitation wavelength of 490 nm and an emission wavelength of 520 nm were used. The maximal fluorescence was obtained by solubilization of the vesicles with 1% v/v of Triton X-100. The measured fluorescence was corrected for dilution by Triton X-100. Leakage at time *t* was expressed in percentage relative to the initial fluorescence F_0 and the maximal fluorescence according to $(F_t - F_0)/(F_{TX} - F_0) \times 100\%$. Concentrations of 20 mM calcein²² were used when concentrations of 100 mM were not possible due to the high ionic strength.

Materials. The syntheses of the phosphate analogues of dioleoyl phosphate (DOP), Azo-9P, 4-Azo-6P, and 4-Azo-9P have been described previously.²³

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Chart 1. Structures of the Amphiphiles



Distearyl phosphate (DSP) was prepared from distearyl phosphonate by oxidation with I_2 .²⁴ White crystals were obtained in a 51% yield after crystallization from ethanol; mp 81–83 °C (lit. 82 °C).²⁵ 1H NMR (200 MHz, $CDCl_3$, δ): 0.88 (t, $J = 6.3$ Hz, 6H), 1.25–1.35 (m, 60H), 1.61–1.70 (m, 4H), 3.96–4.06 (m, 4H). ^{13}C NMR (200 MHz, $CDCl_3$, δ): 14.0, 22.7, 25.5, 29.2, 29.5, 29.6, 29.7, 30.3 (d, $J = 7.3$ Hz), 31.9, 67.7. ^{31}P NMR (200 MHz, $CDCl_3$, δ): 1.20(s). Distearyl phosphonate was prepared by same method as described earlier.²³

The sodium phosphates were prepared from the phosphates with the use of sodium ethoxide.²⁶

Results and Discussion

Aggregation Behavior of the Pure Azobenzene Amphiphiles. The structures of the amphiphiles used in this study are shown in Chart 1 together with their acronyms. Azo-9P, 4-Azo-6P, and 4-Azo-5P are only very slightly soluble in water at room temperature. DSC experiments between 30 and 95 °C (data not shown) were performed to obtain the solubilization temperatures. Only for Azo-9P was a solubilization temperature found (93.5 °C). All the azobenzene phosphates were stirred for 1 h at high temperature (≥ 95 °C). After cooling, the dissolved material was examined by electron microscopy (EM) and lamellar sheets were observed (data not shown). Similar results have been found for azobenzene phospholipids.¹⁵

UV–Vis Spectroscopy. 4-Phenylazophenol solubilized in water has a maximal absorbance at 347 nm and in hexane at 336 nm. This difference is attributed to a change of the medium polarity. The solutions of the dissolved pure azobenzene phosphates in water have a maximal absorbance at 302 nm. This blue shift is attributed to H-aggregation involving parallel stacking of the azobenzenes, with the transition dipoles of the azobenzenes aligned in a parallel fashion.⁹

Mixed Bilayer Aggregates. Because the azobenzene phosphates were not vesicle-forming, they were mixed with the vesicle-forming lipids DSP, DOP (100% cis), DOP (75% cis/25% trans), and DOPC. At 25 °C, the DSP bilayer is in the gel state. The phase transition temperature (T_m), measured by DSC, is around 78 °C. By contrast, both DOP

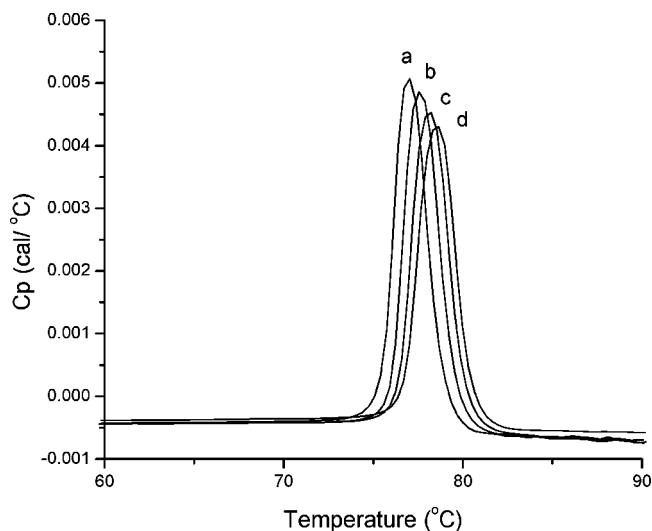


Figure 1. DSC graphs of DSP (2 mM). DSC graphs were taken at (a) day 1, $T_m = 76.8$ °C; (b) day 2, $T_m = 77.6$ °C; (c) day 3, $T_m = 78.0$ °C; (d) day 5, $T_m = 78.5$ °C.

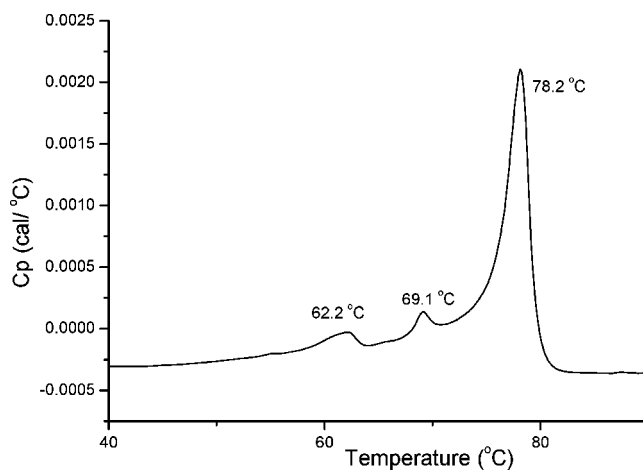


Figure 2. DSC graph of DSP (2 mM). The vesicles were prepared by tip sonication at 85 °C for 30 s.

