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pH-dependent association of SN-38 with lipid bilayers of a novel liposomal formulation

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Abstract

The aim of this study was to determine the location of SN-38 molecules in a liposomal formulation as a function of pH. Steady-state fluorescence polarization anisotropy and gel filtration studies of blank (placebo) liposomes, liposomes containing SN-38 and SN-38 solutions (in some cases suspensions) were conducted before lyophilization and after re-hydration at different pH conditions. SN-38, l-(4-trimethylammoniumphenyl)-6-phenyl-l,3,5-hexatriene *p*-toluenesulfonate (TMA–DPH), N-((4-(6-phenyl-l,3,5-hexatrienyl)phenyl)propyl)trimethylammonium *p*-toluenesulfonate (TMAP–DPH) and l,6-diphenyl-l,3,5-hexatriene (DPH) were used as fluoroprobes in the polarization anisotropy measurements. The localization of SN-38 was governed by the degree of hydrophobicity of the drug molecules. At high pH, SN-38 is in its inactive, hydrophilic form and partitioned into the water phase of the liposome suspensions. In lyophilized LE-SN38 liposomes re-hydrated with low pH buffer, SN-38 was found at the water–lipid interface of the bilayer.

Keywords: SN-38; Liposomes; Lyophilization; Bilayer; Interaction; Fluorescence; Anisotropy

1. Introduction

SN-38 is an active metabolite of irinotecan, a derivative of camptothecin (CPT-11) that inhibits the activity of topoisomerase I (NeoPharm Inc., Waukegan, IL). SN-38 is currently being investigated for use in the treatment of metastatic colon cancer (NeoPharm Inc., Waukegan, IL). As with other camptothecin class of compounds, SN-38 undergoes pH-dependent reversible hydrolysis of the active α-hydroxy-δ-lactone ring to form an inactive carboxylate derivative in aqueous solutions (pH ≥ 7) and plasma (Burke et al., 1992; Burke and Mi, 1993 and Fig. 1). For almost all camptothecins at 37 °C, the half-life of this reversible conversion is about 16 min in PBS and about 12 min in plasma (Burke et al., 1992; Burke and Mi, 1993; Mi and Burke, 1994). Hence, to preserve the anti-tumor activity of SN-38, it is crucial to minimize its conversion to the inactive metabolite.

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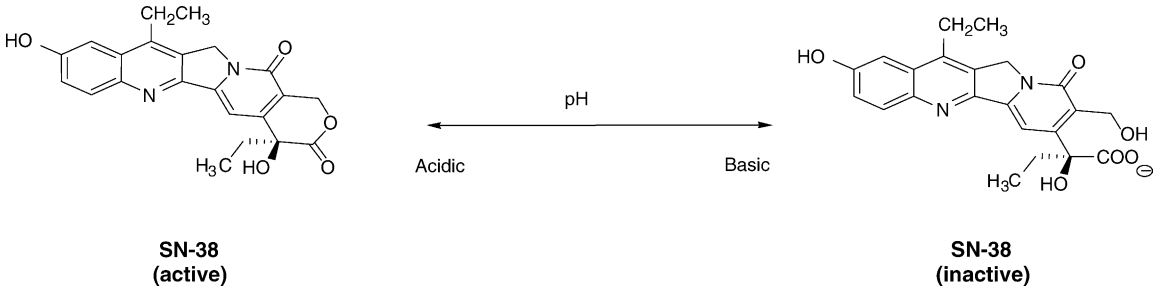

Fig. 1. pH-dependent equilibrium of SN-38 and its inactive metabolite.

SN-38 is very hydrophobic at pH < 7 (the apparent octanol/water partition coefficient ($\log P_a$) at pH 1.5 is 2.09 (unpublished data)) and hydrophilic at pH > 7 ($\log P_a = -2.49$ at pH 10.4 (unpublished data)) due to the ring-closed lactone and ring-opened carboxylate forms, respectively (Kaneda et al., 1997; Wadkins et al., 1999). Despite its hydrophobicity at low pH, SN-38 has low affinity to lipid bilayers resulting in very low drug-to-lipid entrapment in liposomal formulations (Burke et al., 1993; Wadkins et al., 1999). However, once SN-38 is entrapped within the liposomes, the pH-dependent reversible hydrolysis of the lactone ring is significantly reduced (Burke and Gao, 1994).

