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Interaction of 5-Fluorouracil Loaded Nanoparticles with 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine Liposomes Used as a Cellular Membrane Model

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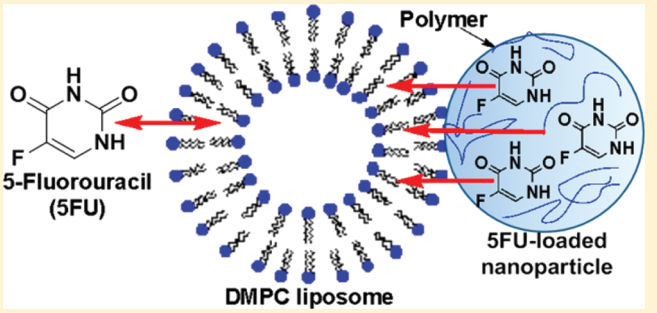
ABSTRACT: Nuclear magnetic resonance (NMR) spectroscopy and steady-state fluorescence anisotropy were used to study the behavior and interaction of 5-fluorouracil, both in a free form (5FU) and included in the polymer matrix of poly(butylcyanoacrylate) nanoparticles (5FUPBCN) with a phospholipid bilayer of large unilammellar vesicles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), as a model system of biomembranes. The results confirm an interaction and penetration of 5FU into the phospholipid bilayer of DMPC liposomes. Different mechanisms of drug transfer from the aqueous environment into the model membrane environment, for the free drug and that incorporated into polymer nanoparticles, are suggested: (i) concentration-dependent reversible diffusion of the free 5FU and (ii) sustained 5FU release from nanoparticles adsorbed on the liposome surface resulting in continuous delivery of the drug into the phospholipid bilayers of the DMPC liposomes.



INTRODUCTION

The 5-substituted pyrimidines comprise a biologically important class of base analogues. 5-Fluorouracil (5FU) is one of the oldest chemotherapy drugs, widely used in the treatment of cancer.[1,2] The mechanism of action of 5FU is thought to be inhibition of DNA synthesis by competitive inhibition of thymidylate synthetase, which is the target enzyme for the drug.[1] The understanding of the mechanism of action of 5FU has led to the development of strategies that may increase its anticancer activity. Despite this, the low drug retention in tumor tissues, the drug resistance, and systemic toxicity, such as severe gastrointestinal toxicities, hematologic side effects, and severe bone marrow disturbances, remain significant limitations to the clinical use of 5FU.[3]

Over the last few decades significant attention has been paid to the design of drug carriers for targeted delivery and controlled drug release that are capable of improving the therapeutic index of drugs through a decrease in toxic side effects, through an increase in drug efficacy, and by overcoming the acquired multidrug resistance of cells. The controlled release of drugs either sustained or pulsatile is important for an achievement of therapeutic success. Drug carriers for sustained drug release involve polymers that release the drug at a controlled rate, by diffusion out of the polymer or by degradation of the polymer over time.[4] Among these polymers, poly(alkylcyanoacrylates) (PACA) have been successfully employed as effective drug delivery systems, due to their biocompatibility, biodegradability, low toxicity, capacity to associate drugs in a stable and reproducible manner, ability to alter the biodistribution of drugs, and easy preparation and purification procedures.[5,6] Different types of drug carriers based on PACA have been developed for targeted delivery and controlled release of 5FU, aiming to improve the therapeutic index of the drug.[7−9]

It is well-known that biological membranes play an essential role in cellular protection and in the control and transport of nutrients. Biological membranes are the primary targets for many drugs of different therapeutic categories, and the knowledge of molecular mechanisms of drug−membrane interaction is of potential practical implications in the discovery, design, and screening of novel therapeutic agents. The study of drug−membrane interactions is usually hampered due to the complexity of the natural cellular membrane structure, and artificial model systems of biomembranes have often been employed. Most of the physicochemical studies have discussed the drug−membrane interactions in terms of induced structural alterations of the cellular membranes.[10,11] However, additional valuable information on the drug−membrane interactions, mechanisms of drug transportation, or penetration can be extracted

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by investigation of physicochemical changes of the drugs themselves.[12]

The most commonly used techniques for biomembrane studies include infrared and Raman spectroscopy, circular dichroism, nuclear and electron magnetic resonance spectroscopy, electron microscopy, X-ray crystallography, and Moessbauer spectroscopy. Among them, NMR spectroscopy is one of the main tools, being particularly sensitive to structural and dynamical changes and intermolecular interactions, and has been successfully applied to study drug delivery and binding processes into cellular membranes.[12−14] The pulsed-field-gradient (PFG) NMR technique offers a chance to directly measure the mobility of lipids and trapped drugs in fluid lipid membranes giving information about the drug and liposome behavior and drug−liposome interactions.[15,16] Changes in the lipid environments produced by drug−liposome interactions can be also detected by steady-state fluorescence anisotropy techniques.[17,18]

In this paper, we report the results from a study, based on NMR spectroscopy and steady-state fluorescence anisotropy, of the behavior and interaction of 5-fluorouracil, both the free drug (5FU) and that included in the polymer matrix of poly(butylcyanoacrylate) nanoparticles (5FUPBCN), with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes, as a simple model system of a biomembrane. The outer layer of the plasma membrane of eukaryotic cells is a complex system composed of a variety of lipids of which phosphatidylcholine lipids are predominant. DMPC is a simple well-characterized choline-containing phospholipid, which can be used to model the main features of the plasma membrane.[19] It thus offers a model system that is less complex than the commonly used natural lipid extracts,[16] allowing for easier monitoring of the main physical and chemical interactions between the membrane and the biologically active species under study. Specific drug−liposome interactions and mechanisms of drug penetration into the lipid bilayer of the liposomes, relevant to the therapeutic index of 5FU, were clarified. The results contribute to the understanding of the therapeutic properties of the 5FU and the role of poly(butylcyanoacrylate) nanoparticles (PBCNs) as a drug carrier, on the drug delivery, penetration, and retention into cellular membranes.