and DOPC possess T_m values below 0 °C and are in the liquid-crystalline state. DSP vesicles were prepared by stirring at 85 °C for 1 h at day 1, and at days 1, 2, 3, and 5, DSC scans were recorded (Figure 1). The results show that it takes several days for the DSP vesicles to reach the thermodynamically most stable equilibrium. In Figure 2, a DSC graph of DSP is given for vesicles after tip sonication at 80 °C. The main phase transition occurs at 78.2 °C, but now two pretransition temperatures are observed.²⁷

EM experiments (negative staining) showed that at a ratio of 95 mol % DSP and 5 mol % 4-Azo-6P vesicles are formed. First the vesicles were formed by stirring of the vortexed solution for 1 h at 85 °C yielding a clear bluish dispersion. Large vesicles were observed (Figure 3a), and most of them were collapsed.

In the UV–vis spectrum, an absorption peak at 310 nm was observed (Figure 4). In a second series of experiments, the vesicles were tip sonicated for 90 s above the T_m and now much smaller vesicles were observed by EM (Figure 3b). Interestingly, the wavelength of maximal absorbance (λ_{max}) was shifted to 350 nm (Figure 4), consistent with the disappearance of H-aggregates.

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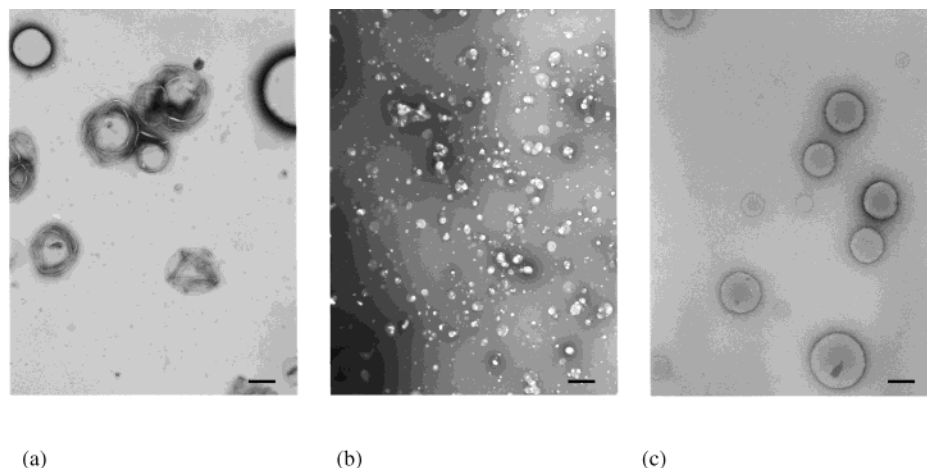


Figure 3. Negatively stained electron micrographs. Total lipid concentration = 2 mM. (a) 95 mol % DSP and 5 mol % 4-Azo-6P, uranyl acetate staining. The bar represents 500 nm. (b) 95 mol % DSP and 5 mol % 4-Azo-6P, after tip sonication, PTA staining. The bar represents 500 nm. (c) 95 mol % DOP (100% cis) and 5 mol % 4-Azo-6P, after bath sonication, uranyl acetate staining. The bar represents 100 nm.

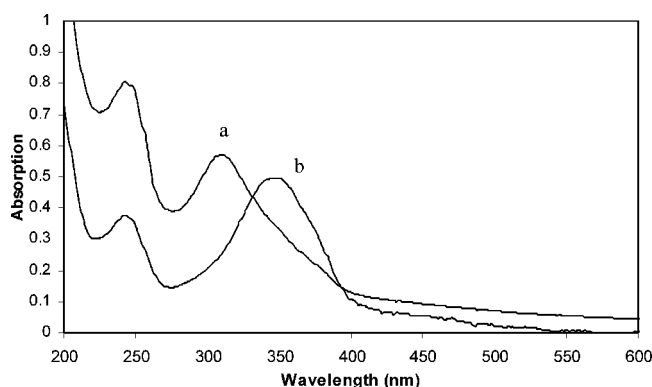


Figure 4. UV-vis absorption spectra of 4-Azo-6P (5 mol %) in DSP (95 mol %): (a) without tip sonication; (b) after tip sonication (90 s).

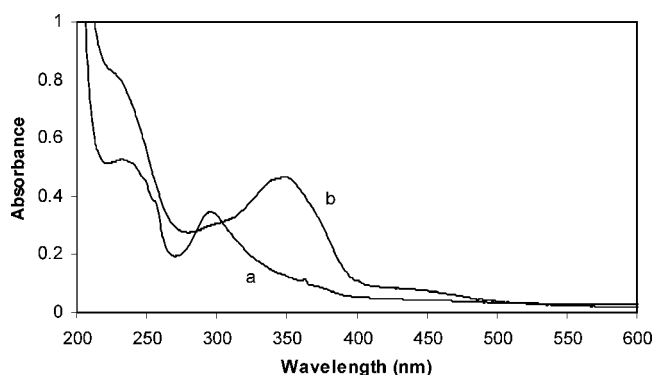


Figure 5. UV-vis absorption spectra of Azo-9P (5 mol %) in DOP (100% cis, 95 mol %): (a) before heating; (b) after heating to 95 °C for 1 h. The small irregularity at 360 nm is an artifact.

Because of the light scattering by the larger vesicles, the spectrum of the mixture without tip sonication shows a higher absorption level.

