

THE USE OF HYDROPHOBIC DYE MOLECULES IN MONITORING THE LIPOSOME BILAYER MICROENVIRONMENT AND LOCATING BLOCK COPOLYMERS ADDED TO ENHANCE LIPOSOME STERIC STABILITY

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ABSTRACT

The dispersion of soybean lecithin in water leads to the formation of multilamellar vesicles (MLVs), which on sonication (4hrs approx.) break down to small unilamellar vesicles of ~50nm diameter. The addition of polymeric molecules in the liposomal system provides increased steric stabilization. The molecules used were (tri-)block copolymers (Synperonics) containing a central hydrophobic part (polypropylene oxide-PPO) and two hydrophilic chains (polyethylene oxide-PEO) extending from either side. The interaction of these molecules with the vesicle bilayer is thought to be of upmost importance to the mechanical stress, thermodynamic restrictions and steric stability that may be induced. The exact localisation of the copolymer molecules was attempted using a multiprobe technique. The full spectrum of two hydrophobic dyes, namely Nile red (NIL) and Pinacyanol chloride (PCYN), were compared while solubilized inside the liposome bilayer. The sensitivity of their spectral characteristics to polarity and molecular mobility produced a monitor of the bilayer micropolarity and fluidity. The relatively high hydrophobicity of Nile red (NIL) provides an accurate polarity sensor of the bilayer microenvironment. The formation of Pinacyanol chloride (PCYN) dimers (and their respective peak) was directly related to the distance between the dye molecules. Shifts of the maximum absorbance (λ_{max}) for both dyes showed that the bilayer environment was becoming more apolar with increasing copolymer concentration. The absorbance intensity decreased with increasing copolymer concentration, denoting a reduction in the solubilization of both dyes and therefore of the bilayer population. The absorbance peak of Pinacyanol chloride (PCYN) due to dimer formation increased at moderate copolymer concentrations, showing signs of possible incorporation inside the bilayer. These experiments provided information about the bilayer

structure. Adding block copolymers at an optimum concentration may increase the stability of the liposome by incorporation, following various models proposed. However, at high content of copolymer some bilayer solubilization and mixed micelle formation may occur.

INTRODUCTION

Phospholipid vesicles (liposomes) are considered to be extremely advantageous colloidal dispersions, because of their ability to solubilise both hydrophilic and lipophilic substances in their inner aqueous phase and their lipidic bilayer, respectively. Their biocompatibility (low toxicity) and biodegradability has attracted numerous applications, mainly in the medical field, as carriers of various drugs.

Steric stabilisation of liposomes was thought to be achieved by the incorporation of surfactant molecules that are known to provide effective steric stabilisation, namely (tri-)block copolymers, which may be incorporated inside their lipophilic bilayer. Importance has been placed on the method of producing the vesicles. It was thought that addition of the copolymer molecules initially, before formation of the multilamellar (MLVs) or the unilamellar (SUVs) vesicles, would allow the polymer to participate in the vesiculation process and bury its hydrophobic component in the lipid bilayer with greater ease than if it was added after the high energy sonication step. In the latter case, the block copolymer is believed to follow a classic physical adsorption mechanism with some gradual penetration onto the liposome surface.

The description of the liposome bilayer is important in providing essential evidence as to whether the hydrophobic component of the copolymer molecule incorporates inside the bilayer or just adsorbs on the phospholipid surface. The employment of two hydrophobic dyes Nile Red (NIL) and Pinacyanol chloride (PCYN) aimed at probing the bilayer.

In this study, by monitoring changes in the respective UV/visible absorbance patterns of the particular chromophores, information may be extracted about their direct microenvironment.