MATERIALS AND METHODS

Materials. The monomer, *n*-butyl cyanoacrylate (*n*-BCA), was purchased from the Research Centre for Specialty Polymers, Bulgaria. Dextran 40 (mol. wt. 40 000 g/mol) was obtained from Pharmachim (Bulgaria) and citric acid monohydrate from Aldrich. 5-Fluorouracil was purchased from Fluka. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexatriene (TMA-DPH) were from Sigma, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was from Avanti Polar Lipids. All other chemicals were from Merck or of laboratory grade purity and used as obtained. Samples for fluorescence anisotropy experiments were prepared with 0.1 M NaCl, pH 7.4.

Poly(butylcyanoacrylate) Nanoparticle (PBCN) Preparation. PBCNs were prepared by in situ anionic polymerization. The monomer, *n*-butyl-2-cyanoacrylate (130.7 mmol/L), was carefully added to the aqueous polymerization medium, containing citric acid (9.5 mmol/L) and a colloidal stabilizer (dextran 40) at concentration of 0.2 mmol/L. Polymerization was carried out for 3 h at room temperature with continuous magnetic stirring. The pH of the resulting polymer colloidal suspension was then adjusted to pH 7 using 1 N sodium hydroxide.

5FU-Loaded PBCN (5FUPBCN) Preparation. 5FUPBCNs were prepared in the same manner as the unloaded PBCNs. Powdered 5-FU was dissolved (3.84 mmol/L) in the aqueous polymerization medium containing the same agents as above before addition of the monomer. After the monomer addition, the polymerization process leading to the formation of drug-loaded nanoparticles was carried out as described above for the unloaded polymer. The pH of the obtained colloidal suspension of drug-loaded nanoparticles was adjusted to 7 using 1 N sodium hydroxide. To perform the NMR analyses, the intact colloidal polymer suspensions of PBCN and 5FU-loaded PBCN were filtered over a 0.1 μm pore-sized VC membrane (Millipore, England), and isolated from the aqueous medium nanoparticles were dried under decreased pressure and kept in a vacuum desiccator over P_2O_5.

Liposome Preparation. A chloroform solution of DMPC containing the appropriate amount of lipid was dried under a stream of argon. The thin film obtained was kept under high vacuum for more than 3 h to remove organic solvent traces. Multilamellar vesicles (MLVs) were obtained after redispersion of the film in 10 mM HEPES buffer (0.1 M NaCl, pH 7.4) and vortexed above the phase transition temperature. Frozen and thawed MLVs were obtained by repeating the following cycle five times: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath. Suspensions of MLVs were then extruded 10 times, on a Lipex Biomembranes extruder attached to a circulating water bath, through polycarbonate filters (100 nm) to produce large unilamellar vesicles (LUVs). 5FU was added to the obtained LUVs to give a final concentration of $\approx$4 μM. The appropriate amount of 5FUPBCN (ensuring the same concentration as of the free 5FU) dissolved in DMSO was added to the chloroform solution of DMPC, and both were dried together. Afterward, the procedure described above was used for LUV preparation. Additionally, samples were prepared by adding the appropriate amount of 5FUPBCN to DPMC LUVs already prepared.

Drug−Liposome Mixtures for NMR Analysis. The 5FU/liposome and 5FUPBCN/liposome samples for NMR spectroscopic analysis were prepared by injection of 50 μL of 0.02 M solution of 5FU in D_2O and 50 μL of suspension of 5FU-loaded PBCN (drug concentration 3.84 mmol/L) in D_2O, respectively, into 0.6 mL of DMPC liposome suspension. After the injection of 5FU and 5FUPBCN into the liposome suspension, the mixtures were vigorously agitated for 10 min at room temperature.

Photon Correlation Spectroscopy. The diameter of the PBCN was measured by photon correlation spectroscopy (PCS) using a laser beam scattered at 173° and temperature of 25 °C (Zetasizer ZS, Malvern Instruments, England). Zeta-potential was measured by laser Doppler electrophoresis on the same apparatus at 25 °C.

Drug Loading Determination (Indirect Estimation). The degree of 5FU incorporation into the polymer matrix of nanoparticles was determined spectrophotometrically using a Perkin-Elmer Lambda 2 UV/vis spectrometer, after filtration of portions of intact drug-loaded polymer suspensions over a 0.1 μm pore-sized VC membrane. Drug concentration in the filtrate was determined by direct spectrophotometric analysis at λ = 265 nm. The amount of drug incorporated into the nanoparticles was determined after subtracting the unincorporated drug amount

from the total known amount of drug added in the polymerization medium. The entrapment efficiency (EE) and drug loading capacity (LC) were calculated by

$$\%\,\mathrm{EE} = 100 \times \frac{(\text{initial concentration} - \text{concentration in filtrate})}{\text{initial concentration}} \quad (1)$$

$$\%\,\mathrm{LC} = 100 \times \frac{(\text{amount of drug incorporated in nanoparticles (mg)})}{\text{polymer mass (mg)}} \quad (2)$$

In Vitro Drug Release from 5FUPBCN. An appropriate amount of 5FUPBCN suspension was enclosed in a dialysis bag (cellulose Visking membrane, MWCO 12 000−14 000, pore size diameter 25 Å, Serva-USA), sealed, and placed in a vessel containing 25 mL of phosphate buffer (PBS) with pH 7.4 and maintained under constant mild agitation in a water bath (37 ± 0.5 °C). The final concentration of 5FU (25 μg/mL) was chosen to ensure sink conditions for the in vitro study. Aliquots of 1.5 mL were taken and subsequently replaced with fresh medium, in predetermined time intervals from 10 to 540 min. Each aliquot was analyzed with UV−visible spectroscopy at 265 nm for 5FU content.