A mixture of DOP (100% cis, 95 mol %) and Azo-9P (5 mol %) was sonicated with the bath sonicator. Vesicles were formed (Figure 3c) exhibiting a λ_{\max} of 296 nm consistent with the formation of H-aggregates. (Figure 5). Heating of the vesicle solution to 95 °C for 1 h led to a shift of λ_{\max} to 350 nm. A similar effect has been reported previously.²⁸ After 3 days at room temperature, the UV-vis spectrum was still unchanged and H-aggregation had not taken place. In other words, the occurrence of H-aggregation is not reversible under these conditions.

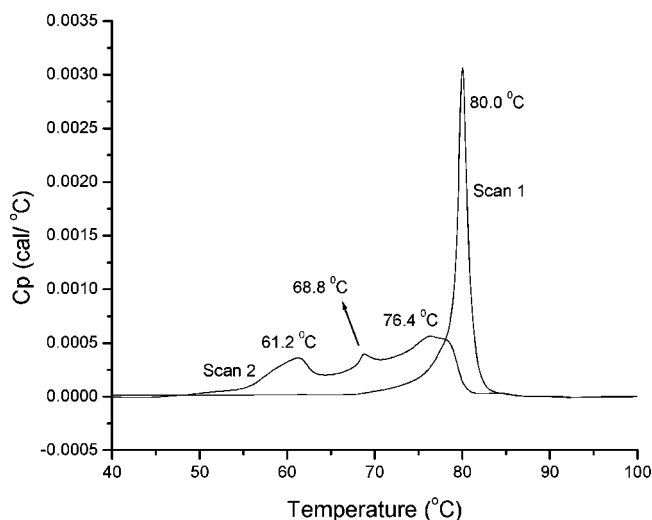


Figure 6. DSC graphs of Azo-9P (5 mol %) in DSP (95 mol %). The vesicles were prepared by stirring at 85 °C for 1 h.

DSC experiments also illustrated the effect of temperature on H-aggregation. A mixture of 95 mol % DSP and 5 mol % Azo-9P was stirred at 85 °C for 1 h, and a λ_{\max} of 310 nm was observed. The sample was subjected to DSC, and the first and second scans of the DSC are shown in Figure 6.

After the second scan, a λ_{\max} of 350 nm was found, which indicates that mixing of the lipids occurred during the first mixing at the higher temperatures (>95 °C). The second scan illustrates a severe disturbance of the DSP membrane packing by the azobenzene phosphates.²⁹

For all combinations of vesicle-forming lipids and azobenzene phosphates, vesicle solutions were prepared with 95 mol % of vesicle-forming lipid and 5 mol % of azobenzene phosphate. In Table 1, λ_{\max} and the preparation methods are given. EM experiments showed vesicle formation in all cases (data not shown).

The data in Table 1 suggest that 4-Azo-5P has the smallest tendency to form H-aggregates. Furthermore, the data indicate that there are roughly three different absorption maxima. One occurs around 350 nm, which is

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Table 1. Wavelengths of Maximal Absorbance for Combinations of Vesicle-Forming Lipids (95 mol %) and Azobenzene Phosphates (5 mol %)

	DSP ^a	DOP (100% cis) ^b	DOP (75% cis/25% trans) ^b	DOPC ^b
Azo-9P	310 347 ^d	296 350 ^d	300, 313 313, ^d 350 ^d	297, 350 350 ^d
4-Azo-6P	310 350 ^c	300 316 ^d	305, 355	350
4-Azo-5P	320 342 ^c	350	350	351

^a Vesicles were prepared by stirring the solutions for 1 h at 85 °C. ^b Vesicles were prepared by bath sonication at room temperature. ^c After tip sonication for 1–3 min at 85 °C. ^d Samples were heated to 95 °C for 1 h.

probably due to azobenzene monomers or dimers in a vesicular aggregate. Then there are two absorptions at lower wavelengths, around 300 and 310 nm, respectively. Presumably, both are due to H-aggregation. The absorption around 300 nm seems to come from H-aggregates, which are strongly packed and, in fact, are due to azobenzene phosphate aggregated in microdomains. The H-aggregates which exhibit the 310 nm absorption apparently represent more loosely packed azobenzene phosphates in their mix with the carrier amphiphile.^{14,28} Some combinations, which lacked the 350 nm absorption band, were heated to 95 °C for 1 h. From the table, it can be seen that this leads to a change in absorbance from 300 or 310 nm to 350 nm. Most likely, heating of the sample breaks up the H-aggregates of the azobenzene phosphates in the vesicle.

A strong indication that the H-aggregates with the 300 nm absorption band are incorporated in vesicles, rather than, for example, forming part of small bilayer fragments, came from an encapsulation experiment. Vesicles (95 mol % DOPC/5 mol % Azo-9P) were prepared by bath sonication in a 20 mM calcein solution and filtrated over a Sephadex column. The UV–vis absorption spectrum of the collected vesicle fraction showed an absorption at 300 nm indicating that the H-aggregates were present in the vesicle fraction from the column.³⁰

Irradiation Experiments. Particular emphasis was placed on the question of how far photochemical trans–cis isomerization of the azobenzene unit in the vesicles affected bilayer packing and formation of H-aggregates. All the samples were irradiated with light of a specific wavelength to induce the trans/cis isomerization. A typical irradiation cycle is given in Figure 7. Initially the absorption spectrum contains a π – π^* transition at 248 nm, a second intense π – π^* transition at 350 nm, and a weak n – π^* transition near 440 nm. Using light of 365 nm, the azobenzenes were isomerized to the cis isomers, leading to an increase in absorbance at 440 nm and a decrease at 350 nm. At 310 nm, a new absorption is observed. For the back isomerization to the trans isomer, light of 436 nm was used.

