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Spectroscopic characterization of nanoErythrosomes in the absence and presence of conjugated polyethyleneglycols: an FTIR and ^{31}P-NMR study

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Abstract

We have recently developed from red blood cells a new delivery system called nanoErythrosomes. These nanovesicles offer a high degree of versatility for the encapsulation of biological or nonbiological compounds and for the binding of targeting agents. In particular, polyethyleneglycols can be conjugated by a covalent link to the basic amino acid residues constitutive of the different proteins. The binding of polyethyleneglycols to the nanoErythrosome membrane could be interesting for the therapeutic use of this delivery system since it could overcome heterologous immunogenicity and reduce rapid clearance from circulation. In the present study, we have investigated the effect of temperature on the nanoErythrosome behavior in the absence and presence of conjugated polyethyleneglycols. More specifically, Fourier transform infrared (FTIR) spectroscopy has been used to evaluate the lipid order and dynamics, the hydration and the degree of protein aggregation of the nanoErythrosomes after covalent binding of polyethyleneglycols having molecular weights of 2000 and 5000 g mol^{-1}. The results indicate that the nanoErythrosome lipid chain order is not significantly affected by heating the nanoErythrosomes at temperatures up to 50 °C. They also indicate that the nanoErythrosome proteins aggregate irreversibly at temperatures above 37 °C, this effect being abolished in the presence of polyethyleneglycols. The presence of polyethyleneglycols decreases the accessibility of water to the lipid head groups. On the other hand, ^{31}P-nuclear magnetic resonance (NMR) and electron microscopy results reveal that the presence of polyethyleneglycols prevents the aggregation of the nanoErythrosome structures.

Keywords: Drug delivery system; nanoErythrosome; Membrane; Polyethyleneglycol; ^{31}P-NMR; FTIR

1. Introduction

For many years, various types of particulate drug carriers have been investigated with the aim of perfecting drug delivery systems that will specifically localize drugs to their desired in vivo target tissue, thus minimizing their deleterious effects. The main goal of the present study is to characterize by Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopies a new drug delivery system called nanoErythrosome (nEryt), which offers great potential for a sustained release system as well as a target drug delivery system.

NanoErythrosomes are microspheres prepared by the extrusion of red blood cell ghosts [1–4]. These ghosts are essentially composed of the red blood cell membrane. Several extrusions of the red blood cell ghosts through filters of defined pore size led to the formation of nanoErythrosomes. About 4000 to 6000 nanoErythrosomes can theoretically be formed from a single red blood cell. These vesicles have an average diameter of 100 to 200 nm. They are buoyant, have a very high surface area to volume ratio (approximately 80-fold higher than that of the parent red blood cells) and remain in suspension for prolonged periods of time. They are naturally biodegradable vesicles without toxicity, are nonimmunogenic in autologous administration, and have a chemical composition that resembles that of the red blood cell membranes. More specifically, their membrane is composed of approximately 50% phospholipids, 25% protein and 25% cholesterol. This feature is of utmost

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importance when comparing these particles to other nanoparticle delivery systems such as liposomes because it allows a high degree of versatility both in the type of molecules that can be encapsulated within the nanoErythrosomes (carbohydrates, proteins, DNA, chromophores, drugs) and in the covalent coupling of various molecules to the nanoErythrosome surface (receptor ligands, antibodies, anchoring proteins, polyethyleneglycols). In addition, passive targeting can be more effective by taking advantage of the difference between normal and tumor vasculature. Normal blood vessels have fenestration of about 100 nm while blood vessels in solid cancer tumors are much more permeable with cutoff fenestration size of about 400–600 nm [5]. Hence, nanoparticles with a size intermediate between these two limits will be extravasated into the tumor tissues and endocytosis becomes advantageous to achieve intracellular delivery of the chemical content of the nanoErythrosome.

Polyethyleneglycol is a nontoxic, amphipatic and chemically inert synthetic polymer that has been grafted onto the surface of liposomes and proteins for the purpose of prolonging blood circulation [6–13]. More specifically, PEG–lipid conjugates have been synthesized and incorporated into liposomes to form a steric polymer surface barrier. The prolonged circulation of liposomes obtained with PEG surface coating has been shown to significantly enhance their therapeutic applications [8,9,14]. More specifically, among the important biological properties of PEG-grafted liposomes are favorable pharmacokinetics and tissue distribution [12,15,16] and the lack of toxicity and immunogenicity [17]. These liposomes persist in the blood, permitting extravasation into the tumor interstitial compartment and into infectious and inflammation sites. Thus, PEG-grafted liposome formulations can deliver encapsulated drugs to these pathological sites, as shown with doxorubicin treatment of tumors [15,18,19] and Kaposi's Sarcoma [20].

