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Journal of Controlled Release 120 (2007) 79–87
journal of controlled release
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Passive diffusion of polymeric surfactants across lipid bilayers

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Received 1 December 2006; accepted 23 March 2007
Available online 31 March 2007

Abstract

Self-assembling polymeric surfactant, mmePEG$_{750}$P(CL-co-TMC) [monomethylether poly(ethylene glycol)$_{750}$-poly(caprolactone-co-trimethylene carbonate)], increases drug solubility and crosses an enterocyte monolayer both in vitro and in vivo. The aims of the present work were to investigate whether mmePEG$_{750}$P(CL-co-TMC) polymers can diffuse passively through lipid bilayer using parallel artificial membrane permeability assay (PAMPA) and affect membrane properties using liposomes as model. The mmePEG$_{750}$P(CL-co-TMC) polymer was able to cross by passive diffusion an enterocyte-mimicking membrane in PAMPA at concentration which did not perturb membrane integrity. A weak rigidification associated with a low increase in permeability of liposomal lipid bilayers was observed. These data suggest that polymeric surfactants can cross the lipid membrane by passive diffusion and interact with lipid bilayers.
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Keywords: Self-assembling polymeric micelles; mmePEG$_{750}$P(CL-co-TMC); PAMPA; Liposomes; Diffusion

1. Introduction

Surfactants are widely used in various applications including as stabilizing excipients for emulsions in cosmetic and pharmaceutical formulations or solubilizing agent for membrane proteins in biochemistry. Besides conventional low molecular weight surfactants, polymeric surfactants that form micelles have been developed to enhance solubility of poorly-soluble drugs by entrapment of materials inside the hydrophobic core. Micelles formation and/or drug entrapment often necessitate a complex manufacture procedure usually requiring organic solvent. Such polymeric micelles have been mainly investigated for parenteral administration of anticancer drugs [1–3].

Recently, it has been demonstrated that mmePEG$_{750}$P(CL-co-TMC) polymers [monomethylether poly(ethylene glycol)$_{750}$-poly(caprolactone-co-trimethylene carbonate)] (weight average molecular weight: ±5500 g/mol) upon gentle stirring in aqueous media spontaneously self-assemble in aqueous solutions. To obtain liquid polymers at room temperature allotting their self-assembling property, a hydrophobic copolymer made of relatively close molecular structures (ε-caprolactone and trimethylene carbonate) was chosen in order to provide homogeneity while avoiding crystalline areas. Furthermore, the mmePEG$_{750}$P(CL-co-TMC) polymers increase drug solubility by 1 to 4 orders of magnitude by entrapment inside the core formed by the lipophilic moiety of the surfactant [4]. Moreover, these polymers are able to cross the intestinal barrier. In vitro, they permeate Caco-2 cell monolayers with an apparent permeability coefficient (Papp) of 2.3×10^{-5} cm s^{-1} below its critical micellar concentration (CMC) and 2.5×10^{-6} cm s^{-1} above its CMC [5]. In vivo, their oral bioavailability is 40% [5].

The mechanism by which mmePEG$_{750}$P(CL-co-TMC) or other polymeric surfactants permeate the intestinal barrier is not

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doi:10.1016/j.jconrel.2007.03.015

well understood [6,7]. It has been suggested that endocytosis could be the main mechanism of their intestinal transport [3,8–10]. Moreover, recent studies demonstrate that polymeric surfactants, such as Pluronics, affect the membrane structure of lipid bilayers [5,8,11,12] and passive diffusion of polymeric surfactants themselves across enterocytes could therefore be possible due to their amphiphilic character but has never been reported.

The aim of this paper is to assess this hypothesis and investigate whether mmePEG$_{750}$P(CL-co-TMC) could cross a lipid bilayer by passive diffusion. Hence, parallel artificial membrane permeability assay (PAMPA) which models the intestinal membrane was performed [13]. This system which is routinely used for screening drug permeability in drug discovery allows elimination of all active transports and focuses only on passive diffusion.

As surfactants are well-known to induce alterations in lipid membranes, mmePEG$_{750}$P(CL-co-TMC) could also induce membrane changes. Its effect on membrane fluidity and permeability as well as the interaction with phospholipid headgroup were investigated using liposomes as model membranes. Different ratios (30:70, 50:50 and 70:30) in hydrophobic monomers (ε-caprolactone and trimethylene carbonate) constituting the lipophilic moiety of mmePEG$_{750}$P(CL-co-TMC) polymers were examined for their effects on lipid membranes. The hydrophilic moiety stayed constant (750 g/mol PEG derivative) and the weight average molecular weight for all different mmePEG$_{750}$P(CL-co-TMC) polymers as well (each around 5500 g/mol).