MATERIALS AND METHODS

Soybean lecithin was purchased by Sigma (~ 48% DMPC purity). The sonication technique (1) using a Kerry ultrasonic bath was used for the preparation of the vesicles. The study involved the use of one block copolymer of the Synperonic family, supplied by ICI Surfactants, Belgium; namely the Synperonic PF127 of the following structure:



Nile red (NIL) and Pinacyanol chloride were purchased from Sigma Chem. Co. All absorbance measurements were carried out using a Lambda 2, Perkin-Elmer UV/vis Spectrometer. All materials were used without further purification.

Preparation of the Vesicles

Importance is placed on the method of preparing the phospholipid-copolymer vesicles. It is thought that the addition of the polymer before the formation of the vesicles (multi- or unilamellar) leads to more stable systems, compared with addition of the copolymer after the MLVs or SUVs have formed. For ease of presentation, the samples denoted (I) refer to vesicle systems where the block copolymer was added Initially, while the samples denoted (A) refer to the systems with addition of the polymer After the liposomes had been formed.

Stock solutions of NIL and PCYN were prepared in a $\text{CHCl}_3:\text{CH}_3\text{OH}$ (4:1) solvent mixture. The required amount of lecithin was also dispersed in the solvent mixture and rotary evaporated at 40 °C and under 1bar pressure. The dry lipid and dye film formed in the round bottomed flask was left under pressure for at least 1hr to ensure the complete removal of solvent traces.

In the case of initial addition, the copolymer was added to the dry lipid-dye film and the mixture was redispersed in water. Sonication followed for approx. 4hrs using a sonibath and the mean vesicle diameter was monitored by dynamic light scattering measurements (PCS). All (I) samples were prepared separately.

In the case of addition of the polymer after vesicle formation, a stock lecithin-dye mixture was dispersed in solvents, rotary evaporated and resuspended in water. Sonication and small unilamellar vesicle formation followed. The liposomal system was titrated with aqueous copolymer solutions to reach the required amounts.

All procedures were performed with minimum exposure to light, keeping the dye solutions wrapped in aluminum foil at all times, to avoid any photodegradability complications. The final concentration of lipid was 2% (wt/wt) and the lipid:dye molar ratio was kept constant for all preparations at 500:1. At this molar ratio, the partition coefficient between membrane-bound and free dye molecules in solution ensures that no significant interference of the spectral data occurs. As has been previously shown (2) not more than 5% of the cyanide dye (PCYN) and only traces of the highly hydrophobic NIL are dissolved in buffer. The corresponding dye-free vesicle preparations were used as reference samples to compensate for turbidity effects.

RESULTS AND DISCUSSION

Nile red is an uncharged benzophenoxazone dye, poorly soluble and strongly quenched in water, with high partition coefficient from water to hydrophobic solvents. In chloroform it gives a light red colour, while solubilised in vesicles it shows a deep red colour. It is photochemically stable. NIL has been used as a fluorescent stain for intracellular lipids (3), and as a polarity sensor deducing protein structure and conformation (4). Its structure (FIG. 1) reveals

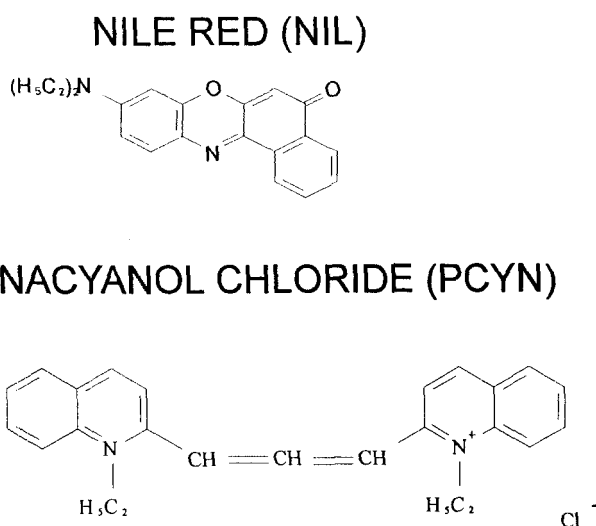


FIG. 1 Molecular structure of the chromophores NIL and PCYN

widely separated potential electron donor and acceptor groups, suggesting that its spectroscopic properties may be sensitive to the polarity of the solvent (5).