Steady State Fluorescence Anisotropy. Steady state fluorescence anisotropy measurements were performed in a Varian Cary Eclipse spectrofluorometer, equipped with a constant-temperature cell holder (Peltier Multicell Holder, Cary Temperature Probe series II). The structural order of a lipid membrane system in interaction with 5FU and 5FUPBCN was investigated by measuring, as a function of temperature, the steady-state fluorescence anisotropy (r_s) of DPH and TMA-DPH incorporated into DMPC liposomes. The suspensions of liposomes were mixed with TMA-DPH or DPH for 30 min, to a final lipid-to-fluorescent probe ratio of 300:1 (mol/mol). 5FU was added to these liposomes at a final concentration of ≈0.4 mM. The excitation and emission wavelengths used were 355/426 and 336/427 nm for TMA-DPH and DPH, respectively. For 5FUPBCN, two different ways of preparing the samples were tested, one by cosolubilization with the lipid and the other by adding it to the liposomes previously prepared, as described above. Similar results were obtained independently of the procedure used. The steady-state fluorescent anisotropy was recorded at 5 °C intervals for all studied systems, using both probes, in the range of temperature from 0 to 55 °C. Samples were left to equilibrate five minutes at each temperature. Five independent experiments, for each system, were made.

The steady state fluorescence anisotropy (r_s) is defined by the following equation[17]

$$r_s = \frac{I_{VV} - I_{VH}G}{I_{VV} + 2I_{VH}G} \quad (3)$$

where I_{VV} and I_{VH} are the intensities measured in directions parallel and perpendicular to the excitation beam, respectively. The correction factor G is the ratio of the detection system sensitivity for vertically and horizontally polarized light, which is given by the ratio of vertical to horizontal components when the excitation light is polarized in the horizontal direction, $G = I_{HV}/I_{HH}$.[17]

The typical temperature dependence of DPH and TMA-DPH anisotropy in liposomes is depicted in Figure 2. The following equation was fitted to the anisotropy versus temperature data

$$r_s = r_{s2} + \frac{r_{s1} - r_{s2}}{1 + 10^{B'(\frac{T}{T_m} - 1)}} \quad (4)$$

where T is the absolute temperature; T_m is the midpoint phase transition; and r_{s1} and r_{s2} are the upper and lower values of r_s. B' is the slope factor which is correlated with the extent of cooperativity (B) by $B = [1 - 1/(1 + B')]$; the introduction of B yields a convenient scale of cooperativity ranging from 0 to 1.[20]

NMR Spectroscopy. All NMR experiments were recorded on a Bruker Avance III 400 spectrometer, operating at 400.15 MHz for protons and 376.52 MHz for fluorine, equipped with pulse gradient units, capable of producing magnetic field pulsed gradients in the z-direction of 50 G/cm. The NMR spectra were acquired using standard Bruker pulse sequences, at a temperature of 30 °C in dimethyl sulfoxide-d_6 (DMSO-d_6) or/and deuterium oxide (D_2O) solution. For the NMR spectra recorded in DMSO-d_6, the solvent resonance peak at 2.49 ppm was used as a chemical shift reference. Chemical shifts in D_2O were referenced to internal sodium trimethylsilyl-[2,2,3,3-d_4]-propionate (TSP) for ^{1}H and to internal KF for ^{19}F NMR. ^{1}H NMR experiments in water solution were performed with water suppression using excitation sculpting with gradients,[21] an acquisition time 5.45 s, and a relaxation delay of 2 s, and 64 transients of a spectral width of 3500 Hz were collected into 32K time domain points. The ^{19}F NMR spectra were recorded with inverse gated proton decoupling, a pulse width of 12 μs, an acquisition time of 1.75 s, a relaxation delay of 1 s, and a spectral width of 37 000 Hz. 128 K time domain points and up to 512 transients were collected.

Proton-registered diffusion-ordered NMR (^{1}H DOSY) experiments were acquired using the bipolar longitudinal eddy current delay (BPPLED − Bipolar Pulsed Field Gradient Longitudinal Eddy Delay) pulse sequence.[22] The experimental conditions (5 mm NMR tubes, amount of the solute and solvent, a temperature of 30 °C, no sample rotation, and high air flow of 535 L/h to avoid convections in the samples) for all DOSY experiments were kept constant. Before the NMR experiments, the temperature was equilibrated and maintained at 30 °C, as measured using the spectrometer thermocouple system. Typically, in each experiment a number of 16 spectra of 16K data points and 128 scans were collected, with values for the duration of the magnetic field pulse gradients (δ) of 2.4 ms for pure 5FU and 4.4 ms for the rest of the samples, diffusion times (Δ) of 110−600 ms, and an eddy current delay set to 5 ms. The pulse gradient (g) was incremented from 2 to 95% of the maximum gradient strength in a linear ramp. The spectra were processed with the Bruker Topspin software package (version 2.1). The diffusion coefficients were calculated by exponential fitting of the data belonging to individual columns of the 2D matrix. The diffusion coefficients (D) were obtained by measuring the signal intensity of the resonances of the methine proton of 5FU and N-methyl protons of the choline residue of liposomes. For 5FU/liposome and 5FUPBCN/liposome samples, three different measurements in the time (30 min, 60 min, and 24 h after injection of 5FU and 5FUPBCN to liposomes) were made. The diffusion coefficient of TSP (5.93×10^{-10} m^2 s^{-1}, calculated standard deviation of 1.07×10^{-2}) obtained from ^{1}H DOSY experiments was used as an internal reference, and all diffusion coefficients are given as a ratio D/D_{TSP} according to a procedure published before.[23]

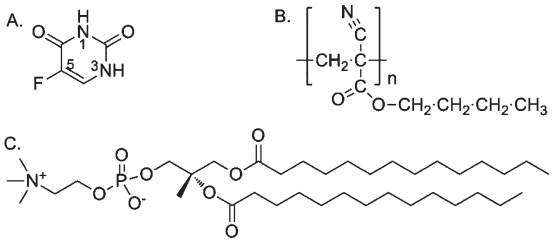

Figure 1. Chemical structures of: (A) 5-fluorouracil (5FU), (B) poly-(butylcyanoacrylate), and (C) DMPC.