2. Materials and methods

2.1. Synthesis and characterization of polymers

The mmePEG$_{750}$P(CL-co-TMC) (30:70, 50:50 and 70:30 molar ratio) polymers were produced by Johnson & Johnson Center for Biomaterials and Advanced Technologies (CBAT, Somerville, NJ, USA). Stannous octoate and toluene were obtained from Aldrich (Milwaukee, WI, USA). Monomethyl-ether-polyetheleneglycol with a molecular weight of 750 g/mol (mmePEG$_{750}$) was purchased from Fluka (Milwaukee, WI, USA). ε-caprolactone (CL) was purchased from Union Carbide (Danbury, CT, USA), and trimethylene carbonate (TMC) from Boehringer Ingelheim (Petersburg, VA, USA). The radiolabelled [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) (50:50 molar ratio) polymer was provided by Perkin Elmer employing ε-[caprolactone-2,6–^{14}C].

The synthesis of mmePEG$_{750}$P(CL-co-TMC) diblock polymer was performed by ring opening polymerization as described earlier by Ould-Ouali et al. [4,14,15]. For the [^{14}C]-labelled mmePEG$_{750}$P(CL-co-TMC), ε-[caprolactone-2,6–^{14}C] replaced the unlabelled ε-caprolactone during the polymerization.

The polymer composition and residual monomer content were analysed by proton NMR (Nuclear Magnetic Resonance). Gel permeation chromatography was utilized to determine the molecular weight and the polydispersity of the polymers [4,8]. The radiochemical purity of [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) was assessed using a HPLC (High Performance Liquid Chromatography) system equipped with radioactivity detector [5].

2.2. Physicochemical characterization of micelles

The nanoparticle size and their Zeta potential were determined by dynamic light scattering (DLS) and laser Doppler velocimetry combined with phase analysis light scattering (PALS), using a Zetasizer® Nano ZS (Malvern Instruments, UK), respectively. The measurements were performed in citrate–citric acid buffer pH 6.0 (300 mOsm/kg). The critical micellar concentrations (CMC) of the polymers were determined based on the emission spectrum of pyrene, as previously described [4].

2.3. Permeation kinetics in the PAMPA model

2.3.1. Formulations

mmePEG$_{750}$P(CL-co-TMC) (30:70; 50:50 and 70:30) were diluted at 10, 3, 0.5, 0.05 and 0.001% (w/v) in citrate/citric acid buffer (pH 6).

Pure radiolabelled mmePEG$_{750}$P(CL-co-TMC) (50:50) was utilized to formulate 0.001 and 0.05% (w/v) polymeric solutions by mixing an appropriate amount of [^{14}C]-polymer with citrate/citric acid buffer pH 6.0 for 1 h. Radiolabelled micellar solutions of 0.5, 3 and 10% were prepared by combining unlabelled polymer with radiolabelled polymer solution.

Polymer-free solutions of either carbamazepine or furosemide (all from Sigma, Bornem, Belgium) at 0.5 mg/ml were exclusively solubilized in pure dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany).

2.3.2. PAMPA assay

The commercial PAMPA "sandwich" is composed of a 96-well microtiter plate nested to a 96-well filter plate (*p*ION Inc., PN 110163) whose filters were coated with 4 μl of a lipid mixture dissolved in dodecane that mimics enterocyte phospholipids (*p*ION Inc., PN 110615). The donor chamber, on the bottom, and the receiver chamber on the top, both containing 200 μl of media, were separated by the phospholipids-coated filter.

Donor chambers filled with the 3 different polymers (50:50, 30:70 and 70:30) at various concentrations (10, 3, 0.5, 0.05 and 0.001% (v/w)) were incubated at 20 °C for 12 h in a water-saturated atmosphere in order to allow the permeation of the different polymer formulations. Next, the plates were rinsed twice with fresh buffer and the integrity of the PAMPA membranes was checked by replacing the contents of the donor chambers with 23 μM D-[1-^{14}C] Mannitol solution (61.0 mCi/mmol) (GE Healthcare Europe GmbH, Diegem, Belgium) and the receiver chambers with fresh buffer. The permeation of mannitol through the lipids-coated filters was measured after additional 6 h incubation. Other chambers were filled with buffer for the first 12 h (polymer-free wells) and were incubated the next 6 h with mannitol and Triton X-100 (1%) (Acros Organics, Geel,

Belgium) or with mannitol alone (positive control and negative control, respectively).

The membrane integrity (I%) can be expressed as:

$$I\% = 100 - \left(\frac{100}{T} * M\right)$$

where T is the theoretical concentration of mannitol found in the receiver wells if the lipids-coated filters were completely disrupted (equals to 11.5 μM which is the half of the donor concentration) and M is the measured concentration of mannitol found in the receiver wells.

The membrane integrity was considered as non-compromised for mannitol concentration below the detection limit ($3*\sigma a_0/a_1$) in the receiver chambers (equals to 0.003 μM). Conversely, a mannitol concentration equals to 11.5 μM in the receiver wells would indicate a complete disruption of the lipid membrane. The experiments were conducted in sextuplicate.