For some mixtures, precipitation was observed which was also confirmed by the UV–vis spectra. There was no clear trend; only for the mixtures with 4-Azo-5P did almost no precipitation occur. Previously, Sakai et al.³¹ also encountered precipitation and attributed this to disruption

(30) At a concentration of 20 mM of encapsulated calcein, the absorption of calcein around 300 nm is significantly lower than that of the azobenzene in these samples. The strong absorbance in Figure 7 at 300 nm can be attributed to H-aggregation. At a concentration of 100 mM of encapsulated calcein, a weak absorption band is observed at 300 nm (see also Figure 14).

(31) Sakai, H.; Matsumura, A.; Yokoyama, S.; Saji, T.; Abe, M. *J. Phys. Chem. B* **1999**, *103*, 10737.

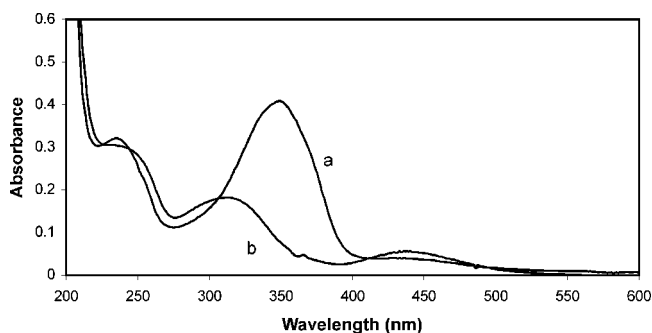


Figure 7. UV–vis spectra of Azo-9P (5 mol %) in DOPC (95 mol %): (a) before irradiation; (b) after irradiation with 365 nm light. After subsequent irradiation with 436 nm light, an absorbance curve identical to the one in curve a was obtained. The vesicles were prepared by heating to 95 °C for 15 min.

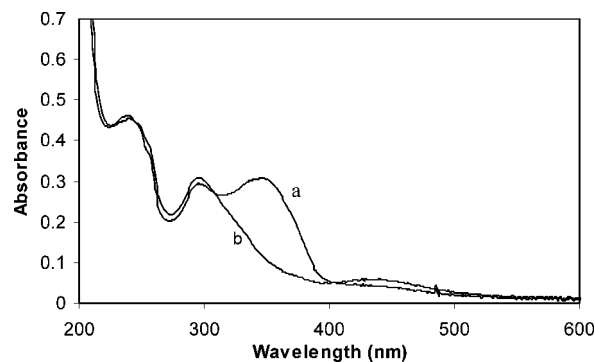


Figure 8. UV–vis spectra of Azo-9P (5 mol %) in DOPC (95 mol %): (a) before irradiation; (b) after irradiation with 365 nm light. After subsequent irradiation with 436 nm light, an absorbance curve identical to the one in curve a was obtained. The vesicles were prepared by bath sonication.

of the vesicles into larger aggregates (precipitate) upon UV-light irradiation.

When vesicles containing H-aggregates were irradiated, some remarkable results were obtained. Irradiation of mixtures showing an absorption band around 300 nm with 365 nm light had no effect on the 300 nm absorption³² (see, for an example, Figure 8). The change in absorbance around 350 nm should be ascribed to trans–cis isomerization of azobenzene amphiphiles not residing in microdomains. This was also found for the combination of DOP (75% cis/25% trans) and Azo-9P (the unheated sample). Irradiation with 313 nm light also did not affect the 300 nm absorption band. We contend that the H-aggregates responsible for the 300 nm absorption band are hard to isomerize. This is in accordance with an increase in the efficiency of the interchain interaction in the bilayer as the result of the formation of tight H-aggregates present in microdomains.

By contrast, vesicle solutions with an absorption around 310 nm could be readily isomerized to the cis configuration by irradiation with 365 nm light. An example which consists of 4-Azo-6P (5 mol %) in DSP is given in Figure 9. After the irradiation cycle, λ_{max} is 336 nm, indicating that weak H-aggregation still takes place. These results confirm the notion (vide supra) that these types of H-aggregates possess weaker interactions than H-aggregates with an absorption band around 300 nm. Irradiation of a sample with an absorption band around 310 nm with light of 313 nm induced a decrease in absorbance at around 310 nm and an increase at around

(32) Nakahara, H.; Fukuda, K.; Shimomura, M.; Kunitake, T. *Nippon Kagaku Kaishi* **1988**, 1001.

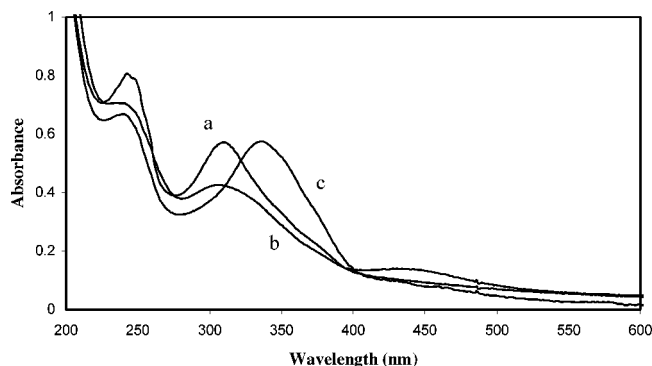


Figure 9. UV-vis spectra of 4-Azo-6P (5 mol %) in DSP (95 mol %): (a) before irradiation; (b) after irradiation with 365 nm light; (c) after subsequent irradiation with 436 nm light.