Since it is highly desirable to prolong the circulation time of nanoErythrosomes, it is of considerable interest to investigate the physicochemical properties of nanoErythrosomes with polyethyleneglycols grafted onto their membrane surface. In addition, polyethyleneglycols could mask antigens responsible for the immunological recognition of foreign red blood cells. This aims to the production of universally injectable nanoErythrosomes.

In the present study, PEGs with different molecular weights (2000 and 5000 g mol^{-1}) have been covalently linked to lysine residues present on the proteins of the nanoErythrosome membrane. More specifically, we have investigated the structure and dynamics of both the lipid and protein molecules of the nanoErythrosomes in the absence and in the presence of polyethyleneglycols using a combination of FTIR and ^{31}P solid-state NMR spectroscopies. FTIR is well suited to the study of biological membranes since it allows the investigation of the phospholipid molecules at different levels in the lipid bilayers [21,22] and it can also provide information on the secondary structure and aggregation of proteins [23–25]. On the other hand, the ^{31}P-NMR spectral line shapes are very characteristic of the phases formed by the lipid molecules, such as the gel and the liquid-crystalline lamellar phases, the inverted hexagonal phase and isotropic phases such as small vesicles and micelles [26,27].

2. Materials and methods

2.1. Materials

Horse blood was purchased from the St-Hyacinthe Veterinary School (St-Hyacinthe, QC). The salts used in the preparation of the buffers were of analytical grade and were purchased from Aldrich (Milwaukee, WI, USA). The methoxy PEG derivatives were synthesized as described before [9,28]. D_2O was purchased from CDN Isotopes (Pointe-Claire, QC).

2.2. Preparation of nanoErythrosomes

The method used to produce nanoErythrosomes in the initial small-scale laboratory experiments employed osmotic shock and centrifugation and was described elsewhere [1]. However, the time required and the inability to scale-up industrial production have necessitated the development of a different methodology. Various options were considered for compatibility with large-scale production, including osmotic shock with dialysis and osmotic shock with exclusion chromatography. The later was successfully assessed. Briefly, 500 ml of heparinized horse blood were centrifuged at 500×*g* for 15 min at 4 °C to remove the plasma and the buffy coat. The packed cells were resuspended in a saline Baxter solution 0.9% and centrifuged. After three successive resuspensions and centrifugations, the final supernatant was removed and the white ghosts were prepared by depleting the red blood cells of their hemoglobin content through a passage on a prototype size exclusion chromatography column under alkaline and hypotonic conditions. The white ghosts were then heated to 37 °C in a water bath and converted to nanoErythrosomes under nitrogen pressure extrusion through polycarbonate filters of defined pore sizes. NanoErythrosomes were obtained by six consecutive extrusions through a filter having pores of 1 μm in diameter (Integra or Nucleopore Corp., Plesanton, CA) and one through a 0.2 μm filter (Integra) placed in a Amicon apparatus (model 8400). The nanoErythrosome concentrations were determined by quantification of the protein content using the Bio-Rad protein assay kit (Bio-Rad, Mississauga, ON). The cholesterol content was also determined using the cholesterol assay kit (Randox Laboratories Ltd., Mississauga, ON) to know the cholesterol/protein ratio, which is necessary to evaluate the protein content after the grafting of polyethyleneglycol. The quality of each nanoErythrosome production was assessed by the incorpo-

ration of FITC–dextran in the nanoparticles and controlled under microscopical examination. All manipulations were performed under sterile conditions.

2.3. Coupling of polyethyleneglycol derivatives onto the nanoErythrosomes

The methoxy PEG derivatives were dissolved in a minimum amount of anhydrous dimethylsulfoxide (DMSO) and kept frozen at 4 °C prior to their coupling to nanoErythrosomes to avoid their hydrolysis. The coupling of polyethyleneglycols was achieved by the addition of the appropriate quantity of PEG to the nanoErythrosomes to yield approximately 50% pegylated nanoErythrosomes. These reactions were performed in 0.1 M sodium carbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH=10.0) followed by incubation at room temperature for 1 h. After pegylation, the unreacted PEG was removed by chromatography on a Sepharose gel CL6B (Amersham Pharmacia Biotech, Piscataway, NJ) using a citrate buffer (5 mM, pH 7.0) as the eluant. To determine the exact ratio of pegylation, the cholesterol content was determined using the cholesterol assay kit (Randox Laboratories). With this value and the ratio obtained for the pure nanoErythrosomes, it is possible to evaluate the protein concentration, which could not be determined by the Bio-Rad protein assay kit after pegylation.