The amount of radiolabelled polymer (50:50) that crossed the phospholipids-coated filter during 12 h was assessed by removing 150 μl of media from the acceptor compartments. Each sample was placed in 4 ml of Aqualuma® (Lumac, Groningen, The Netherlands) and counted in a Wallac 1410 Liquid Scintillation Counter (Pharmacia) with automatic external standardization. Standard curves were generated for high and low concentrations of [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) (from 0.00001 to 0.001%; $R^2 \sim 1$ and from 0.001 to 0.05%; $R^2 \sim 1$, respectively). The same procedure was applied for mannitol quantification. Standard curves for high and low concentrations were established (from 0.023 to 0.75 μM; $R^2 \sim 1$ and from 0.75 to 7.5; $R^2 \sim 1$, respectively).

Control samples containing furosemide or carbamazepine were analyzed using published methods [16] and [17], respectively, which were modified to render them adaptable to our laboratory conditions. In both cases, analyses were performed on a HPLC Agilent 1100 series (Agilent Technologies, Diegem, Belgium) equipped with a quaternary pump, a vacuum degasser and a diode array detector, all from Agilent. The thermostatted column (Hypersil BDS C18, 3 μm, 100×4.6 mm protected by a guard column Hypersil ODS, 5 μm, 4.0×20 mm (Alltech, Lokeren, Belgium)) was set at 30 °C. Standard curve for furosemide ranged from 0.005 to 0.1 mg/ml ($R^2 \sim 1$) and for carbamazepine, from 0.001 to 0.01 mg/ml ($R^2 \sim 1$) and from 0.01 to 1 mg/ml ($R^2 = 0.998$). The coefficients of variation (CV) for intra- and inter-assay were all within 4.3%. The accuracy was approximately 100% for all analyzed drugs ([measured amount/actual amount]×100).

The transport rate (dQ/dt) was calculated by plotting the amount of polymer or drug penetrating into the acceptor chamber versus time (12 h) and then determining the slope of this relationship. The permeability coefficient (P, cm s^{-1}) was calculated from the following equation:

$$P = \frac{dQ}{dt} * \frac{1}{C_o * A} * I$$

where dQ/dt is the transport rate (mg/s), I is the percentage of the membrane integrity, C_o is the initial polymer or drug concentration in the donor chamber (mg/ml), and A is the surface area of the membrane filter in cm^2 [8,18].

2.4. Liposomal models

2.4.1. Production and characterization of liposomes

Liposomes used to assess the influence of the polymers on membrane fluidity and permeability were produced by extrusion which formed Large Unilamellar Vesicles (LUV) [19,20]. All liposome constituents were purchased from Lipid Products (Surrey, UK) except for cholesterol and sphingomyelin which were obtained from Sigma (Bornem, Belgium). Briefly, a dry lipid film constituted of cholesterol, sphingomyelin, phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine (21%, 13%, 26%, 22% and 18% (w/v), respectively) was rehydrated over a period of 1 h with citrate/citric acid buffer with or without 30 mM of highly pure calcein (Molecular Probes, Leiden, The Netherlands) at 37 °C. This colloidal solution containing Multilamellar Large Vesicles (MLV) underwent 5 cycles of freeze–thawing followed by 10 cycles of extrusion through 200-nm polycarbonate filters (Nucleopore, Corning Costar, NY) in a 10 ml Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada) under a nitrogen pressure of 20 bars. Phosphorus assay was performed to determine phospholipid concentration which was adjusted to 10 mg/ml in total lipid [21]. The size of LUV liposomes was determined before storage under nitrogen.

NMR experiments were performed with Multilamellar Large Vesicles of the same composition than Large Unilamellar Vesicles. Lipid concentration was adjusted to 45 mg/ml in order to enhance ^{31}P-signal.

2.4.2. Membrane fluidity studies

Membrane fluidity was evaluated by measuring the rotational diffusion coefficients of a fluorescent probe inserted in the membrane, 1,6-diphenyl-1,3,5-hexatriene (DPH) [22] (Molecular Probes, Leiden, The Netherlands) at 4 and 37 °C using Perkin Elmer LS50 (Beaconsfield, UK) equipped for polarization measurements and operating at excitation and emission wavelengths of 365 nm (slit-width: 5 nm) and 427 nm (slit-width: 4 nm), respectively [20,23].

Three different mmePEG$_{750}$P(CL-co-TMC) polymers (30:70, 50:50 and 70:30) solubilized in citrate/citric acid buffer were tested at three different final concentrations (0.1%, 0.01% and 0.0005%). The stock solution of 0.3 mM DPH was constituted in dimethylformamide (Acros Organics, Geel, Belgium).

The liposome solution was first preincubated with DPH (1 mol/150 mol of lipids) over a period of 180 min at 37 °C. The different polymer solutions (and the buffer control solutions) were then incubated with DPH-probed liposomes (600 μM lipid concentration) over 30 min at 37 °C in vials protected from light and under gentle stirring. The polymer to lipid molar ratios for polymer concentrations at 0.1, 0.01 and 0.0005% were 0.28, 0.028 and 0.0014, respectively. Samples were monitored for fluorescence polarization by illuminating them with a monochromatic polarized light beam, and recording the emitted

intensity in the same plane and at an angle of 90 °C. The degree of polarization can be expressed as:

$$\text{Degree of polarization} = \frac{[I_{par} - I_{per}]}{[I_{par} + I_{per}]}$$

where I_{par} and I_{per} are the intensities of the lights emitted in the planes parallel and perpendicular to that of the polarized excitation light, respectively. All conditions were conducted at least in triplicate.