Recently, we reported that unilamellar liposomes of DOPC, cholesterol and cardiolipin (volume-weighted mean diameter ~150 nm) in the presence of SN-38 (LE-SN38) can be formed in high pH medium (pH > 10) (Zhang et al., 2004). At this pH, the entrapment efficiency of SN-38 is less than 10% (Zhang et al., 2004). However, upon lyophilization of the liposomes and re-hydration in acidic medium (pH < 3), SN-38 entrapment efficiency significantly increased to greater than 95%. The re-hydrated LE-SN38 liposomes were stable and showed no drug crystallization or precipitation for up to 8 h after re-hydration or after dilution in normal saline (Zhang et al., 2004).

This study aims to determine the location of SN-38 in LE-SN38 liposomes before lyophilization and after re-hydration in acidic medium by steady-state fluorescence polarization anisotropy technique using DPH derivatives and SN-38 as fluoroprobes and by gel filtration method. In aqueous medium, DPH and its derivatives partition exclusively into the lipid bilayer. The partitioning is accompanied by up to 1000-fold increase of the fluorescence of the fluoroprobes. The fluorescence polarization anisotropy of a fluoroprobe depends on the microstructure or "fluidity" of the medium (bilayer) surrounding the probe (Lentz, 1989). This makes the steady-state fluorescence anisotropy measurements one of the most sensitive methods for quantitative study of the structural order in lipid membranes in the presence or absence of drug molecules (Balasubramanian and Straubinger, 1994; Ben-Yashar and Barenholz, 1989; Bernsdorff et al., 1999; Burke et al., 1992, 1993; Lentz et al., 1976a,b; Pottel et al., 1983; Shinitzky and Barenholz, 1978). Different DPH derivatives preferentially partition and consequently probe the microstructure of different regions of the bilayer. The three fluoroprobes employed in this study, TMA–DPH, TMAP–DPH and DPH, probe the water–lipid interface, intermediate region of the bilayer and deeper region of the bilayer, respectively (Bernsdorff et al., 1999; Lentz et al., 1976a,b; Lentz, 1989). The presence of drug molecules in the lipid bilayer will affect the local order and movement of the lipid molecules. The variations in the microstructure of the lipid bilayer due to drug–lipid interactions can be detected by comparing polarization anisotropy data obtained in the presence and absence of drug molecules. The differences in polarization anisotropy between active and placebo formulations will be the most pronounced for the fluoroprobe that partitions in the same region of the bilayer where the drug molecules are located. Consequently, the polarization anisotropy measurements can reveal the approximate position of the drug molecules within the lipid bilayer.

2. Materials

SN-38 was purchased from Qventas Inc. (Newark, DE, USA). 1,2-Dioleoyl-sn-glycero-3-phosphocholine

(DOPC), cholesterol and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Hydrochloric acid and sodium hydroxide were obtained from EM Science (Gibbstown, NJ, USA). Sucrose NF grade was obtained from Mallinckrodt (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Sodium lactate was obtained from Fisher Scientific (Fairlawn, NJ, USA). Nitrogen, NF was obtained from BOC Gases (Carol Stream, IL, USA). TMA–DPH, TMAP–DPH and DPH were purchased from Molecular Probes (Eugene, OR, USA). Sephadex G-50 beads were obtained from Sigma (St. Louis, MO, USA). All chemicals used in the fluorescence measurements were of spectroscopic grade. All chemicals were used as received.