Nil's absorbance spectrum shows a single broad peak at 556 nm. Studies were carried out in order to elucidate the dependence of NIL's maximum absorption (λ_{\max}) on the polarity of the solvent. Increasing the polarity of the solvent mixture chloroform/methanol, causes a moderate increase in the λ_{\max} of NIL (FIG.2). This finding correlates well with previous work (4) in which NIL λ_{\max} shifted to higher wavelength as the polarity of dioxane/water solvent mixtures was increased. Attempts to solubilise NIL inside block copolymer F127 micelles failed, indicating the chromophore's highly apolar character and also the relatively increased polarity of the hydrophobic PPO micelle core (FIG.3).

Pinacyanol chloride (PCYN) was also used to probe the vesicle bilayer. PCYN is a cationic cyanine dye, less hydrophobic than NIL. It is a large molecule compared to other cyanines (FIG.1) like methylene blue, MER 540 (the latter being the most extensively studied cyanine dye). PCYN, similarly to NIL, shows solvatochromic sensitivity. In the preliminary study undertaken (FIG.2), its λ_{\max} drops as polarity of the solvent mixture chloroform : methanol is increased.

PCYN's absorbance spectrum exhibits the strong dependence of its spectroscopic properties on the extent of aggregation occurring between the dye molecules. The sharp peak

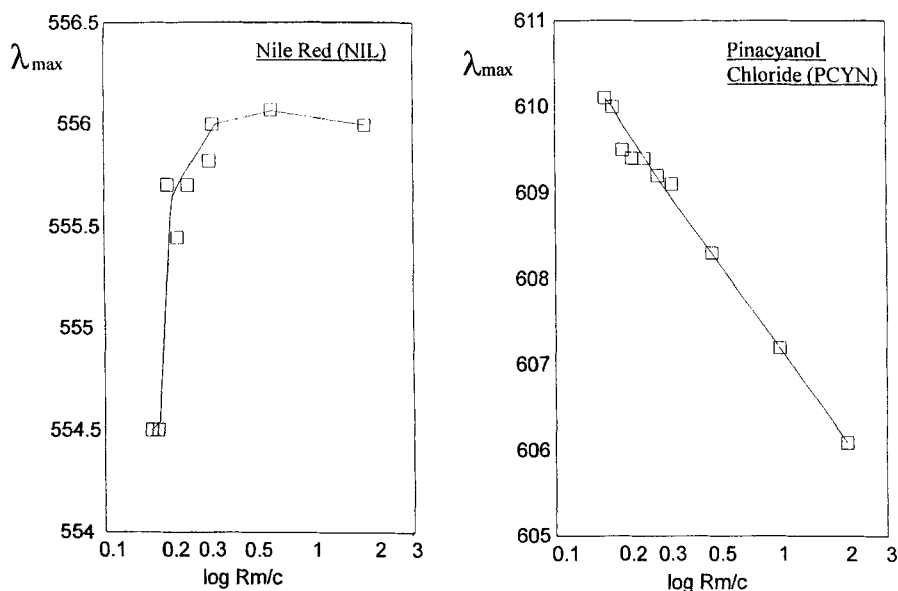


FIG. 2 Solvatochromic behaviour of NIL and PCYN when increasing the polarity of the solvent mixture chloroform:methanol. $R_{m/c}$ is the ratio (w/w) of methanol:chloroform