2.4.3. Membrane permeability studies

Membrane permeabilization was evaluated by measuring the increase of fluorescent signal upon release of calcein entrapped at a self-quenching concentration in liposomes [24].

To include calcein within liposomes, the dry lipid film was resuspended in citrate/citric acid buffer supplemented with 30 mM of highly pure calcein at the beginning of liposome production [25]. The unencapsulated dye was eliminated by the minicolumn centrifugation technique [26]. Finally, the osmolarity was checked by the freezing point technique with a semimicro osmometer type L (Knauer, Berlin, Germany).

The solution of calcein-loaded liposomes (50 μM lipid concentration) was incubated at 37 °C with solutions of different concentrations in mmePEG$_{750}$P(CL-co-TMC) (50:50; in citrate/citric acid buffer) to equal volume (1:1) during 210 min. The polymer to lipid molar ratios for polymer concentrations at 20, 10 and 5% were 0.69, 0.345 and 0.17, respectively. All fluorescence determinations were performed in triplicate at 37 °C on a Perkin Elmer LS30 using an excitation and emission wavelengths of 472 and 516 nm, respectively. The percentage of calcein release was defined as:

$$\text{Percentage of calcein release} = \frac{[F_t - F_{contr}]}{[F_{tot} - F_{contr}]} * 100$$

where F_t represents the fluorescence signal recorded at time t, F_{contr} is the fluorescence signal measured at the same time in the control liposomes, and F_{tot} is the total fluorescence signal obtained upon complete disruption of liposomes by sonication. Melittin at 1 μM (Sigma, Bornem, Belgium), a well-known porogenic agent [27], was utilized as positive control.

2.4.4. ^{31}P-NMR experiments

Interaction between mmePEG$_{750}$P(CL-co-TMC) and the phosphate group of phospholipids was analysed by ^{31}P-NMR [28]. To this end, 100 μl of mmePEG$_{750}$P(CL-co-TMC) (30:70) at 30 or 60% (w/v), solubilized in citrate/citric acid buffer pH 6, were placed in 5-mm NMR tubes containing 500 μl of MLV liposomes. One hundred microliters of D_2O were also added for calibration. ^{31}P-NMR spectra were acquired at 202.5 MHz on a Bruker AVANCE 500 spectrometer. The Fourier transform parameters were as follows: 50.5 kHz of spectral width, a flip angle of 45° (6 μs), 0.6 s of pulse interval, 3000 scans and 8 K data points. A line broadening of 70 Hz was applied to the free induction decay before Fourier transformation. Powergated ^{1}H decoupling was applied for minimizing dielectric heating. Experiments were performed at several temperatures with 30 min equilibration times between each temperature.

2.5. Statistical analysis

For all experiments, data are presented as the mean ± SD. Statistical analyses were done using JMP software (SAS Institute Inc., version 4.0.2). Significance was tested using Anova Oneway (Tukey test). Values of $p < 0.05$ and $p < 0.001$ were considered statistically significant and highly significant, respectively.

3. Results

3.1. Characterization of polymers and micelles

Polymer characteristics are summarized in Table 1. The mmePEG$_{750}$P(CL-co-TMC) diblock polymers had a weight average molecular weight in the range of 5800 g/mol and were liquid at room temperature. The chemical composition of mmePEG$_{750}$P(CL-co-TMC) as determined by proton NMR was in good agreement with the ratio of the monomers charged. The residual monomer content was less than 1 mol%. The [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) polymer had a radiochemical purity of ≥97% with a specific activity of 1.13 μCi/mg.

mmePEG$_{750}$P(CL-co-TMC) block polymers formed micelles spontaneously on contact with aqueous media. The size of the micelles (22 to 24 nm) was not influenced by the composition or by the concentration of the polymer. The Zeta potentials were all around zero. The CMC were in the range of 20 μg/ml (0.002%) [4,8].

Table 1
Properties of mmePEG$_{750}$P(CL-co-TMC) polymers (CL = ε-caprolactone; TMC = trimethylene carbonate)

CL-to-TMC ratio	Mw [a]	Mw PDI [a]	CMC (μg/ml) [b]	CL-to-TMC ratio in final polymer [c]	Size (nm) [d]	Size PDI [d]	ζ potential ± SD (mV) [d]
30:70	5448	1.5	30	30.6/69.2	21.5	0.024	−2.8 ± 56.1
50:50	5320	1.9	12	49.3/50.1	24.1	0.052	−2.7 ± 13.2
50:50-[^{14}C]	5188	1.6	ND	ND	ND	ND	ND
70:30	6185	1.6	14	70.1/29.7	23.0	0.019	3.9 ± 69.9

ND: not determined.
[a] Mw = weight average molecular weight and PDI (polydispersity index) determined by gel permeation chromatography.
[b] CMC = critical micellar concentration determined by fluorescence spectroscopy ($n \geq 3$).
[c] The monomer composition was evaluated by NMR.
[d] Size and Zeta potential determined with Nanosizer ZS.