The fluorescamine test was also used to determine the exact amount of free amino groups prior and after the pegylation step. The percentage of pegylation (%P) is calculated according to the following equation: $\%P=(1-(x/y))\times 100$, where x is the amount of amino groups (mmol) not pegylated and y is the total amount of amino groups (mmol) before the pegylation step [29]. The percentages of pegylation obtained were 59% and 71% for PEG-2000 and PEG-5000 derivatives, respectively.

2.4. Lyophilization and resuspension

The nanoErythrosomes were cooled down to −80 °C for 3 h prior to overnight lyophilization to give a stable and storable form. The appropriate amounts of nanoErythrosomes or PEG–nanoErythrosomes were then dispersed in D_2O (10% w/w for FTIR and 30% w/w for NMR measurements). The samples were vortexed to ensure a homogeneous suspension.

2.5. FTIR measurements

Infrared spectra were recorded with a Nicolet Magna 550 Fourier Transform spectrometer equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. Samples were inserted between BaF_2 windows (Wilmad Glass Co. Inc, Buena, NJ) using 13-μm Mylar spacers. Two hundred fifty interferograms were recorded with a resolution of 4 cm^{-1} and each spectrum was corrected for the water contribution by subtracting appropriate polynomial functions. The spectra in the amide and carbonyl regions were deconvolved using the Grams software (Galactic Industries, Salem, NH), which uses the deconvolution technique of Griffiths and Pariente [30] with narrowing parameters (γ) of 5.45 and 2.67 and apodization filters of 80.9% and 76%, respectively.

2.6. ^{31}P-NMR measurements

^{31}P-NMR spectra were acquired at 121.5 MHz on a Bruker ASX-300 (Bruker Canada Ltd., Milton, ON) operating at an ^{1}H frequency of 300.0 MHz. The experiments were carried out with a broadband/^{1}H dual frequency 4-mm probehead. The free induction decays (2 K data points) were recorded with a spin echo sequence (2000 or 4000 scans) with a 4 to 7 s repetition time and under conditions of proton decoupling. The ^{31}P 90° pulse length was typically 5 μs and the interpulsive delay was set to 30 μs to avoid anisotropic T_2 effects in the powder spectra. The temperature was controlled to within ±0.5 °C and the chemical shifts expressed in parts per millions (ppm) were referenced relative to the signal of phosphoric acid at 0 ppm.

2.7. Spectral simulations

The ^{31}P-NMR spectra obtained for the nanoErythrosomes in the absence and presence of polyethyleneglycols have been simulated using different proportions of powder spectra and isotropic peaks. Line broadening was used in the simulations in order to fit the experimental spectra. The quality of the fit was determined by visual comparison of the experimental and simulated spectra. The error on the calculated proportions of each phase is estimated to be of the order of ±2%.

2.8. Electron microscopy measurements

The morphology of the nanoErythrosomes in the absence and presence of PEG has been studied by negative staining electron microscopy. Small aliquots of the nanoErythrosome suspensions (6 μg/ml) were deposited onto a Formvar-Carbon coated grid and negatively stained with 1% uranyl acetate in water. The samples were observed under a Philips EM 208/20 transmission electron microscope at 80 kV.

3. Results and discussion

3.1. Infrared spectroscopy results

The conformational order of the lipid acyl chains of the nanoErythrosomes in the absence and in the presence of polyethyleneglycol has first been characterized by the investigation of the acyl chain C–H stretching mode region as a function of temperature. This spectral region is domi-

nated by two strong bands at 2920 and 2850 cm^{-1}, assigned to the methylene antisymmetric and symmetric stretching modes, respectively [21]. The two methylene bands exhibit the same behavior as the temperature is increased: they become broader and they shift to higher frequency due to the increase of conformational disorder in the acyl chains [22].

Fig. 1 presents the evolution of the frequency of the methylene symmetric stretching mode band of the lipid acyl chains as a function of temperature for the nanoErythrosomes in the absence (Fig. 1A) and presence of polyethyleneglycol (Fig. 1B). The data points represented by filled symbols have been obtained by increasing the temperature from 20 to 50 °C while the hollow points have been obtained by decreasing the temperature down to 20 °C. These results first indicate that the lipid chain order of the nanoErythrosomes is slightly decreased with increasing temperature up to 50 °C, as reflected by the increase in the frequency of the symmetric methylene stretching mode band. However, this behavior is completely reversible. This indicates that heating the sample up to 50 °C does not irreversibly modify the order of the lipid acyl chains in the nanoErythrosomes.