RESULTS

The structures of the compounds studied are presented in Figure 1, and the methods of preparation of 5FUPBCN, liposomes, and mixtures of 5FU and 5FUPBCN with liposomes are described in the Materials and Methods. Important characteristics of 5FUPBCN, such as particle size, Z-potential, EE, LC, and the in vitro 5FU release profile from PBCN were determined. NMR spectroscopy and steady state fluorescence anisotropy were employed to study drug−liposome interactions and mechanisms of 5FU penetration into liposomes.

Physical Characterization of 5FUPBCN. The amount of 5FU included in PBCN, estimated as % EE, was found to be 31% of the total amount added, while the LC was found to be 0.78%. The low values for EE and LC at the concentration of 5FU used for preparation of 5FUPBCN are not surprising. It is well-known for 5FU that drug concentration is among the main factors determining the drug incorporation into the polymer matrix of nanoparticles with positive effect (e.g., with the increase of concentration, the degree of incorporation increases).[24,25] The *z*-average (hydrodynamic) diameters of PBCN and 5FUPBCN determined by PCS were 138.2 nm (polydispersity index, PDI = 0.037) and 158.1 nm (PDI = 0.085), respectively. The low PDI values obtained (<0.1) confirm that the suspensions are highly size monodisperse. Both nanoparticles, unloaded and 5-FU-loaded, are negatively charged with Z-potential (−5.05 ± 5.6) and (−4.79 ± 5.85) mV, respectively.

Steady State Fluorescence Anisotropy. Two fluorescent probes, DPH and TMA-DPH, were used to evidence possible modifications in membrane fluidity induced by 5FU or 5FUPBCN. The probes were incorporated in DMPC LUVs, and then 5FU was added in a 300:1 lipid/drug ratio, at physiological conditions. Pure DMPC lipid bilayers exhibit a mean gel-to-fluid ($P_{\beta'}-L_{\alpha}$) phase transition temperature (T_m) of 24 °C.[26] The data obtained are presented in Table 1 and Figure 2 and show that both forms of the drug, free and incorporated in PBCN, interact with DMPC bilayers and that this interaction induces a change in the gel−fluid T_m phase coexistence region with a markedly decreased conformational order of the lipid acyl chains below the gel−fluid phase T_m of DMPC. There is no observable change in the DMPC T_m revealed by the TMA-DPH probe, which is embedded near the lipid head groups. However, for DPH, which is more deeply localized in the bilayer, the DMPC T_m decreased from 24 to 17 °C, in the presence of both 5-FU and 5FUPBCN. In both cases, a fluidification of the membrane was induced.

NMR Spectroscopy. The structure of 5FU and 5FUPBCN in DMSO-d_6 and D_2O solutions was determined by ^1H and ^{19}F NMR spectroscopy. The ^1H and ^{19}F resonance signals of the free 5FU in D_2O appear at 7.66 and −172.5 ppm and in DMSO-d_6 at 7.72 and −171.5 ppm, respectively. The ^1H NMR spectra of 5FUPBCN in both solutions are dominated by broad resonance signals at 0.9 (CH_3), 1.4 (CH_2), 1.6 (CH_2), 4.1 (CH_2), and 2.6 ppm (CH_2), corresponding to the protons belonging to the *n*-butoxycarbonyl group and those of methylene protons from the polymer chain of poly(butylcyanoacrylate), respectively. Resonance signals characteristic for 5FU were registered in ^1H and ^{19}F NMR spectra of 5FUPBCN in DMSO-d_6 and D_2O solutions. Typical 400.15 MHz ^1H and 376.52 MHz ^{19}F NMR spectra of 5FUPBCN in DMSO-d_6 are presented in Figures 3 and 4, with spectra obtained for 5FU and PBCN, included for comparison.

Table 1. Mean Lipid Transition Temperatures (T_m/°C) Obtained for the Different Lipid Systems Studied, Using TMA-DPH and DPH Steady-State Fluorescence Anisotropy[a]

	5FU	5FUPBCN
TMA-DPH	23.5 ± 0.4	24.2 ± 0.4
DPH	17.1 ± 0.4	17.0 ± 0.8

[a] All lipid mixtures were studied in 10 mM HEPES buffer, pH 7.4, NaCl 0.1 M. The values represent the mean value of the five independent measurements.

In the ^1H and ^{19}F NMR spectra of 5FUPBCN in DMSO-d_6, the appearance of three sets of weak signals belonging to H4 and NH protons and F5 fluorine of 5FU was clearly observed (Figures 3 and 4). The results from analysis of the spectral data, verified by $^1H/^1H$ COSY spectroscopy, confirmed the existence of three forms (one physically entrapped and two polymer chain-bound forms) of 5FU present in the polymer matrix of PBCN. The resonance peaks at 7.75, 7.96, and 8.07 ppm in the ^1H NMR and those at −171.4, −169.0, and −168.5 ppm in the ^{19}F NMR spectra of 5FUPBCN were attributed to the physically entrapped, N-1 and N-3 covalently bonded to the polymer chain 5FU, respectively. These results are in agreement with data published before.[7,24] A significantly greater amount of 5FU was found physically entrapped in the polymer matrix (66%) than covalently bonded to the polymer chain, N-1 (29%) and N-3 (5%), as determined by the integral intensity of the signals in the ^1H NMR spectrum. In ^1H and ^{19}F NMR spectra of 5FUPBCN acquired in D_2O, due to the poor solubility of the sample and low fractions of 5FU covalently bonded to the polymer chain, only the resonance signals of the 5FU physically entrapped in the polymer matrix were observed (H4, 7.66 ppm, and F5, −172.0 ppm).