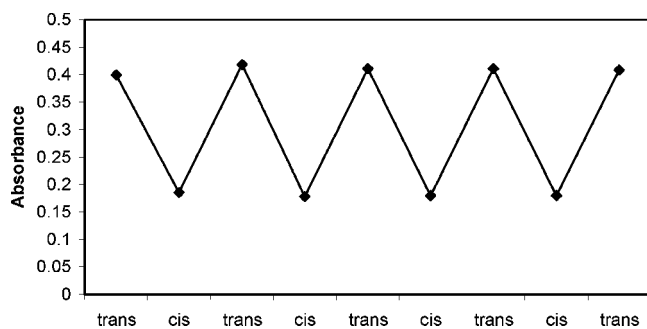


Figure 10. Repeated cycles of the isomerization of Azo-9P in a vesicle composed of 95% DOPC and 5% lipid Azo-9P. For the trans configuration, the absorbance at 349 nm is given, and for the cis configuration, the absorbance at 313 nm is given.

350 nm (data not shown). This apparently shows that the H-aggregates with an absorbance around 310 nm can be broken up by irradiation with light of 313 nm but no isomerization occurs.

The isomerization of Azo-9P (5 mol %) in DOPC (95 mol %) with light of 365 nm was examined in greater detail. Vesicles were prepared by waterbath sonication, and subsequently they were heated to 95 °C for 1 h. UV-vis spectrometry revealed that there was no H-aggregation ($\lambda_{\text{max}} = 349$ nm). Results given in Figure 10 show that the isomerization cycle could be repeated several times without decomposition of the material.

In a further experiment, the vesicle solution (DOPC 95 mol %/Azo-9P 5 mol %) was subjected to irradiation (at 365 nm) for 30-s intervals and the UV-vis spectrum of the sample was taken between each irradiation cycle (Figure 11). The same type of experiment was carried out for isomerization from cis to trans. In both cases, isobestic points were observed. This means that there are no side reactions and that decomposition does not take place.

Calcein Encapsulation Experiments. The strength of bilayer packing in the mixed phosphate amphiphiles was further investigated by performing leakage experiments. Thus, the permeabilities of DOP, DSP, and DOPC vesicles were tested using calcein as a fluorescent probe.³³ Calcein was encapsulated at a sufficiently high, self-quenching concentration, and free calcein was removed by filtration over a Sephadex G75 column. Leakage of the fluorescent probe was monitored for at least 30 min by fluorescence spectroscopy. DOPC vesicles showed no leakage of encapsulated calcein. Vesicles of DOP (100% cis and 75% cis/25% trans) could not be formed using

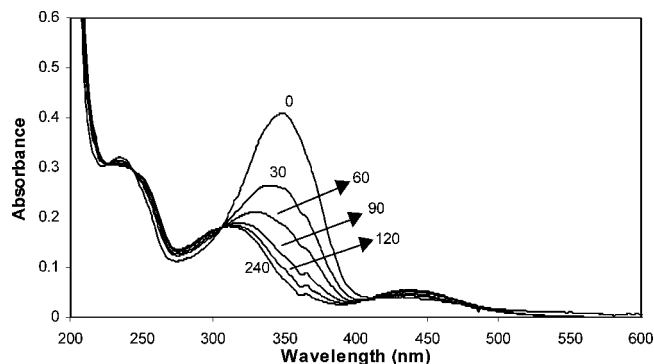


Figure 11. UV-vis spectra of Azo-9P (5 mol %) in DOPC (95 mol %) at different stages of the isomerization to the cis isomer. The sample was irradiated with 365 nm light. The numbers refer to seconds. The small irregularity at 360 nm is an artifact.

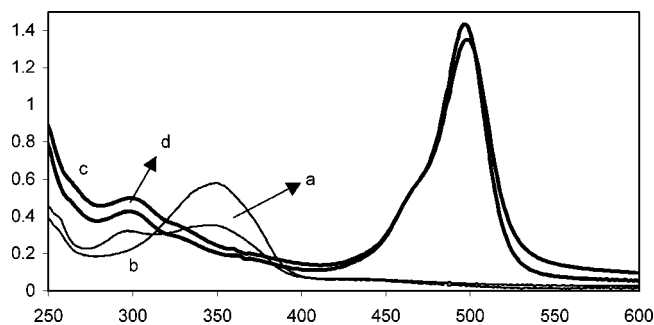


Figure 12. UV-vis spectra of Azo-9P (5 mol %) in DOPC (95 mol %). Vesicles were prepared by bath sonication until clear. (a) Vesicles in pure water. (b) Vesicles in pure water after heating to 95 °C. (c) Vesicles in a solution containing 100 mM calcein, 5 mM HEPES, 5 mM NaAc, and 1 mM EDTA. (d) The same as for curve c but now after heating to 95 °C.

calcein concentrations of 100 mM, but at a calcein concentration of 20 mM vesicles could be prepared. Under these conditions, there was no leakage of calcein. Vesicles of DSP could not be prepared at either low or high concentrations of calcein. Apparently the high ionic strength inhibits the formation of vesicles.

Introduction of the azobenzene-functionalized phosphates into vesicular dispersions containing calcein and buffer gave rise to an interesting phenomenon. Two vesicle solutions (DOPC 95 mol %/Azo-9P 5 mol %) were simultaneously prepared, one with pure water and the other one with an aqueous solution of calcein and a buffer. The two solutions were bath sonicated until clear. Free calcein was removed by filtration over a Sephadex column. As evidenced by the UV-vis spectra (Figure 12), H-aggregation (300 nm absorbance) occurs more extensively for vesicles containing calcein and buffer.