3. Methods

3.1. SN-38 and cholesterol assays

SN-38 was quantitated according to a previously reported HPLC method (Zhang et al., 2004). Cholesterol was quantitated by an HPLC method (unpublished data). Briefly, the HPLC system consisted of an Agilent 1100 module (Wilmington, DE, USA), a quaternary pump, mobile phase degasser, auto-sampler with thermostat and a column heater compartment. Agilent software, Chemstation, was used for data acquisition and analysis. UV variable detector set at a wavelength of 205 run and Kromasil C-18 (4.6 mm × 250 mm, 5 μm) column were utilized. The mobile phase consisted of a mixture of isopropanol and acetonitrile in 75:25% v/v ratio. During the analysis, 50 μl samples were injected in duplicate into the HPLC system at mobile phase flow rate of 1 mL/min and column temperature of 40 °C.

3.2. Vesicle size measurements

Mean vesicle size was measured by dynamic light scattering technique, using Nicomp 380 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA, USA). Prior to sample measurement, polystyrene beads of standard size were used to verify the performance of the instrument. All samples were measured in duplicate. The data were analyzed by ZW380 Application Version 1.60 software (Particle Sizing Systems, Santa Barbara, CA, USA), assuming that vesicles are spherical. The data were reported as volume-weighted Gaussian mean diameter.

3.3. Preparation of lyophilized LE-SN38 liposomes

LE-SN38 liposomes composed of DOPC: cholesterol:cardiolipin in molar ratio 50:40:10 (60 mg/mL total lipids) in 10% sucrose solution containing 2 mg/mL SN-38 at pH 10.4 were prepared by the ethanol injection method as described elsewhere (Zhang et al., 2004). Briefly, the lipid components (DOPC, cholesterol and cardiolipin) were solubilized in ethanol. The solubilized lipid mixture was diluted into an aqueous solution of SN-38 in 10% sucrose at pH 10.4. The dispersion was then extruded through polycarbonate filter membranes with pore size of 0.4, 0.2 and 0.1 μm until a mean size of 150 nm was achieved. Ethanol was removed by evaporation under vacuum. Placebo liposomes with identical lipid composition were prepared using the same procedure. The liposomes were lyophilized immediately after preparation and stored at 2–8 °C (Zhang et al., 2004).

3.4. Preparation of samples for fluorescence measurements

Pre-lyophilized liposomes (pH 10.4) and lyophilized liposomes re-hydrated with acidic buffer (10 mM sodium lactate buffer, pH 1.5) were used. These samples were further diluted with 10% sucrose (pH 10.4) and with lactate buffer (pH 1.5), respectively, to a final concentration of 0.3 mM total lipids. The equivalent concentration of SN-38 was 15.3 μM (6 μg/mL). Dynamic light scattering measurements confirmed that the liposomes were present and stable in the diluted samples for the duration of the experiments (data not shown).

Solutions/suspensions of SN-38 (15.3 μM (6 μg/mL)) in 10% sucrose (pH 10.4) and 10 mM lactate buffer (pH 1.5) were also used.

3.5. Labeling of the liposome samples with DPH derivative fluoroprobes

Fluoroprobes were not added during the preparation of the liposomes because DPH derivatives are known to partition easily into lipid bilayers (Bernsdorff

et al., 1997). Instead, the fluoroprobes were dissolved in organic solvents and added to the prepared liposomes. TMA–DPH and TMAP–DPH were dissolved in ethanol at concentration of 1 mM. DPH was dissolved in THF at concentration of 2 mM. Small volumes (between 0.4 and 0.6 μL) of the fluoroprobe stock solutions were mixed with the diluted liposomal samples from Section 3.4 in fluorometer cells. After mixing, the fluoroprobes were incubated with the liposomes at room temperature for more than 30 min. The incubation time was long enough to attain equilibrium partitioning of the probe into the lipid bilayer as confirmed by fluorescence measurements (data not shown) following an experimental procedure described previously (Lentz et al., 1976a). The final probe-to-lipid molecular ratio was 1:1500 for TMA–DPH and TMAP–DPH, and 1:1000 for DPH. Due to the high sensitivity of the instrument, these ratios were sufficient to obtain reliable anisotropy data. At the low probe-to-lipid molecular ratios used in our study, DPH derivative fluoroprobes have been shown not to disturb the overall structure of the lipid bilayer (Lentz, 1989).