To study the interactions of 5FU and 5FUPBCN with liposomes, ^1H NMR spectra of liposomes and ^1H and ^{19}F NMR spectra of 5FU/liposome and 5FUPBCN/liposome mixtures in D_2O were recorded and analyzed. The data obtained for 5FU/liposome and 5FUPBCN/liposome samples were compared to the corresponding values of the free 5FU, 5FUPBCN, and liposomes in D_2O. Typical broad resonance signals corresponding to the lipid methyl (0.90 ppm), methylene (1.28 ppm), and phosphatidylcholine (3.20 ppm) protons were registered in the ^1H NMR spectra of liposomes, 5FU/liposome, and 5FUPBCN/liposome samples. In both ^1H and ^{19}F NMR spectra of 5FU/liposome and 5FUPBCN/liposome samples, resonance signals for 5FU were shifted with respect to those of the free 5FU and 5FUPBCN. However, the analysis of the spectra showed a significant difference in the spectral behavior of 5FU in 5FU/liposome and 5FUPBCN/liposome samples, with time-dependent alterations of some NMR spectral parameters in the former. Selected areas from ^1H and ^{19}F NMR spectra of 5FU/liposome

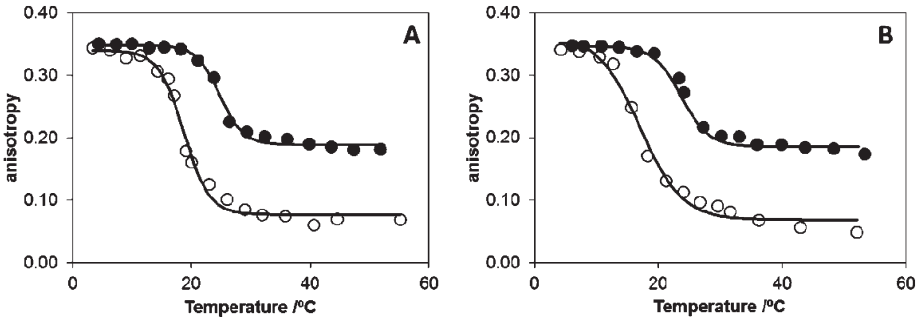

Figure 2. Representative results of steady-state fluorescence anisotropy vs temperature showing the thermothropic lipid phase transition of DMPC liposomes in HEPES buffer 10 mM, pH 7.4, 0.1 M NaCl with TMA-DPH (filled circles) and DPH (open circles) in the presence of 5FU (A) and of 5FUPBCN (B). Solid lines represent the fit of eq 1 to the data.

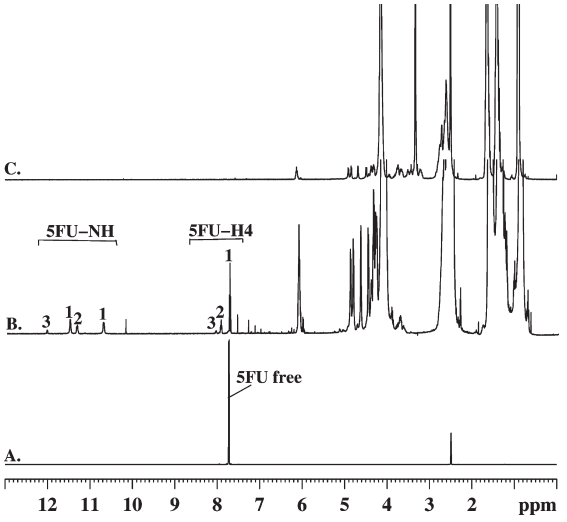

Figure 3. 400.15 MHz ^{1}H NMR spectra: (A) 5FU free; (B) 5FUPBCN; (C) PBCN. Indication of the different (immobilized) forms of 5FU: 1 free (physically entrapped); 2 covalently immobilized at the N-1 position; and 3 covalently immobilized at the N-3 position.

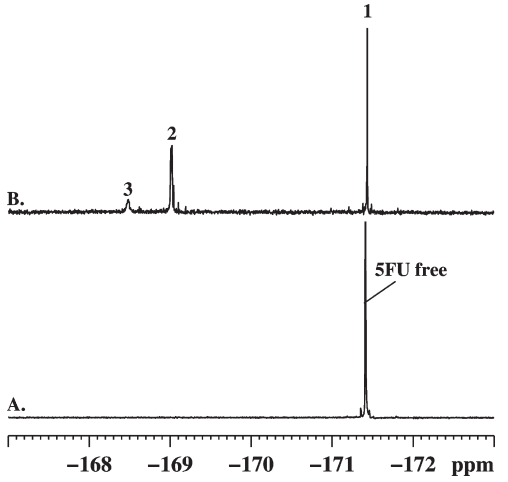

Figure 4. 376.46 MHz ^{19}F NMR spectra: (A) 5FU (free) and (B) 5FUPBCN. Indication of the different (immobilized) forms of 5FU: 1 free (physically entrapped); 2 covalently immobilized at the N-1 position; and 3 covalently immobilized at the N-3 position.

and 5FUPBCN/liposome samples, covering the methine proton (H4) and fluorine (F5) resonances of 5FU, are presented in Figure 5. The spectra recorded 30 min, 60 min, and 24 h after 5FU and 5FUPBCN injection to liposomes are shown, with the spectra of the free 5FU and 5FUPBCN in D_2O included for comparison. The chemical shifts of H4 and F5 of 5FU in ^{1}H and ^{19}F NMR spectra of the samples studied are presented in Table 2.

Relative to the samples of the free 5FU and 5FUPBCN, an upfield shift of the resonance signal of H4 in the ^{1}H NMR spectra of 5FU/liposome ($\Delta\delta$ = 0.02 ppm) and 5FUPBCN/liposoms ($\Delta\delta$ = 0.06 ppm) was observed 30 min after injection of the drugs into liposomes. Insignificant changes ($\Delta\delta \approx 0.01$ ppm) in resonance frequency of *N*-methyl protons of the choline residues ($-N(CH_3)_3$) of liposomes were detected in the spectra of 5FU/liposome.

Besides the weak chemical shift of H4 and $-N(CH_3)_3$ proton resonances, additional changes in ^{1}H NMR spectra of 5FU/liposome, recorded within 30 min, 60 min, and 24 h were observed. After the mixing of 5FU and liposomes, the resonance signal of H4 was first upfield shifted and then broadened and split into two signals (at 7.642 and 7.640 ppm) within 60 min (Table 2). The new slightly upfield shifted signal represents 30% of the total amount of the drug in the sample, as found from the integral intensity of the two resonance signals of H4 detected in ^{1}H NMR spectra of 5FU/liposome. The chemical shift alterations observed in ^{1}H NMR spectra of 5FU/liposome were attributed to an entrapment of the hydrophilic drug molecules at the polar interfacial side of the liposomes and the resulting splitting of the resonance signal consistent with the presence of at least two states of 5FU defined by different environmental locations.