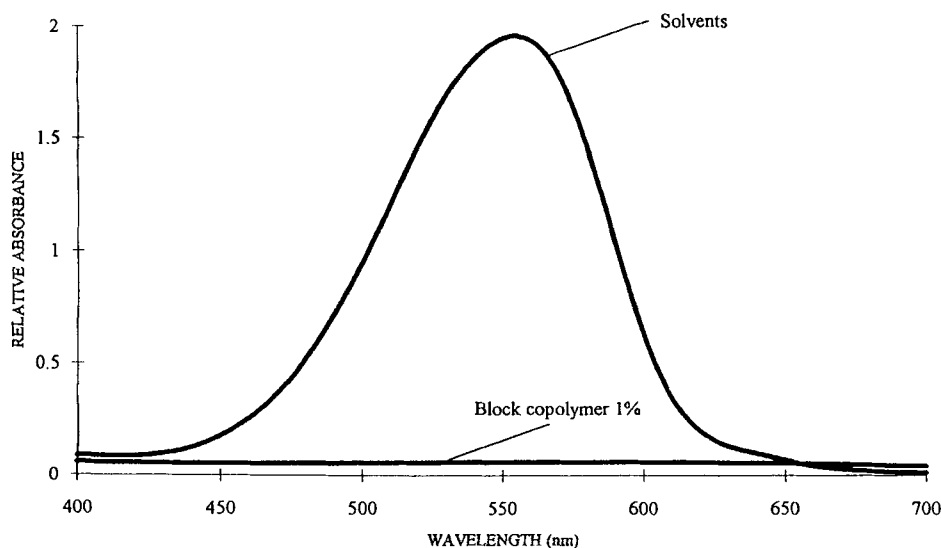


FIG. 3 Absorbance spectra of NIL in the solvent mixture chloroform:methanol (4:1) compared with the unsuccessful attempt to solubilize NIL in 1% (w/w) F127 block copolymer

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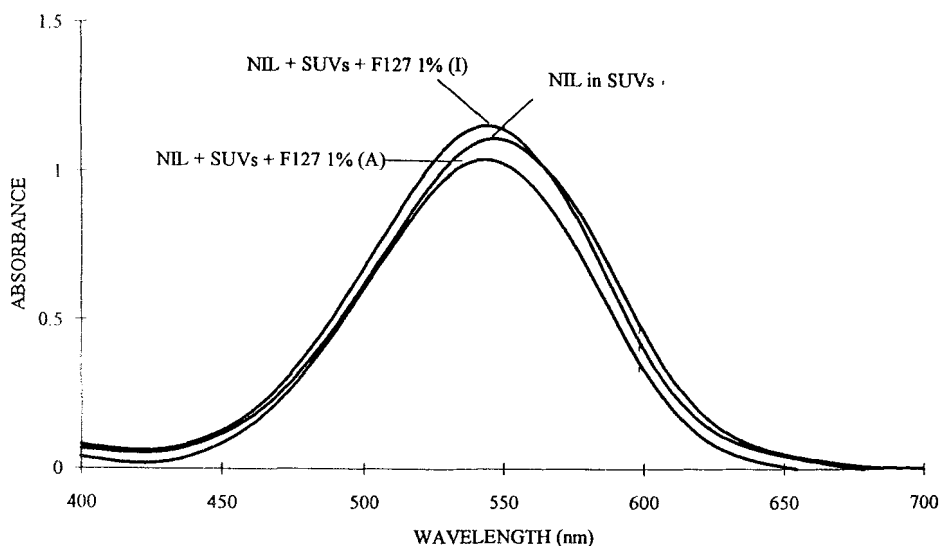


FIG. 4 Absorbance spectrum of PCYN in solvents (chl:meth-4:1) compared with the spectra obtained when solubilizing PCYN in increasing concentration F127 aqueous dispersions. The inset table depicts the λ_{\max} of the dye monomer peak in the different preparations.

at 600 nm is assigned to the absorbance of monomer dye molecules. The peak at 560 nm is due to absorbance of the dimeric form of PCYN. The third component, usually observed as a shoulder to the dimer peak, at about 525 nm is thought to be due to the polyaggregate form of the chromophore (6). Figure 4 shows the absorbance spectrum of PCYN in a solvent mixture. The solubilisation of the dye molecules in block copolymer F127 dispersions was achieved (FIG.4). The ratio between the dimeric : monomeric absorbance components, increases evidently at 1% F127, when compared with the absorbance patterns produced in solvents. The decrease of the latter ratio as block copolymer concentration increases, with the concomitant shift of λ_{\max} towards higher wavelengths, suggest a more apolar environment (see also FIG.2). These results may be used to throw some light on the controversy surrounding the micellisation behaviour of block copolymers and specifically F127 (7).