The results obtained for the nanoErythrosomes in the presence of polyethylenegycol (PEG 2000) are presented in Fig. 1B. For reasons of clarity, only the results obtained with

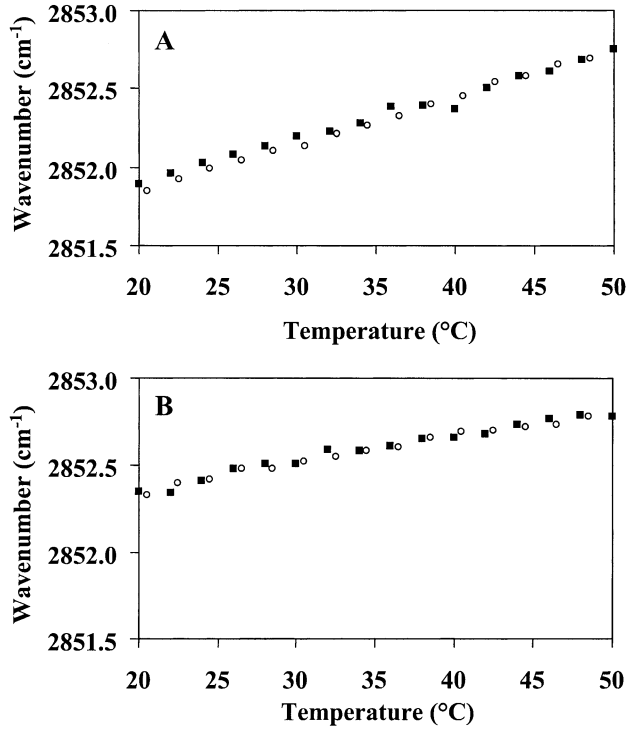

Fig. 1. Temperature dependence of the CH_2 symmetric stretching vibration of the lipids of pure nanoErythrosomes (A) and of the nanoErythrosomes in the presence of polyethyleneglycols (molecular weight of 2000 g mol^{-1}) (B). The data points represented by filled symbols have been obtained by increasing the temperature from 20 to 50 °C while the hollow points have been obtained by decreasing the temperature down to 20 °C.

PEG 2000 are shown in this figure but very similar results have also been obtained with the PEG 5000 sample. These results first indicate that as observed for the pure nanoErythrosome sample, the order of the lipid acyl chains is not irreversibly modified by heating the sample up to 50 °C. However, the comparison of the results presented in Fig. 1A,B indicates that the CH_2 methylene stretching frequencies obtained at low temperatures for the PEG–nanoErythrosome sample are slightly higher, indicating that the lipid acyl chains are slightly more disordered.

The interfacial region of the lipid bilayer of the nanoErythrosomes and the PEG–nanoErythrosomes has been investigated from the carbonyl stretching vibrations in the infrared spectra. These vibrations are sensitive to the degree of hydration of the lipid head group and to the formation of hydrogen bonds [32,33]. The deconvolved spectra are presented in Fig. 2A,B for the nanoErythrosomes in the absence and in the presence of polyethyleneglycol (PEG 2000), respectively. The spectra have been obtained at 20 and 50 °C, and after the temperature was decreased down to 20 °C. Two bands are clearly distinguishable after resolution enhancement by Fourier deconvolution [31]. It has been shown that the band at about 1742 cm^{-1} is attributed to the free lipid carbonyl groups while the band at about 1727 cm^{-1} is associated to hydrogen-bonded carbonyl groups [32,33].

The results presented in Fig. 2 first indicate that the frequencies of the two lipid carbonyl bands (1742 and 1727 cm^{-1}) obtained for the nanoErythrosomes in the absence and in the presence of PEG are not significantly affected by the presence of PEG. In addition, for the pure nanoErythrosome sample (Fig. 2A), we observe that the ratio between the intensity of the free carbonyl group component (1742 cm^{-1}) and the hydrogen-bonded carbonyl component (1727 cm^{-1}) decreases slightly with increasing temperature, going from 0.90 at 20 °C to 0.85 at 50 °C. This behavior can be explained by the fact that the hydrocarbon chains become more disordered at high temperature. This disorder facilitates the penetration of water in the interfacial region and thus favors hydrogen bonding between the solvent (D_2O) and the carbonyl groups. The proportion of bonded C=O to water molecules is therefore more important at high temperature. This behavior is, however, totally reversible when the temperature is decreased down to 20 °C.