The existence of drug molecules adsorbed at the membrane–water interface (at 7.642 ppm) and others with some degree of penetration into the membrane (at 7.640 ppm) was assumed. The NMR signal broadening reflects the 5FU partition into membrane and also suggests exchange processes between the different drug states.

When 5FUPBCN was injected into liposomes, similar ^{1}H NMR spectra were recorded within 30 min, 60 min, and 24 h, where a broad resonance signal for H4, strongly shifted to high field with respect to those of pure 5FU and 5FUPBCN, was observed (Table 2). Changes of the chemical shift of H4 in the time interval studied were not registered. The data suggest deeper penetration and retention of the drug molecules into the membrane. The spectral line width of H4 was found to be broader relative to that of the free 5FU but rather narrow

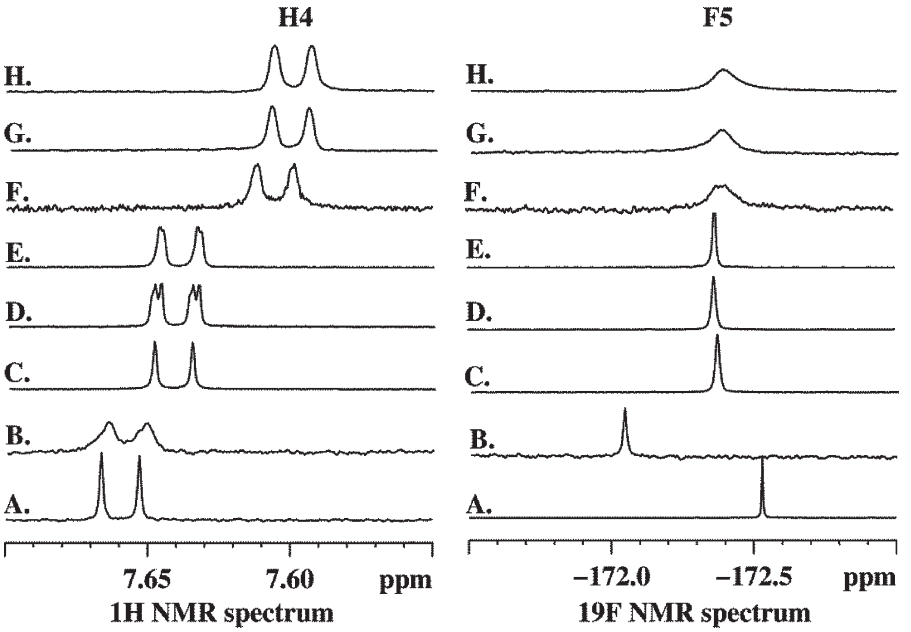

Figure 5. Selected spectral areas from 400.15 MHz ^{1}H and 376.46 MHz ^{19}F NMR spectra in D_2O of: (A) 5FU; (B) 5FUPBCN; (C) 5FU/liposome (after 30 min); (D) 5FU/liposome (after 60 min); (E) 5FU/liposome (after 24 h); (F) 5FUPBCN/liposome (after 30 min); (G) 5FUPBCN/liposome (after 60 min); and (H) 5FUPBCN/liposome (after 24 h).

Table 2. Chemical Shifts of H4 and F5 Resonances of 5FU in 400.15 MHz ^{1}H and 376.46 MHz ^{19}F NMR Spectra in D_2O of the Samples Studied

	5FU (H4)			5FU (F5)		
sample	30 min	60 min	24 h	30 min	60 min	24 h
5FU	7.661			−172.50		
5FUPBCN	7.660			−172.04		
5FU/liposome	7.642	7.642	7.640	−172.37	−172.37	−172.37
		7.640	7.638			
5FUPBCN/liposome	7.605	7.602	7.601	−172.40	−172.38	−172.39

compared to that of 5FUPBCN. The decrease of the line width of H4 in ^{1}H NMR spectra of 5FUPBCN/liposome compared to that of 5FUPBCN was attributed to the release of 5FU from the polymer matrix of the PBCN attached to the liposome surface and diffusion toward the hydrophobic lipid bilayers of liposomes.

In ^{19}F NMR spectra of 5FU/liposome and 5FUPBCN/liposome samples, the fluorine resonance signal was downfield shifted with respect to those of 5FU. These results are in agreement with previous observations, where the ^{19}F NMR resonances from the solute populations in the intracellular compartments are downfield shifted with respect to those of the extracellular species.[27,28] However, relative to the samples of 5FUPBCN, a stronger upfield shift of the ^{19}F resonance of 5FU in ^{19}F NMR spectra of 5FUPBCN/liposome ($\Delta\delta$ = 0.35 ppm) was detected. The line broadening of the resonance signals in ^{19}F NMR spectra of 5FU/liposome and especially of the 5FUPBCN/liposome sample with respect to the pure 5FU reveals drug−membrane interactions and penetration of drug molecules from the water to the more restricted liposomal environment.