3.6. Fluorescence measurements

Steady-state fluorescence anisotropy measurements were performed on ISS-PCl Photon Counting Spectrophotometer in L-shape configuration equipped with two monochromators (ISS, Champaign, IL, USA). One centimeter rectangular quartz fiuorometer cells were used. Temperature was controlled within ±0.1 °C with EcoLine RE120 water bath (Lauda-Brinkmann, Germany). Prior to each measurement, the fluorescence cell was kept for at least 10 min in the spectrophotometer to allow for temperature equilibration. Samples were continuously mixed with a magnetic stirrer placed inside the cell.

Fluorescence measurements were performed with 4 or 8 nm waveband excitation and emission slits. In the case of DPH derivative fluoroprobes, the excitation and emission wavelength were set at 355 and 430 nm, respectively (Balasubramanian and Straubinger, 1994; Campbell et al., 2001). The fluorescence anisotropy was measured over a temperature range from 15 to 45 °C (above the T_m of the bilayer). The polarization anisotropy results were corrected for the intensity of the light scattered from the liposomes, as described elsewhere (Litman and Barenholz, 1982). In lactate buffer (pH 1.5), the results were also corrected for the depolarization due to light scattering (Lentz et al., 1979).

Due to its strong fluorescence, SN-38 was also used as a fluoroprobe (Burke et al., 1992; Burke et al., 1993). In this case, the polarization anisotropy was measured at 25 °C using excitation and emission wavelengths of 370 and 550 nm, respectively.

3.7. Samples for gel filtration

The following samples were analyzed: pre-lyophilized LE-SN38 liposomes, pH 9.68; pre-lyophilized LE-SN38 liposomes, pH reduced to pH 1.75; lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, initial pH 1.82, after 30 min readjusted to pH 10.4; lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, final pH 1.76.

3.8. Gel filtration procedure

Three mL syringes were filled with Sephadex G-50 previously hydrated with 0.9% sodium chloride. The excess amount of liquid was removed by centrifugation for 2 min at 2000 rpm (rcf of 800 × *g*) at 4 °C on a Centra CL3R centrifuge, rotor 243 (Thermo IEC, Needham Heights, MA, USA). To facilitate the gel filtration, some of the samples in lactate buffer (pH 1.5) were diluted two-fold with 0.9% NaCl immediately prior to centrifugation. Sample (250 μL) was placed on the top of each syringe and centrifuged. To completely elute and recover the liposomes from the column, 250 μL of 0.9% NaCl was added to each syringe and centrifuged again. The washing procedure was conducted twice. All samples were run in triplicate. The filtrates were collected, pulled together and analyzed for SN-38 and cholesterol by HPLC as described in Section 3.1.

This gel filtration procedure ensured that the liposomes along with SN-38 embedded into the bilayer or encapsulated in the water compartment of the liposomes were eluted with the filtrate. SN-38 dissolved in the water phase outside the liposomes and SN-38 aggregates were retained in the gel. The gel filtration procedure was validated with placebo liposomes and SN-38 solutions in 10% sucrose at pH > 10 and with SN-38 suspensions in lactate buffer, pH 1.5. Ninety-nine percent of the placebo liposomes was recovered with the filtrate. Ninety-eight and hundred percent of

SN-38 was retained in the gel filtration columns at high and low pH, respectively (data not shown).

4. Results and discussion

4.1. Fluorescence polarization anisotropy of SN-38

Earlier reports have shown that the camptothecin's excited-state lifetime is relatively insensitive to alterations in microenvironment, such as solvent viscosity or binding to phospholipids (Burke et al., 1993). Consequently, an increase or decrease of the rotational correlation time of the drug molecule will lead to an increase or decrease of the steady-state fluorescence anisotropy (Burke et al., 1993). Because of instrumental limitations we were not able to measure the excited-state lifetime of SN-38 and we assumed that the observations by Burke et al. (1993), would hold for SN-38 as well. This assumption allowed us to interpret the variations of the fluorescence anisotropy as an indication of changes in the microviscosity and local order surrounding the drug molecules.