Liposome-bound Dye Molecules

Dye molecules were solubilised inside the lipid bilayer of small unilamellar vesicles (SUVs), following the procedure described above. The transparency of the latter systems renders them as a sensitive tool for absorbance studies, overcoming the large errors that were introduced using the multilamellar structures.

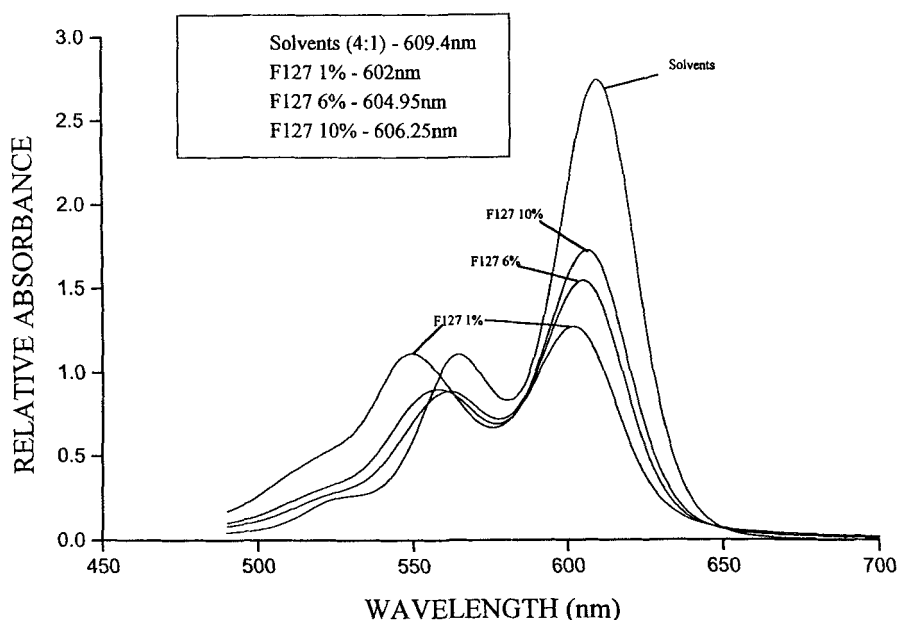


FIG. 5 Absorbance spectra of NIL when bound to small unilamellar vesicles (SUVs). Note the slight shift of λ_{max} when the block copolymer was added, irrespective of the way of addition.

NIL solubilised inside the bilayer of SUVs (FIG.5). The addition of copolymer before or after liposome formation seems to cause a shift to lower values of absorbance by 3-4 nm, indicating that the lipid bilayer polarity is slightly decreasing in the presence of copolymer, irrespective of the way the F127 was added.

PCYN was used to systematically study the interaction between the block copolymer molecules and the SUVs, comparing the two different ways of copolymer addition, i.e. before and after vesicle formation. The sensitivity of its visible absorbance towards homoaggregation provided strong evidence of direct interaction between the copolymer and dye molecules only when adding the F127 initially. The absorbance pattern was different to the one observed when the dye was solubilised in copolymer/water alone (see FIG.4). The initial addition of F127 at low concentrations (0.05, 0.5, 1, 2 %) causes a considerable depression in the total absorbance intensity. When increasing the molar ratio copolymer:lipid even further, the intensity and features of the PCYN absorbance recover (FIG.6). Comparing with the PCYN visible absorbance for the (A) samples, where intensity and features remain almost identical with increasing copolymer concentration, seems that the vesicle bilayer interacts with the copolymer