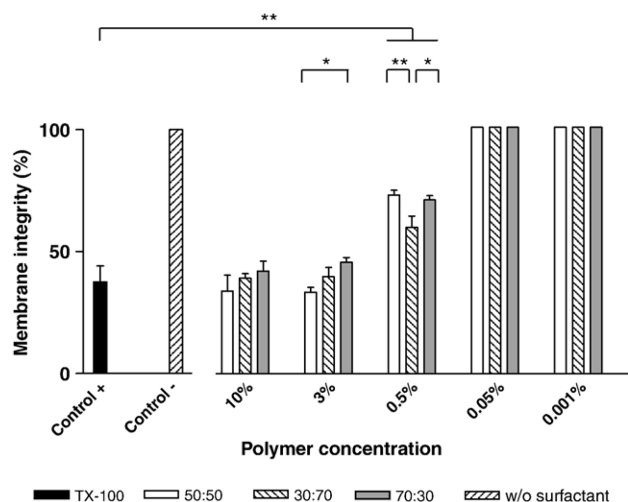

Fig. 1. Effect of various concentrations of mmePEG$_{750}$P(CL-co-TMC) (50:50, 30:70 and 70:30) on the PAMPA membrane integrity (based on the mannitol permeability) after 12 h incubation (mean ± SD, $n=6$) (*= $p<0.01$; **= $p<0.001$).

3.2. PAMPA experiments

Before investigating whether the polymeric surfactants mmePEG$_{750}$P(CL-co-TMC) could cross a lipid bilayer by passive diffusion, an experiment was performed in order to evaluate their impact on the membrane integrity of the PAMPA system. To this end, permeation of mannitol, a highly polar probe that does not cross lipid membranes, was carried out upon exposition of the membranes with various concentrations of the different polymers. As illustrated in Fig. 1, PAMPA membrane integrity was affected by the polymers in a dose-dependent manner.

Indeed, the membrane integrity decreased to approximately 40% upon incubation with polymers (50:50, 30:70 or 70:30) at 3 or 10% and with 1% Triton X-100 (negative control). At lower polymer concentration (0.5%), the membrane integrities for the 3 polymers reached approximately 67% and became significantly different ($p<0.001$) from the negative control and from the polymer concentrations at 3 and 10%. Nevertheless, the mmePEG$_{750}$P(CL-co-TMC) 30:70 was more deletery for the lipid layer than the two other polymers at that concentration. Finally, for lower polymer concentrations (0.05 and 0.001%) the integrity of the membranes remained intact after exposition to polymers.

The analysis in the receiver chambers of the amount of [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) 50:50 indicates that the polymer was capable of going through the lipids-coated filter by passive diffusion (Fig. 2). The permeability coefficient (P) for polymer at 0.001% was significantly higher ($p<0.0001$) than for the 2 more concentrated polymeric solutions (0.05 and 0.5%); the latter ones being not significantly different ($p>0.05$) to each other. All P values were corrected with the percentage of membrane integrity. Data for higher polymer concentrations (10 and 3%) was excluded from the experiment because of membrane damage.

Polymer-free formulations containing either carbamazepine (BCS class II) or furosemide (BCS class IV) solubilized at 0.5 mg/ml in DMSO were used as controls of PAMPA membrane relevance (in term of selective permeability) and were recovered in the upper compartments in amounts of 43.9 ± 1.8 and 3.3 ± 0.9 µg/well, respectively.

3.3. Polymer effect on membrane fluidity

Because the polymeric surfactants mmePEG$_{750}$P(CL-co-TMC) can cross the lipid bilayers, their interaction with liposomes as model lipid bilayers was investigated. Both their effect on membrane fluidity and permeability were evaluated.

In order to quantify the effect of mmePEG$_{750}$P(CL-co-TMC) on membrane viscosity, increasing concentrations of three different polymers mmePEG$_{750}$P(CL-co-TMC) (30:70, 50:50 and 70:30) were incubated with DPH-probed liposomes. The higher the probe polarization (anisotropy), the lower the membrane fluidity. Indeed, polarized light is slightly scattered when impinging upon membrane-inserted dye with low rotational motion, due to the membrane fluidity [22]. In order to avoid light scattering from the colloidal dispersion of micelles, low polymer concentrations had to be used.

At concentration below the CMC (0.0005%), where only unimeric form of polymers was present, no effect on probe polarization was detectable regardless of the polymers used, as illustrated in Fig. 3 A. For the first polymer concentration above the CMC (0.01%), the mmePEG$_{750}$P(CL-co-TMC) (30:70) and (50:50) rigidified lipid bilayers as compared to the control ($p<0.001$). In contrast, mmePEG$_{750}$P(CL-co-TMC) (70:30) did not modify membrane viscosity below and above the CMC.