The polarization anisotropy of the fluorescence of SN-38 was measured in diluted LE-SN38 formulations and SN-38 solutions at two different pH as described in the Section 3. The results of these measurements are given in Table 1. The very low values of the polarization anisotropy at pH 10.4 suggested that SN-38 was free and dissolved in the solution. No difference was observed between SN-38 solutions and LE-SN38 formulations indicating that the drug did not associated with the lipid bilayer due to its high degree of hydrophilicity at this pH condition. This observation was confirmed by gel filtration (see the Section 4.3 below) and was in agreement with previous studies that reported low encapsulation efficiency of SN-38 at high pH (Zhang et al., 2004). At low pH however, the fluorescence anisotropy in the LE-SN38 liposomes was significantly higher than in SN-38 solutions. This suggested an increased viscosity of the microenvironment of SN-38 molecules in the LE-SN38 liposomes and could be interpreted as an indication of association of SN-38 with the lipid bilayer (Burke et al., 1992; Burke et al., 1993). The association could be driven by the increased hydrophobicity of SN-38 molecules associated with the closing of the lactone ring at low pH. The change of fluorescence anisotropy from 0.020 at high pH to 0.130 at low pH observed for SN-38 solutions could be caused by self-aggregation or stacking of SN-38 molecules that is possible at low pH (Burke et al., 1993).

Table 1
Effect of the pH on the polarization anisotropy of the fluorescence of SN-38 at 25 °C

Probe	Formulation	Polarization anisotropy[a]	
		Lactate buffer, pH 1.5	Sucrose, pH 10.4
SN-38	SN-38 solutions	0.130 ± 0.007	0.020 ± 0.002
	Liposomes with SN-38	0.185 ± 0.005	0.021 ± 0.002

[a] Averaged values and standard deviations of 10 measurements.

4.2. Fluorescence polarization anisotropy of DPH derivative fluoroprobes

The effect of the pH on the polarization anisotropy of interface probe TMA–DPH in blank liposomes and in LE-SN38 at various temperatures is shown in Fig. 2. There and in the next two figures data were averaged for 10 measurements and the standard deviation is shown for each datapoint. At high pH, there was no significant difference between the polarization anisotropy measured in placebo and LE-SN38

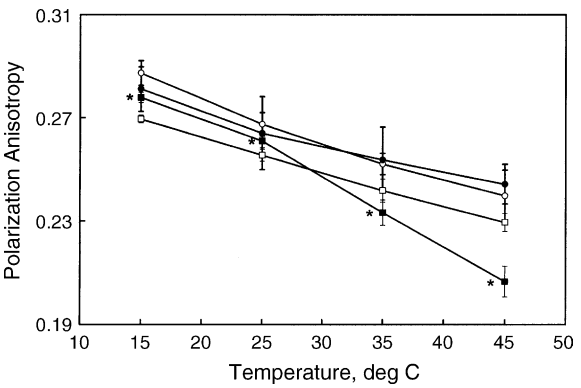

Fig. 2. Effect of the pH on the polarization anisotropy of TMA–DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (◯, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5. Statistical significance in the difference of polarization anisotropy for liposomes with SN-38 compared to blank liposomes is shown on the figure: $*p < 0.001$; $n = 10$.

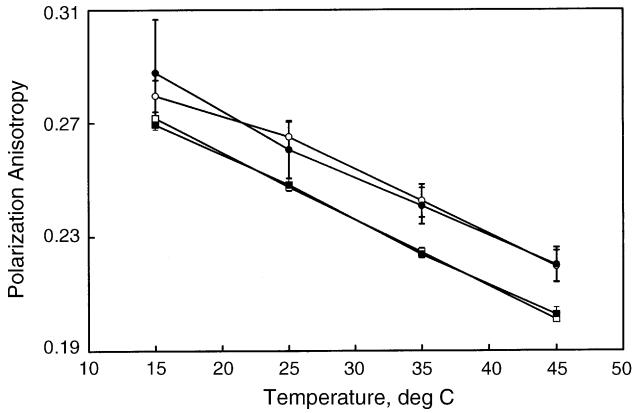

Fig. 3. Effect of the pH on the polarization anisotropy of TMAP–DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (◯, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5.