After heating the samples, the pure water sample exhibits no H-aggregation anymore but the sample containing calcein and buffer still displays H-aggregation (no peak around 350 nm). It was already shown previously that microdomain formation in biomembranes and liposomes can be induced by addition of ionic solutes.³⁴ However, the H-aggregation in the samples containing calcein could be broken up by tip sonication. Figure 13 shows the UV-vis spectra of a mixture of DOPC (95 mol %) and Azo-9P (5 mol %) in the presence of 0, 20, or 100 mM of calcein after tip sonication in different solutions. Nonencapsulated calcein was removed by filtration over Sephadex because otherwise no UV-vis spectra could be taken.

(33) Allen, T. M. In *Targeted Drug Delivery and Biological Interaction*; Gregoriadis, G., Ed.; Liposome Technology, Vol. III; CRC Press: Boca Raton, FL, 1984; pp 177–182.

(34) (a) Ohnishi, S. *Adv. Biophys.* **1975**, *8*, 35. (b) Mayer, L. D.; Nelsestuen, G. L. *Biochemistry* **1981**, *20*, 2457.

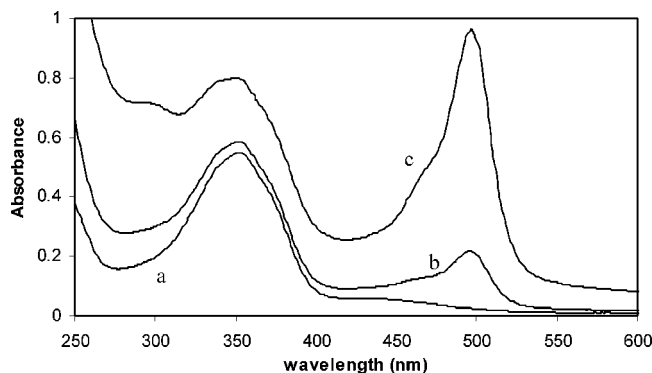


Figure 13. UV-vis spectra of Azo-9P (5 mol %) in DOPC (95 mol %). Vesicles were prepared by tip sonication until clear. (a) Vesicles in pure water. (b) Vesicles in a solution containing 20 mM calcein, 5 mM HEPES, 5 mM NaAc, and 1 mM EDTA. (c) The same as for curve b but now for 100 mM calcein.

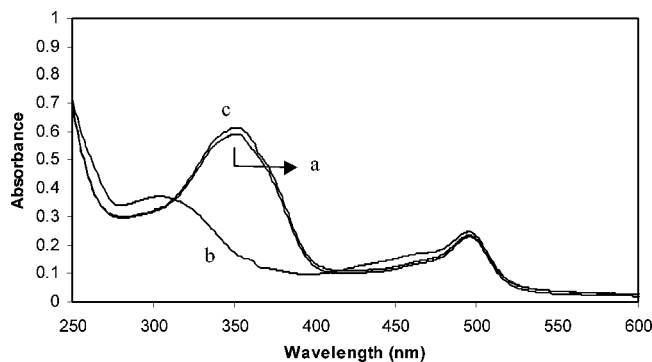


Figure 14. UV-vis spectra of DOPC (95%) and Azo-9P (5%) containing 20 mM calcein: (a) before irradiation; (b) after irradiation with 365 nm light; (c) after irradiation with 436 nm light.

Comparison with the calcein spectrum in pure DOPC vesicles suggests that the absorption around 300 nm for the vesicle solution with 100 mM of calcein can be mainly attributed to the absorption of calcein. The three solutions were monitored for a few days, and no changes in UV-vis spectra were observed. The solutions were stable for at least 4 days. The leakage of the fluorescent probe was monitored by fluorescence spectroscopy for the combination of DOPC (95 mol %)/Azo-9P (5 mol %) containing 20 mM of calcein (after tip sonication). After an hour, no significant leakage was observed.

Combined Calcein Release and Irradiation Experiments. Vesicle solutions of DOPC (95%)/Azo-9P (5%) (tip sonicated) containing 20 mM of calcein were irradiated with the suitable wavelength to induce a trans to cis isomerization (365 nm). The leakage before and after irradiation was compared. In Figure 14, the UV-vis spectra of the different stages of the irradiation cycle are given. The azobenzene was kept for a few minutes in the cis configuration before irradiation with light of 436 nm was initiated.

No significant leakage was observed before and after irradiation, indicating that no leakage occurred during the irradiation. These results agree with those of Sandhu et al.³⁵ (11% (w/w) Azo-PC in DPPC). In other studies,^{15,19} leakage was observed (5–20% mol/mol Bis-Azo PC in various phospholipids), but unfortunately, UV-vis spectra (or λ_{\max}) were not (completely) given and the aggregation state of the azobenzenes was therefore unclear. It was

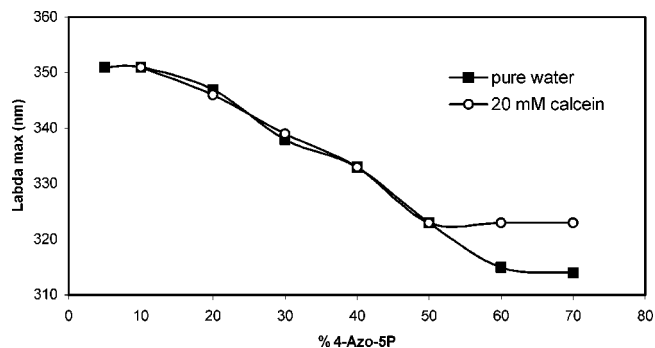


Figure 15. The wavelengths of maximal absorbance (λ_{\max}) against mol % 4-Azo-5P. 4-Azo-5P was mixed with DOPC, and the total lipid concentration was kept constant. The lines drawn are just a guide to the eye. Preparation methods: pure water containing 5 mol % of 4-Azo-5P; bath sonication at room temperature; for 10 and 20 mol % of 4-Azo-5P, no sonication was applied. Clear solutions were obtained after vortexing. For 30, 40, 50, 60, and 70 mol % of 4-Azo-5P, tip sonication was applied for 1–2 min at 50–70 °C; for 20 mM calcein, all the solutions were tip sonicated for 2 min at 70 °C.