A temperature modification from 37 to 4 °C in the experimental conditions led to an expected global enhancement in the anisotropy of DPH, as shown in Fig. 3 B. In fact, control condition allowed us to verify the ability of the membrane to rigidify in a cold environment. The three mmePEG$_{750}$P(CL-co-TMC) polymers decreased membrane fluidity compared to the experiment at 37 °C, except for the (70:30) polymer at 0.1% which exerted a significant rigidification of lipid bilayers.

3.4. Polymer effect on membrane permeability

Calcein-entrapped liposomes were utilized to assess the influence of mmePEG$_{750}$P(CL-co-TMC) (50:50) on membrane

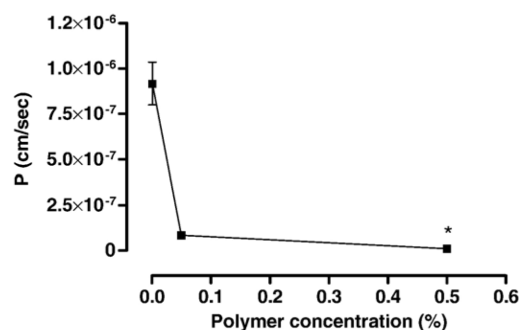

Fig. 2. Permeability coefficient (P) of [^{14}C]-mmePEG$_{750}$P(CL-co-TMC) (50:50) across PAMPA membrane as function of rising concentrations in polymer during 12 h incubation. *P for polymer at 0.5% was corrected by the membrane integrity percentage (mean ± SD, $n=6$).

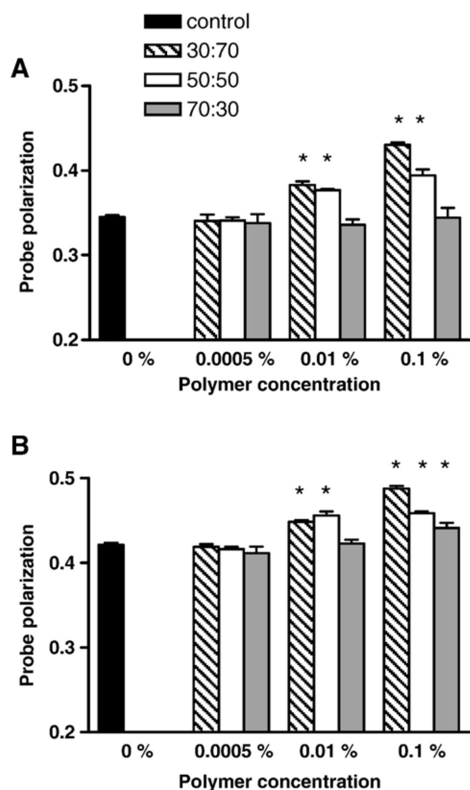

Fig. 3. Variation in polarization of DPH fluorescence incorporated in liposome bilayers according to concentration and composition of mmePEG$_{750}$P(CL-co-TMC) (30:70, 50:50 or 70:30) at 37 °C (A) and 4 °C (B). Probe polarization is proportional to membrane rigidity. Significant difference ($p<0.001$) from the control is represented by * (mean±SD, $n=3$).

permeability. The mean percentages of released calcein during the course of time were negligible for low polymer concentrations (from 0.0005% to 0.1%) but became statistically different from the control for higher polymer concentrations: 5.3±1.4, 11.9±2.3 and 22.7±5.0% in presence of 5, 10 and 20% of polymer, respectively (Fig. 4). After 30 min of incubation, steady states of calcein release were already achieved for polymer concentrations from 5 to 20%. Release of calcein from liposomes was proportional to the applied polymer concentrations for each time point (mean $R^2=0.995$). These releases were low and slow compared to melittin used as a positive control which induced 87.4±4.2% of calcein leakage from liposomes within 2 min upon its addition (inset graph in Fig. 4).

3.5. Polymer effect on polar head group of lipids

To further characterize the interaction of the most rigidifying polymer mmePEG$_{750}$P(CL-co-TMC) (30:70) with the lipid bilayers, ^{31}P-NMR was performed to check interactions with the polar headgroup of the lipids. ^{31}P-NMR spectra of liposomes alone and incubated with mmePEG$_{750}$P(CL-co-TMC) (30:70) at final concentrations of 8.6% and 17.2% (Fig. 5) revealed that the polymer mmePEG$_{750}$P(CL-co-TMC) (30:70) did not interact with liposome phosphate heads. None of spectra suggested a significant difference in effective chemical shift anisotropy compared to the control. Spectral shapes were also