liposomes suggesting that SN-38 did not associate with the lipid bilayer. This finding was supported by the polarization anisotropy results for SN-38 (see above) and by previous studies (Zhang et al., 2004). At low pH, however, there was a significant difference between the polarization anisotropy measured in placebo and LE-SN38 liposomes. The difference was more pronounced at higher temperatures (30–45 °C). The difference could be interpreted as indication that SN-38 was located near the TMA–DPH fluoroprobe. Because TMA–DPH predominantly occupies the water–lipid interface region of the bilayer our results suggested that after re-hydration with lactate buffer (pH 1.5) SN-38 was located close to the interface.

The effect of the pH on the polarization anisotropy of TMAP–DPH and DPH in blank liposomes and in LE-SN38 at various temperatures is shown in Figs. 3 and 4, respectively. There was no significant difference between the polarization anisotropy in liposomes containing or not SN-38, at high pH, confirming that SN-38 did not associate with the lipid bilayer. Furthermore, there was no significant difference at low pH, suggesting that even though SN-38 was located close to the water–lipid interface it did not penetrate into the intermediate or deeper regions of the bilayer where TMAP–DPH and DPH are located.

4.3. Gel filtration studies

From the fluorescence data at low pH it was difficult to determine if SN-38 was located inside the lipid bilayer close to water–lipid interface or formed aggregates in the water phase close to the interface. To further investigate these possibilities, we carried out gel filtration studies.

The experimental results of the gel filtration study are presented in Table 2. The table shows the amount of SN-38 and cholesterol recovered in all filtrates as a percentage of the amount initially placed on the columns. Cholesterol was used as a marker for liposome loss/recovery during the experiment. In the pre-lyophilized liposomes at high pH, 9.1% of SN-38 was associated with the liposomes. This was most probably due to passive entrapment of SN-38 inside the

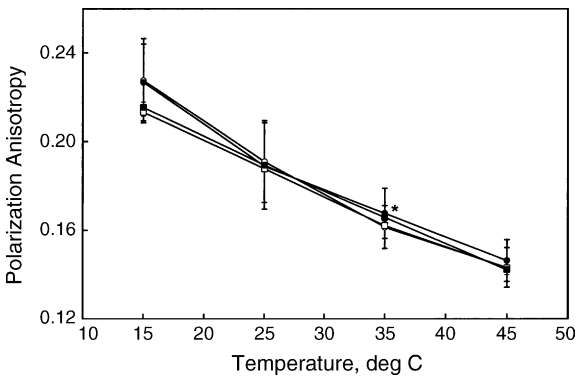

Fig. 4. Effect of the pH on the polarization anisotropy of DPH in blank liposomes (open symbols) and in liposomes with SN-38 (solid symbols): (◯, ●) pH 10.4; (□, ■) lactate buffer, pH 1.5. Statistical significance of the difference of polarization anisotropy for liposomes with SN-38 compared to blank liposomes is shown on the figure: $*p < 0.001$; $n = 10$.

Table 2
Percent SN-38 associated with liposomes and percent recovery of cholesterol (as a measure of liposome recovery)

Sample	High pH (pH ≥ 10)		Low pH (pH ≤ 2)	
	SN-38 associated (%)	Cholesterol recovered (%)	SN-38 associated (%)	Cholestrol recovered (%)
Pre-lyophilized LE-SN38	9.1	99	0.8[a]	96[a]
Lyophilized LE-SN38 re-hydrated with lactate buffer	1.7[b]	102[b]	72	95

[a] Pre-lyophilized LE-SN38 liposomes, pH reduced to pH 1.75.
[b] Lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, initial pH 1.82, after 30 min readjusted to pH 10.4.

liposomes during the extrusion. The result confirmed the data from the fluorescence measurements and previous findings of low encapsulation efficiency at high pH (Zhang et al., 2004). For lyophilized LE-SN38 liposomes re-hydrated with lactate buffer, 72% of SN-38 was associated with the liposomes. The remaining 28% of SN-38 was separated from the liposomes by the gel filtration procedure employed in this study.