already stated by Whitten et al.⁶ that the consequences of photoisomerization are strongly dependent on the aggregation state of the azobenzene amphiphile. When an azobenzene was present as monomer or dimer, irradiation resulted in little release of entrapped fluorophore. However, when H-aggregates could be photoisomerized, a rapid release was observed.¹⁵

Vesicles from DSP could only be obtained in the presence of less than 5 mol % of azobenzene phosphate. Conversely, DOPC could be mixed with 4-Azo-5P up to at least 70 mol % and still vesicles were formed. Mixtures up to 70 mol % 4-Azo-5P were prepared in pure water, and λ_{\max} was examined by UV-vis spectroscopy (Figure 15). A gradual shift is observed upon increasing the concentration of 4-Azo-5P. This suggests that the H-aggregation develops gradually and that at concentrations between 5 and 70 mol % no microdomain formation occurs. Moss et al.³⁶ performed a similar experiment. A double-chained lipid with an ammonium headgroup and one azobenzene-functionalized tail was mixed with the corresponding saturated dialkyl lipid. The azobenzene was connected at both sides to the rest of the lipid chain by oxygen bridges. A nongradual shift of λ_{\max} was observed from 364 to 336 nm going from 10 to 100 mol % of azobenzene-containing lipid. In more detail, going from 10 to 66 mol % a total shift of 8 nm was observed and then from 66 to 75 mol % a shift of 12 nm was reported. By contrast, a gradual increase in H-aggregation was reported recently for a DNA strand,³⁷ the effect increasing with an increasing number of azobenzene units.

Returning to our amphiphiles, calcein was encapsulated at different concentrations of 4-Azo-5P in DOPC. First, vesicle preparations were performed using 100 mM of calcein, but already at 10 mol % of 4-Azo-5P in DOPC no stable vesicle solutions were formed. At a lower calcein concentration (20 mM), vesicles could be prepared till at least 70 mol % 4-Azo-5P. After filtration over Sephadex, no leakage was observed for at least 30 min for all samples. In Figure 14, the λ_{\max} values of the samples are given. Going from 10 to 50 mol % 4-Azo-5P, similar values were found as for the pure water samples. The reason for the deviation at the 60 and 70 mol % 4-Azo-9P is not clear at the moment. Interference of the absorption of calcein seems

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(36) Moss, R. A.; Jiang, W. *Langmuir* **1995**, *11*, 4217.
(37) Asanuma, H.; Shirasuka, K.; Takarada, T.; Kashida, H.; Komiyama, M. *J. Am. Chem. Soc.* **2003**, *125*, 2217.

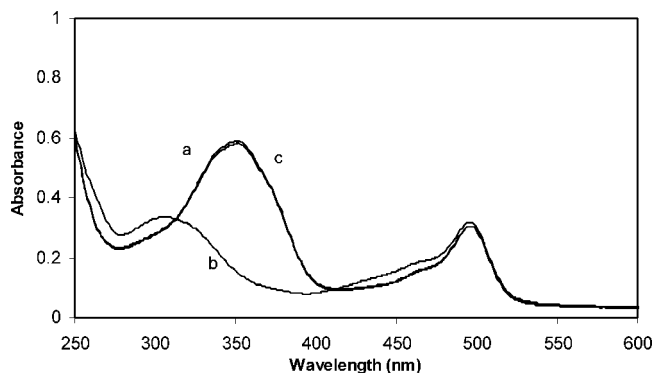


Figure 16. UV-vis spectra of DOPC (90%) and 4-azo-5P (10%) containing 20 mM calcein: (a) before irradiation; (b) after irradiation with 365 nm light; (c) after irradiation with 436 nm light.

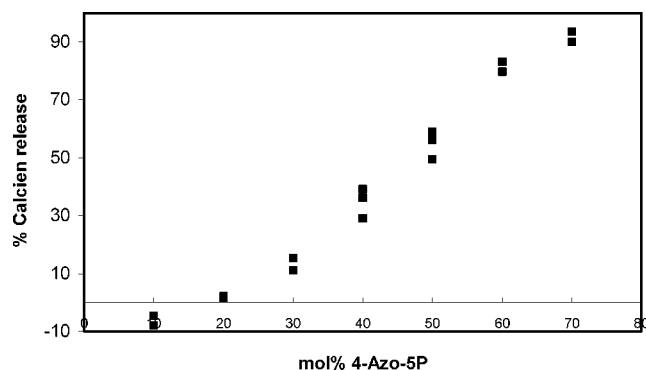


Figure 17. Irradiation-induced calcein release as a function of the concentration of 4-Azo-5P. 4-Azo-5P was mixed with DOPC, and the total lipid concentration was kept constant. The vesicles contained 20 mM calcein. The solutions were tip sonicated for 2 min at 70 °C. The solutions were irradiated at 365 nm for 15 min and, immediately afterward, 90 s at 436 nm.

not to be the case because the absorption of calcein between 300 and 350 nm is considerably lower than that of the azobenzenes in these samples.