Table 3. Relative Diffusion Coefficients (D/D_{TSP}) of 5FU and Liposomes in D_2O at 30 °C for the Samples Studied

	5FU			liposome		
sample	30 min	60 min	24 h	30 min	60 min	24 h
5FU	1.63					
5FUPBCN	1.45					
Liposome				0.04	0.03	0.02
5FU/liposome	1.61	1.61	1.54	0.13	0.08	0.04
5FUPBCN/liposome	1.56	1.55	1.57	0.10	0.09	0.10

Diffusion NMR spectroscopy was further applied to study the 5FU−liposome interactions and mechanisms of 5FU transfer into the liposomal membrane concerning 5FU/liposome and 5FUPBCN/liposome samples. From ^{1}H DOSY experiments, the diffusion coefficients of 5FU and liposomes in 5FU/liposome and 5FUPBCN/liposome samples were measured and compared with the corresponding values obtained for the free 5FU, 5FUPBCN, and liposomes. Self-diffusion coefficients (D) of 5FU and liposomes in the samples studied were determined from the resonance signals of the methine proton of 5FU and *N*-methyl protons of the choline residues of liposomes. Since modifications of the solution composition could induce changes in the viscosity and these changes affect equally all species in the solution, we found it useful to use TSP as an internal diffusion reference, and all diffusion coefficients presented in Table 3 are given as a ratio D/D_{TSP} according to a procedure published previously.[23]

As expected, the D/D_{TSP} for the free 5FU was found to be higher than the corresponding value for 5FU entrapped in the PBCN due to a more restricted environment. However, when 5FUPBCN was injected to liposomes, the D/D_{TSP} of 5FU increased and maintained the same value up to 24 h. In contrast,

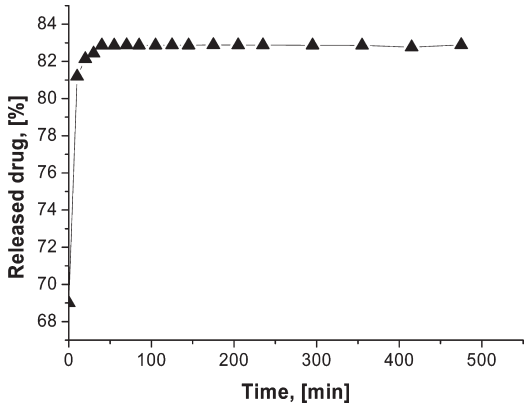

Figure 6. In vitro release profile of 5FU from 5FUPBCN in PBS with pH 7.4 at 37 °C.

similar D/D_{TSP} values were determined for 5FU in water solution and in the presence of liposomes (5FU/liposome sample) even 60 min after injection of the drug. With time, the relative diffusion coefficient of 5FU in the 5FU/liposome sample decreased and after 24 h almost reached the value registered for the 5FUPBCN/liposome sample.

A significant increase of the relative diffusion coefficient (D/D_{TSP}) of liposomes was observed after addition of either 5FU or 5FUPBCN, attributed to the disruption of their well-organized structure and improved mobility. For the time interval studied, the D/D_{TSP} of liposomes remained constant in the 5FUPBCN/liposome sample but decreased significantly in the 5FU/liposome sample, reaching values close to those found for the liposomes themselves. The observed significant changes of D/D_{TSP} in the 5FU/liposome sample 24 h after injection of the drug are most probably due to a provoked liposomal aggregation at the high working concentration used.

In Vitro Drug Release from 5FUPBCN. The 5FU release profile from PBCN as a function of the time in an incubation medium (PBS) of pH 7.4 is presented in Figure 6. A biphasic process occurs: first, an early rapid release up to around 82% took place within 30 min, while the remaining quantity was slowly liberated during the following 8 h. The rapid release probably represents the loss of surface-associated (adsorbed) and poorly entrapped 5FU. Drug release during the slower release phase is probably associated with the particle erosion.

DISCUSSION

To explore the interactions of 5FU, free and included into the polymer nanoparticles, with cellular membrane and the mechanisms of drug penetration into membranes, large unilammellar vesicles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were used as a biomembrane model. A practical challenge faced in the study was to distinguish specific drug−membrane interactions and the role of poly(butylcyanoacrylate) nanoparticles as a drug carrier, on the drug delivery, penetration, and retention into membranes, phenomena that affect the therapeutic index of drugs.

Steady-State Fluorescence Anisotropy. The results from steady-state fluorescence anisotropy indicate that the presence of 5FU and 5FUPBCN affected lipid properties, thus suggesting a drug−liposome interaction. TMA-DPH and DPH were chosen as fluorescent probes since their biophysical characteristics are well-known and they have been extensively used to detect lipid phase transitions, independently of which phase is under study.[29] DPH molecules are incorporated near the center of the bilayers, with an average location of ≈8 Å from the center of the bilayer.[30,31] TMA-DPH, a DPH derivative, is located in a shallow position, near the lipid−water interface, with its DPH moiety located ≈11 Å from the center of the bilayer, thus reporting near the lipid headgroup region of the membrane.[31] Therefore, DPH is embedded in the bilayer, while TMA-DPH is anchored at the aqueous interface, with its DPH moiety intercalated between the phospholipid acyl chains. Consequently, it is possible to gather information preferentially from the core or from the interface regions of the bilayer. The perturbation by the 5FU and 5FUPBCN of the phase transition is significant but only observable by DPH probe, indicating a penetration in the fatty acid chain region. Additionally, altering the membrane's properties, making it more fluid, could lead to lateral membrane heterogeneity in certain membrane domains, that exist in vivo and that the drug may use to more easily perturb the membrane and internalize.[32,33] These results point to a deeper drug penetration, although it is not possible to conclude about the kinetics and retention times in the lipid bilayers.

NMR Spectroscopy. The sensitivity of a number of NMR parameters, such as chemical shift, line shape, and translational diffusion to structural alterations and intermolecular interactions, allows for analysis of drug−liposome interactions and the mechanisms of drug delivery and penetration into liposomes. The results from the present NMR spectroscopic analysis of 5FU/liposome and 5FUPBCN/liposome model systems confirm the existence of drug−membrane interaction and drug delivery from the water environment or polymer matrix of nanoparticles to the membrane. The difference in the spectral behavior of 5FU in 5FU/liposome and 5FUPBCN/liposome samples with time-dependent alterations of some NMR parameters in the former suggests different mechanisms of drug delivery and penetration into the phospholipid bilayer of liposomes.