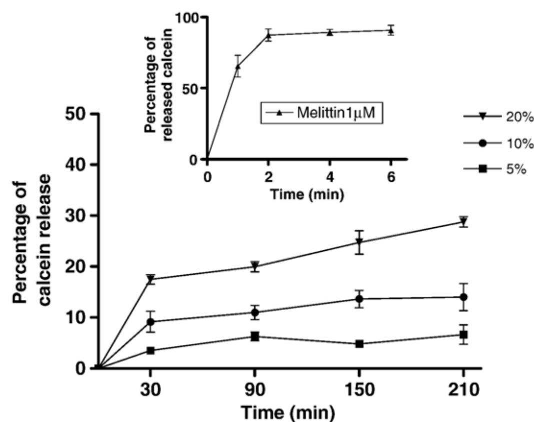

Fig. 4. Percentage of calcein release from liposomes in presence of different concentrations of mmePEG$_{750}$P(CL-co-TMC) (50:50) as a function of time. Inset, positive control carried out with melittin (mean±SD, $n=3$).

identical during a thermal cycle (25, 35, 45 and 55 °C) (data not shown).

4. Discussion

The objective of this paper was to determine whether a polymeric surfactant could cross the lipid bilayer by passive diffusion and whether they could interact with the lipid membrane. The rationale for this research was the recent demonstration that mmePEG$_{750}$P(CL-co-TMC) polymeric surfactant developed to enhance dissolution of poorly-soluble drugs was capable of overcoming the intestinal barrier when delivered orally. The bioavailability of this polymer was 40% in rats [5]. Several previous researchers have suggested that polymeric micelles undergo endocytosis in order to penetrate enterocytes. Some have hypothesized that passive diffusion of

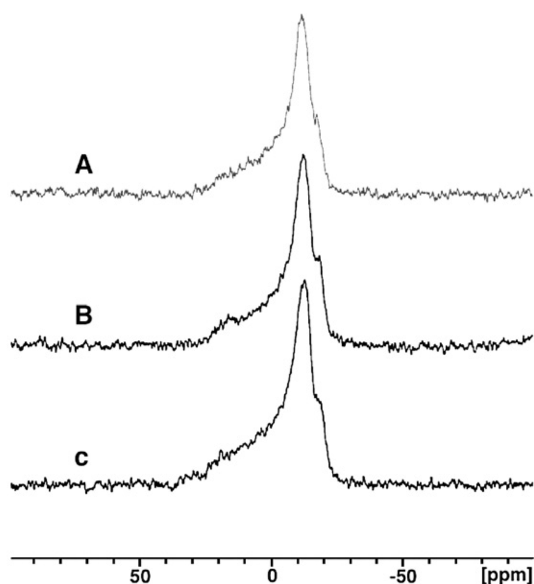

Fig. 5. ^{31}P-NMR spectra of MLV liposomes at 25 °C. (A) Control liposomes; (B) in presence of 8.6% mmePEG$_{750}$P(CL-co-TMC) (30:70), (C) in presence of 17.2% mmePEG$_{750}$P(CL-co-TMC) (30:70).

polymeric surfactants could also be involved but no proof was provided [3,9,10,29]. The present study aimed to provide evidence of passive permeation of polymeric surfactants across the intestinal membrane. To this end, the PAMPA in vitro model mimicking intestinal barrier was chosen for its ability to study exclusively passive diffusion of compounds.

As assessed by the radiolabeled-mmePEG$_{750}$P(CL-co-TMC) (50:50), the polymer at low concentrations was able to diffuse passively at a significant rate in the PAMPA (Fig. 2) without compromising the membrane integrity (Fig. 1). Moreover, at a polymer concentration just below the CMC (0.001%), where only unimers (free polymers) are present, the permeability coefficient (P) was well above those for higher polymer concentrations (0.05 and 0.5%). In agreement with Fick's law, this could be logically explained by the fact that unimers in equilibrium with micelles, should permeate more easily than micelle-made polymers across membrane. Polymeric unimers in equilibrium with micelles were assumed to be the permeated fraction rather than entire micelles. The reliability of the PAMPA system to predict in vivo oral absorption of drugs and excipients was assessed by the fact that the amount of furosemide (BCS class IV, low solubility–low permeability) crossing PAMPA membrane was >10 times lower than carbamazepine (BCS class II, low solubility–high permeability).

This ability of polymers to penetrate membranes of lipid bilayers could be explained by their amphiphilic character as well as by their low hydrogen-bonding capacity relative to their molecular weight (Mw). In fact, for the same mmePEG$_{750}$P(CL-co-TMC) Mw, PEG-5000 exhibits twice as high H-bonding capacity based on the calculation of Diamond and Wright [30] where each alcohol (C–O–H) group and each ether or hemiacetal (C–O–C) group counts for 2.0 and 0.8 H-bonding capacities, respectively.