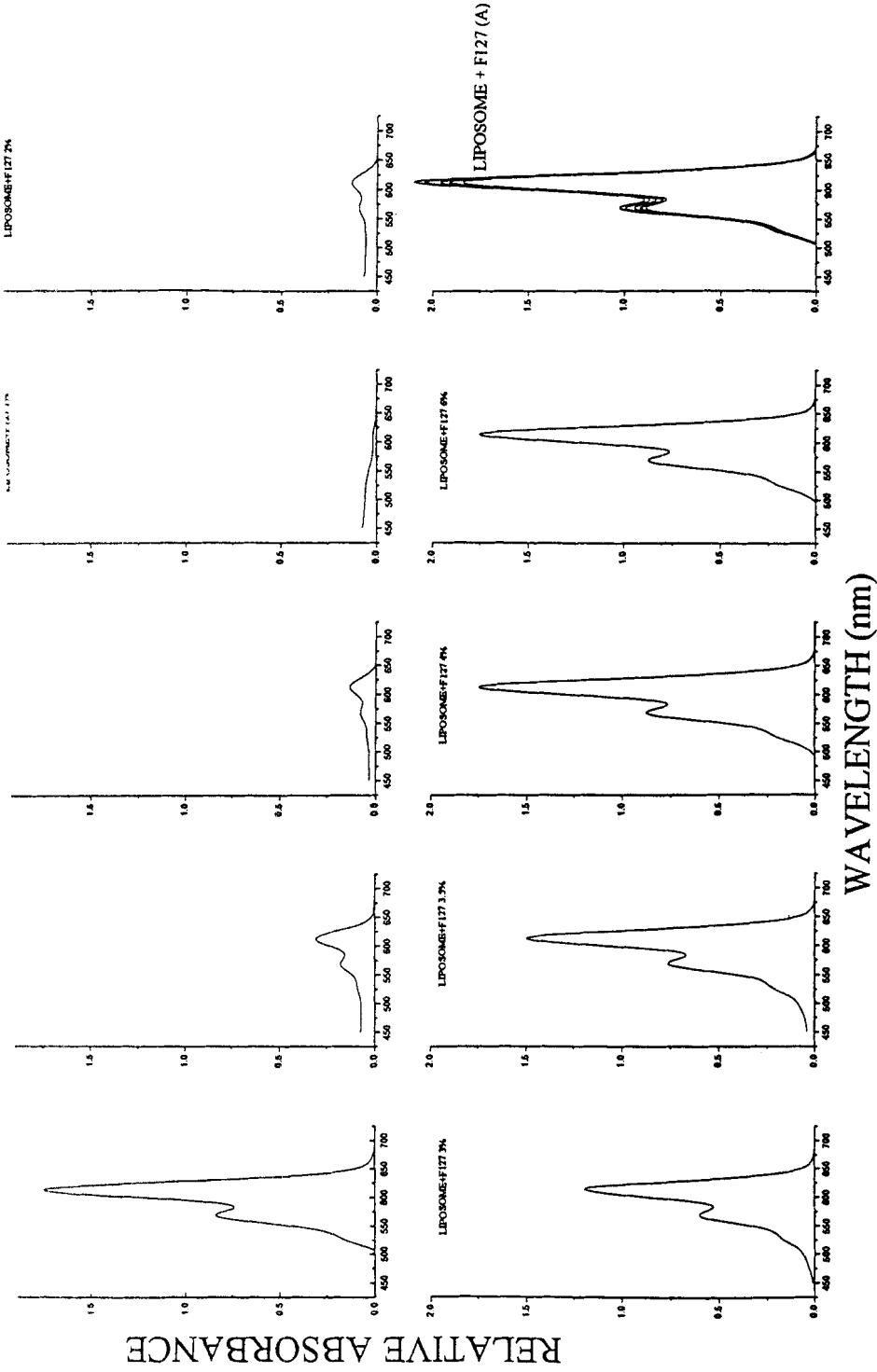


FIG. 6 Absorbance spectra obtained when PCYN was bound to SUVs. The single peak spectra refer to (I) samples, where absorbance intensity almost disappears at 1% and gradually recovers on higher concentrations of copolymer. The last graph in the series shows the (A) samples where intensity fluctuates marginally.