Simple reduction of the pH in the pre-lyophilized liposomes to 1.7 did not increase the association of SN-38 with the liposomes. Instead an aggregation occurred and only 0.8% of SN-38 remained associated with the liposomes. The aggregation was apparent by the visually observed sedimentation and by the thickening of the samples. In contrast, no aggregation was observed by dynamic light scattering or by optical microscopy (data not shown) in the lyophilized liposomes re-hydrated with lactate buffer (pH 1.5).

The lyophilization by itself did not increase the association of SN-38 with the liposomes, either. Only 8.7% of SN-38 was associated with the liposomes after lyophilized LE-SN38 liposomes were re-hydrated with water and the pH was readjusted to 10.4 (cholesterol recovery 101%). The result was similar to the case of pre-lyophilized liposomes and represented the percent of SN-38 passively entrapped in the liposomes.

These results showed that the combination of lyophilization and re-hydration with an acidic buffer was the reason for association of SN-38 with the lipid bilayer. The close proximity of the drug molecules and lipid bilayers during the re-hydration allowed SN-38 to interact with the lipid bilayers. During this process, SN-38 could form micro aggregates containing several drug molecules at or very close to the water–lipid interface. SN-38 micro aggregates may adsorb at the interface which will considerably slow down further crystal growth. We speculate that the main factor facilitating the formation of such micro aggregates was the presence of the large surface area of the lipid bilayer during the re-hydration at low pH. Similar micro aggregates, called quantum dots, have already been observed on the vesicle surface in liposome suspensions (Correa and Schelly, 1998; Correa et al., 2000).

Based on the experimental results presented here we propose the following model for the localization of SN-38 in the liposomes suspensions at different pH conditions:

Case 1. High pH, pre-lyophilized SN-38 liposomes. SN-38 is soluble in water and does not associate with the lipid bilayer. Some SN-38 is passively entrapped inside the liposomes.
Case 2. Low pH, pre-lyophilized SN-38 liposomes. Upon pH reduction, SN-38 converts into its lactone hydrophobic form, its water solubility decreases and it rapidly aggregates forming bulk particles of micrometer size. These particles do not associate with the liposomes but instead sediment.
Case 3. High pH, lyophilized SN-38 liposomes. Upon re-hydration of lyophilized LE-SN38 liposomes with high pH medium, SN-38 remains soluble in water and does not associate with the bilayer. Some SN-38 remains passively entrapped inside the liposomes.
Case 4. Low pH, lyophilized SN-38 liposomes. Upon re-hydration of lyophilized LE-SN38 liposomes with low pH buffer (for example, lactate buffer), SN-38 converts into its lactone hydrophobic form. Due to the close proximity of SN-38 molecules and lipid bilayers during the re-hydration, SN-38 interacts with the lipids and forms micro aggregates close to the water–lipid interface. The micro aggregates adsorb on the liposome surface which prevents further crystal growth and aggregation.

5. Conclusions

The steady-state fluorescence polarization anisotropy and gel filtration experiments revealed that the localization of SN-38 molecules and their interactions with the lipid bilayer were governed by the hydrophobicity of the drug molecules at different pH conditions and by the preparation procedure. In the case of lyophilized SN-38 liposomes re-hydrated with low pH buffer medium (lactate buffer), the active closed-ring form of SN-38 was associated with the water–lipid interface region of the bilayer. No precipitation or sedimentation of SN-38 was observed for up to 8 h. At high pH, the inactive open-ring form of SN-38 partitioned into the water phase of the liposome suspensions. Some SN-38 was entrapped inside the aqueous core of the liposomes due to passive entrapment.

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