All samples with calcein were irradiated with 365 nm light for 15 min. Directly afterward, the sample was irradiated with 436 nm light to obtain a complete isomerization back to the trans azobenzene. The leakage was then monitored immediately by fluorescence spectroscopy. An example of an irradiation cycle is given in Figure 16.

If leakage of calcein occurred at higher concentrations of 4-Azo-5P (>20 mol %), it only took place during the irradiation cycle. After the irradiation cycle (azobenzene groups completely back into the trans configuration), no leakage was observed in time. The irradiation-induced calcein leakage as a function of the concentration of 4-Azo-5P is shown in Figure 17. The small negative value at 10 mol % 4-Azo-9P could be caused by photo-

bleaching of the calcein during the irradiation with 436 nm light. At a concentration of 70 mol % 4-Azo5P, 90% of the calcein was released. It is clear that an increase in the concentration of azobenzene leads to an increase in leakage. Most likely, the chain packing in the cis configuration of the azobenzene is less effective, leading to more leakage of calcein. This is in agreement with the results of Whitten et al.¹⁵ They measured the release of 5(6)-carboxyfluorescein (CF) from DPPC vesicles, which contained approximately 5 or 9 mol % of azobenzene phospholipids (APL). In the case of 9 mol % APL, an almost complete release of CF was observed after 40 s of irradiation. A concentration of 5 mol % APL led to a release of about 20% after 9.5 min of irradiation. CF has a higher ability for leakage than calcein due to its lower molecular weight and its lower net negative charge.³³ Whitten et al.¹⁵ suggested that photoisomerization of aggregates of azobenzenes (H-aggregates) promotes more calcein release than irradiation in the presence of non-aggregated azobenzene or an isolated dimer. At this point, we suggest a distinction between H-aggregation that is due to clustering of the azobenzene amphiphiles in microdomains (Figure 18c) and H-aggregation at higher concentrations of azobenzene amphiphiles in which the lipids are equally distributed in the bilayer (Figure 18b).

When at lower azobenzene amphiphile concentrations (0–10 mol %) a λ_{\max} is observed which is clearly blue shifted, it can be assumed that a clustering of the azobenzene amphiphiles has taken place. Isomerization of the azobenzene will lead in this case sooner to fluorescent probe release than in the case of nonclustered azobenzene amphiphiles.

Conclusions

Phosphate amphiphiles containing azobenzene groups have been studied, and they show interesting features when they reside in a vesicular membrane. UV-vis spectra can give indirect information about the aggregation state within in the membrane. Experiments indicate that a λ_{\max} between 297 and 305 nm can be attributed to maximal H-aggregation and in case of mixed bilayers to extensive microdomain formation. Maximal H-aggregation means the closest/strongest interaction possible between the azobenzenes with a concomitant strong interchain interaction in the vesicular bilayer. Trans-cis isomerization of the azobenzenes in this type of aggregate is not possible. A λ_{\max} around 350 nm can be attributed to the monomeric or dimeric form of the azobenzene, and now trans-cis isomerization is easy. A single absorption band between 320 and 350 nm (weak H-aggregation) is consistent with a homogeneous distribution of the amphiphiles in mixed systems or a clustering of the azobenzenes (at low concentrations of the azobenzene amphiphiles). We find that for a vesicular system containing a low concentration of azobenzene phosphate and showing a λ_{\max} between 305

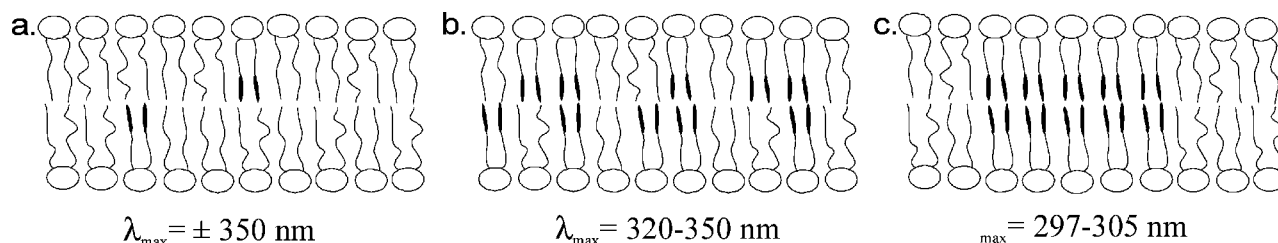


Figure 18. Schematic presentations of the type of aggregates of the azobenzenes and the corresponding wavelengths of maximal absorbance (λ_{\max}): (a) low concentration of azobenzene amphiphile, no H-aggregation; (b) H-aggregation but no microdomain formation; (c) H-aggregation and microdomain formation.

and 320 nm clustering of the amphiphiles does occur and the H-aggregates are more loosely packed than the H-aggregates responsible for the 300 nm absorption band.

Upon increasing the concentration of azobenzene phosphate, a gradual decrease in the λ_{\max} was observed and photochemical switching can occur readily.

No leakage of calcein was observed for mixtures containing DOPC and 10–70% 4-Azo5P for at least half an hour. Isomerization to the cis isomer led to calcein release at concentrations above 20 mol % of 4-Azo-5P. Upon increasing the concentration of azobenzene phosphate to 70 mol %, leakage becomes more extensive.

The present results show that the method of vesicle preparation has a large influence on H-aggregation. Sonication, irradiation, and heating of the sample can disrupt the H-aggregation. Addition of (organic) ions can induce (additional) H-aggregation. Determination of the aggregation state of azobenzenes by UV–vis appears to be a prerequisite in all detailed studies of these types of vesicles.

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