The weak changes in the resonance frequencies and self-diffusion coefficients of 5FU and liposomes observed in ^{1}H, ^{19}F, and DOSY NMR spectra of 5FU/liposomes (Tables 2 and 3) suggest an entrapment of the small, hydrophilic drug molecules at the polar interfacial side of the membrane and further diffusion of some of them through the membrane. The inclusion of 5FU into liposomes is probably initiated by the drug concentration difference outside and inside of the liposomes. The appearance of two resonance signals for H4 indicates the presence of 5FU in two different environments and the coexistence of drug molecules adsorbed at the membrane−water interface with constant access to the aqueous phase (at 7.642 ppm) and others with some degree of penetration into the membrane (at 7.640 ppm) being assumed. The assumption is consistent with the moderate values determined for the partition coefficient (log $P = -0.824$) and polar surface area (PSA = 65.7 Å^2) of 5FU published recently in the literature.[34] These two parameters correlate with passive molecular transport through membranes and allow prediction of transport properties of drugs.[34,35] The NMR signal broadening in the spectra reveals the existence of nuclear exchange processes between drug molecules in the different environmental locations. The results are consistent with data published recently from an investigation on 5-fluorouracil binding and mobility in fluid lipid bilayer membranes of egg phosphatidylcholine.[16,36] The observed time-dependent changes in the liposome diffusivity in 5FU/liposomes (Table 3) indicate that the drug transport through the membrane is

reversible and probably depends on the drug concentration in the bulk water environment. The results reflect good membrane permeation but poor retention of the small drug molecules within the membrane. This means that in real biological systems continuously new amounts of 5FU should be added to the system to reach the desired concentration in the cellular interior and could explain the limited 5FU exposure time and necessity of continuous therapy with 5FU to achieve optimal therapeutic benefits in patients.[37,38]

The stable and more significant changes in the resonance frequencies and self-diffusion coefficients of 5FU and liposomes observed in ^{1}H, ^{19}F, and DOSY NMR spectra of 5FUPBCN/liposomes (Tables 2 and 3) provide evidence for deeper drug penetration and retention into the hydrophobic lipid bilayers of liposomes. The results suggest different mechanisms of drug delivery and penetration into the lipid bilayer of liposomes, when 5FUPBCN are used. In the 5FUPBCN/liposome sample, the zwitterionic headgroups from the outer layer of liposomes should facilitate the delivery of the charged 5FUPBCN species from the water to the liposome surface. The 5FUPBCN are able to adsorb on the liposome surface, due to weak charge−dipole interactions between the negatively charged nanoparticles and the zwitterionic headgroups of liposomes.[39,40] Upon binding of 5FUPBCN to the liposome surface, the drug is exchanged from the PBCN polymer matrix to the lipid bilayers of the liposomes through contact facilitated drug delivery. The contact facilitated mechanism of targeted drug delivery has been recently demonstrated with perfluorocarbon nanoparticles.[41,42] The decrease of the resonance frequency and line width of H4 in ^{1}H NMR spectra of 5FUPBCN/liposome compared to that of 5FUPBCN indicates the release of 5FU from the polymer matrix of the PBCN attached to the liposome surface and diffusion toward the hydrophobic lipid bilayers of the liposomes. The adsorbed 5FUPBCN do not allow access and diffusion back of drug molecules to the bulk water environment. The 5FUPBCN serves most likely as a reservoir of 5FU, continuously "pumping" the drug into the liposomes.

Furthermore, the existence of two polymer chain-bound forms of 5FU in 5FUPBCN should facilitate the deeper drug penetration and retention into lipid bilayers of the membrane due to the lipophilic character of the species. This process provokes a continuous disruption of the well-organized structure of the liposomes and explains the significant increase of the relative diffusion coefficient (D/D_{TSP}) of the liposomes (Table 3). At the same time, the degradation of the polymer nanoparticles could further contribute to the alteration of the liposomal structure due to penetration and retention of oligomer chains and/or alkanols into the membrane. It is well-known that under biological conditions poly(alkylcyanoacrylates) undergo biodegradation (more exactly bioerosion) by means of enzymatic hydrolysis of the side alkyl chain groups accompanied by rapid depolymerization of the "parent" polymer and simultaneous formation of lower-molecular-weight repolymerized "daughter" polymers.[43] The products of the biodegradation process can act as drug penetration enhancers and improve the drug delivery through the membrane by causing some physicochemical changes within the lipophilic membrane barrier.[44,45] Indirect evidence for such a role for polymer chains/polymer nanoparticles could be drawn from the reported results of the local treatment of patients with superficial basal cell carcinoma,[8] suggesting a role of PBCN as skin penetration enhancers for 5-FU.

The results show that the application of 5FUPBCN facilitates the delivery and deep penetration of 5FU into liposomes, permanently destroying their well-organized structure. The nanoparticles adsorbed on the liposome surface prevent the drug diffusion from the liposome to the water environment, protecting in such way the higher drug concentration levels in the former. The results obtained for the in vitro drug release from PBCN are consistent with the above-discussed existence of 5FU both covalently bonded to the polymer chain as well as physically entrapped. The rapid drug release probably represents the loss of physically entrapped 5FU, while the slower release phase is probably associated with particle degradation and consequent breaking of the covalent linkages. The results suggest that through the administration of 5FUPBCN higher drug concentrations in the cellular membranes could be achieved by sustained drug release, and the therapeutic index of 5FU could be improved.

CONCLUSION

The results obtained from the NMR and steady-state fluorescence anisotropy study of the behavior and interaction of 5-fluorouracil, free and included into the polymer matrix of poly(butylcyanoacrylate) nanoparticles, with DMPC liposomes, indicate the interaction and penetration of 5FU into the phospholipid bilayer of liposomes. Different mechanisms of drug transfer from the aqueous into a model membrane environment, for the free drug and that incorporated into polymer nanoparticles, are suggested: (i) concentration-dependent reversible diffusion of the free 5FU and (ii) sustained 5FU release from nanoparticles adsorbed on the liposome surface, resulting in continuous delivery of the drug into the phospholipid bilayers. It is likely that the drug penetration and retention in the membrane are enhanced when 5FUPBCN are used. The results demonstrate that by the application of 5FUPBCN in cancer therapy the efficacy of 5FU can be increased, due to the higher drug concentration in the cellular membranes that can be achieved by sustained drug release.

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