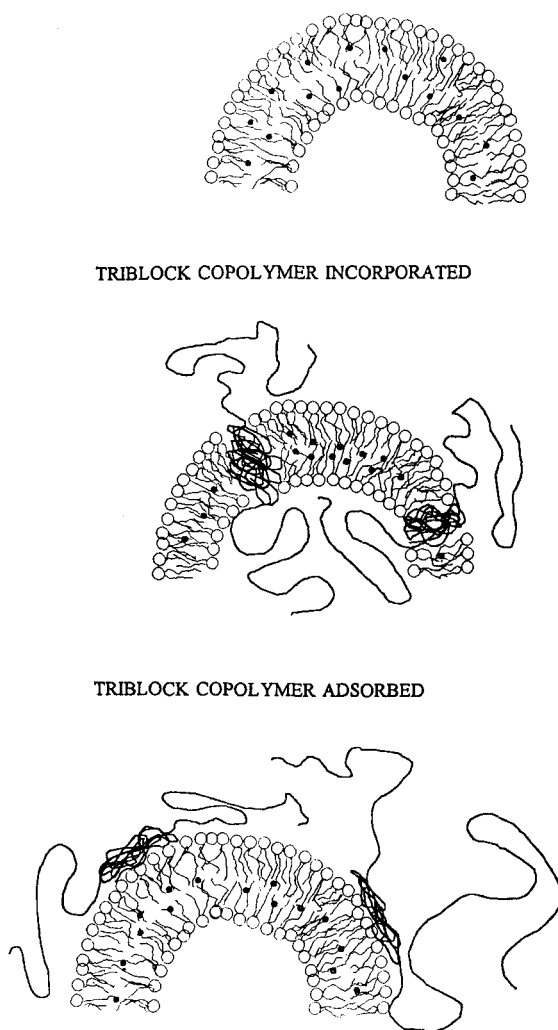


FIG. 7 Models on the interaction between liposomes and copolymers proposed

only when the latter is added before the SUVs have been formed. The dye's deep blue colouring disappears in the (I) samples, most probably due to the formation of the non-absorbing dye homoaggregates, as reported elsewhere (6, 8).

The results obtained fit well in the description of the proposed model (FIG.7). The PCYN molecules solubilize readily inside the vesicle bilayer. In the case where the block copolymer molecules are added initially, incorporation can occur. Therefore, compartments of bilayer will be formed in which the dye molecules will reside. This reduction of the available

area will in fact increase the local dye concentration, so the formation of the non-absorbing dye polyaggregates will be favoured. On the other hand, when the block copolymer is added after the formation of the vesicles, it just adsorbs on the liposome surface. The bilayer-bound PCYN molecules will not be particularly affected in this case, other than favouring their solubilization deeper into the bilayer core, to more hydrophobic environment.

CONCLUSIONS

Monitoring the vesicle bilayer by the observed changes in the absorbance behaviour of two hydrophobic dyes, Nile Red and Pinacyanol Chloride, provided us with:

- a) indications that the local dye microenvironment is becoming more apolar in the presence of triblock copolymer molecules, irrespective of the way the copolymer is added to the vesicle, and
- b) strong evidence of copolymer vesicle incorporation from the direct interaction between SUV-bound Pinacyanol Chloride (PCYN) molecules and block copolymer molecules added before vesiculation.

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