Passage across lipid bilayers of amphiphilic polymer such as the mmePEG$_{750}$P(CL-co-TMC) should logically involve some perturbations in the lipid barrier structure. Demina et al. [11] enumerated some special characteristics polymeric surfactant must present in order to affect lipid bilayer including a large hydrophobic block, a molecular weight equal to or exceeding that of lipids in bilayer and a moderate hydrophobicity in order to avoid shifting the equilibrium in favor of micellization instead of membrane inclusion. All these features were fulfilled by the mmePEG$_{750}$P(CL-co-TMC) polymer. Moreover, the surface tension exhibited by the mmePEG$_{750}$P(CL-co-TMC) (48 mN/m) was at least twice as high [4] as than required for membrane accumulation which was estimated at 25 mN/m [31], depending on the membrane composition.

mmePEG$_{750}$P(CL-co-TMC) polymers influence membrane permeability with a dose-dependent relationship as evidenced by disturbance of the membrane integrity of the PAMPA system (Fig. 1) and by the calcein release on the liposomal model (Fig. 4). However, this increase in permeability was observed at rather high polymer concentrations ($\geq 0.5\%$) and only on membrane in vitro models. Indeed, previous studies on Caco-2 cells have shown that trans-epithelial electrical resistance (TEER), MTT test (methyl thiazol tetrazolium cytotoxic assay) and sodium fluorescein flux were not significantly affected by incubating Caco-2 cells with 10% mmePEG$_{750}$P(CL-co-TMC) during several hours [8]. Moreover, due to both gastrointestinal motility, lowering the exposure time to enterocytes, and dilution in the gastrointestinal media upon oral delivery of mmePEG$_{750}$P(CL-co-TMC), the likely disturbance effect should become negligible.

Other studies also revealed that correlation between release of liposome-entrapped calcein and vesicle lysis cannot be established (e.g. Cetylpyridinium chloride surfactant [32]) as well as between alterations in membrane fluidity and biocidal activity, as demonstrated with several surfactants [33]. This weak effect of mmePEG$_{750}$P(CL-co-TMC) is probably due to cholesterol incorporated within lipid bilayers ensuring a protective effect against perturbations in membrane fluidity [34] and permeability [35] induced by surfactants. Indeed, cholesterol acts as a buffer by regulating membrane fluidity and its association with sphingomyelin allows the lipid membranes to move into the liquid-ordered state (Lo) situated between the gel phase and the liquid-crystalline phase [35,36]. This intermediate state (Lo) can be assimilated to the so-called detergent-resistant membranes and provides a protection against surfactant aggression [37].

Beside the majority of surfactants which fluidize the lipid membrane, some surfactants like vitamin E TGPS [38] or Pluronics P85 and L61 in certain conditions [39] show rigidification effects on membrane viscosity. It was also the case for mmePEG$_{750}$P(CL-co-TMC) which caused a rigidification of the lipid bilayers, especially the polymer containing the highest proportion of trimethylene carbonate (30:70) in its composition. Its tendency to self-aggregate into micelles is reduced (relative higher CMC than the 50:50 and 70:30), this could explain a more efficient membrane insertion. This phenomenon of membrane rigidification probably resulting of raft formation made up of polymers within the bilayer featuring polymer tight packing due to a gain in entropy by mmePEG$_{750}$P(CL-co-TMC) self-aggregation.

Due to this polymer-induced rigification effect on the lipid membrane, the way that mmePEG$_{750}$P(CL-co-TMC) polymeric surfactants cross the lipid bilayers is assumed to process by passive diffusion instead of flip-flop mechanism (translocation from the outside leaflet to the inside leaflet of lipid bilayer). Conversely to the majority of polymeric surfactants which can accentuate the interleaflet lipid exchange by their fluidization effect on lipid bilayer [40], the mmePEG$_{750}$P(CL-co-TMC) polymers rigidify significantly the lipid membranes and then are assumed to abolish the flip-flop process. In addition, it has been recently showed that presence of only 10% of cholesterol in lipid membranes (10 to 40% in biological membranes) led to a drastically decrease in the Pluronic-induced acceleration of lipid flip-flop [41].

Nevertheless, mmePEG$_{750}$P(CL-co-TMC) do not act at the membrane surface as assessed by two different experiments. When liposomes were incubated with mmePEG$_{750}$P(CL-co-TMC), signals from ^{31}P-NMR focussing on the phosphate heads did not change (Fig. 5). The signals from TMA-DPH which inserted in the external layer of liposome membrane due to its charged nitrogen atom was also not modified (data not shown) [42].

In conclusion, we demonstrated that a polymeric surfactant mmePEG$_{750}$P(CL-co-TMC) very likely as unimer can cross the lipid membrane by passive diffusion. Interaction with lipid bilayers was evidenced by a slight membrane rigidification as well as by a membrane permeabilization effect at high polymer concentrations.

Acknowledgements

The authors express their acknowledgements to the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A.) for the financial support of Frédéric Mathot as well as the Fonds de la Recherche Scientifique Médicale (F.R.S.M.). The authors also wish to thank Cor Janssen (Johnson & Johnson, Beerse, Belgium) for the purity analysis of [^{14}C]-labelled mmePEG$_{750}$P(CL-co-TMC). Additionally, the authors are grateful to CBAT (Somerville, NJ, USA) for the polymer syntheses.

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