For the nanoErythrosomes in the presence of polyethyleneglycols (Fig. 2B), the results indicate that the ratio of the intensity of the free carbonyl group component (1742 cm^{-1}) to the hydrogen-bonded carbonyl component (1727 cm^{-1}) is higher (0.94) than that observed for the pure nanoErythrosomes, which suggests that the presence of polyethyleneglycols at the surface of the nanoErythrosomes decreases the water accessibility to the carbonyl groups. In addition, these spectra do not vary significantly as a function of temperature, in agreement with the hypothesis that the presence of the polyethyleneglycol molecules at the surface of the nanoErythrosomes prevents the access of water to the carbonyl groups. It is also important to note that similar

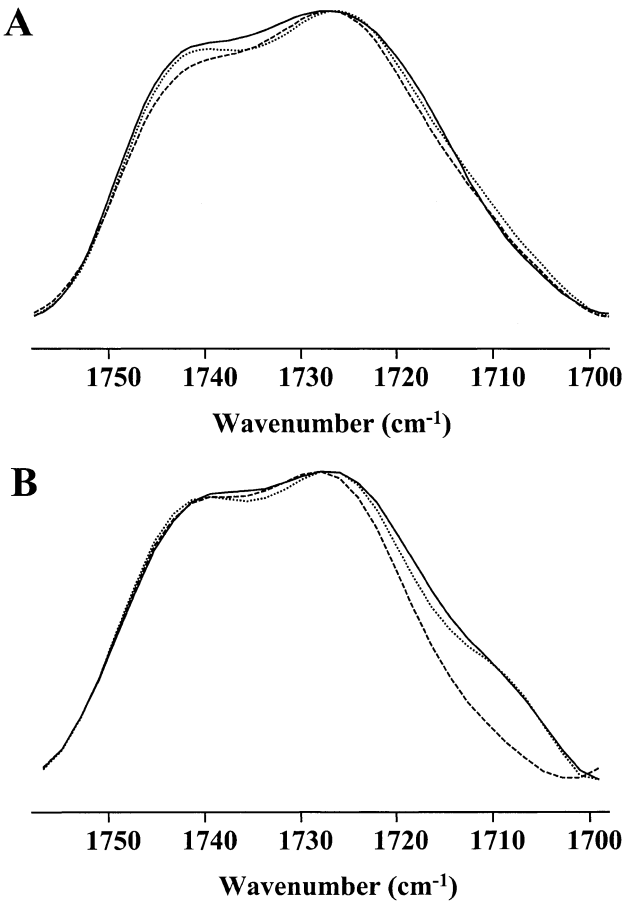

Fig. 2. Deconvolved infrared spectra in the lipid carbonyl stretching mode region for the nanoErythrosomes in the absence (A) and in the presence of polyethyleneglycols (molecular weight of 2000 g mol^{-1}) (B). The spectra have been recorded at 20 °C (—), 50 °C (---) and back to 20 °C (· · ·).

results have been obtained for PEG with molecular weights of both 2000 and 5000 g mol^{-1}.

It has been shown that the PEG 2000 molecule, which has a degree of polymerization of 46, binds approximately 142 molecules of water. This calculation, based on volumetric studies, is in good agreement with DSC results [34]. In addition, the hydration number of a PEG molecule depends on the configuration of the PEG. This has been demonstrated by DSC measurement for free PEG (in the random coil configuration) compared with PEG covalently attached to the headgroup of a phospholipid (in the brush configuration). These results indicate that in the brush configuration, PEG is more accessible to water, binding approximately 30% more water than PEG in the random coil configuration [34]. These results are in agreement with the decreased water accessibility of the lipid carbonyl groups observed in the present study for the nanoErythrosomes in the presence of polyethyleneglycol.

The amide I region of the spectra obtained for the nanoErythrosomes as a function of temperature has also been investigated to monitor the protein thermal stability. The destabilization of protein tertiary structures as a function of temperature has been widely investigated by infrared spectroscopy [23–25]. More specifically, the spectral changes in the amide I region have been monitored for different proteins as a function of temperature. At high temperature, a well-defined amide I band at a wavenumber of about 1616 cm^{-1} has been observed for all the proteins investigated. This band is highly characteristic of thermally denatured proteins and represents hydrogen-bonded extended structures, which are formed upon aggregation of the thermally unfolded proteins.

The deconvolved infrared spectra in the amide I region obtained as a function of temperature for the pure nanoErythrosome sample are shown in Fig. 3A. These spectra show the presence of different secondary structure elements in the nanoErythrosome proteins. More specifically, the spectrum obtained at 20 °C reveals the presence of four bands at 1677, 1656, 1637 and 1616 cm^{-1} with relative intensities of approximately 2%, 54%, 43% and 1%, respectively. The small band at 1677 cm^{-1} is assigned to the β-turn conformation and the band at 1656 cm^{-1} is assigned to

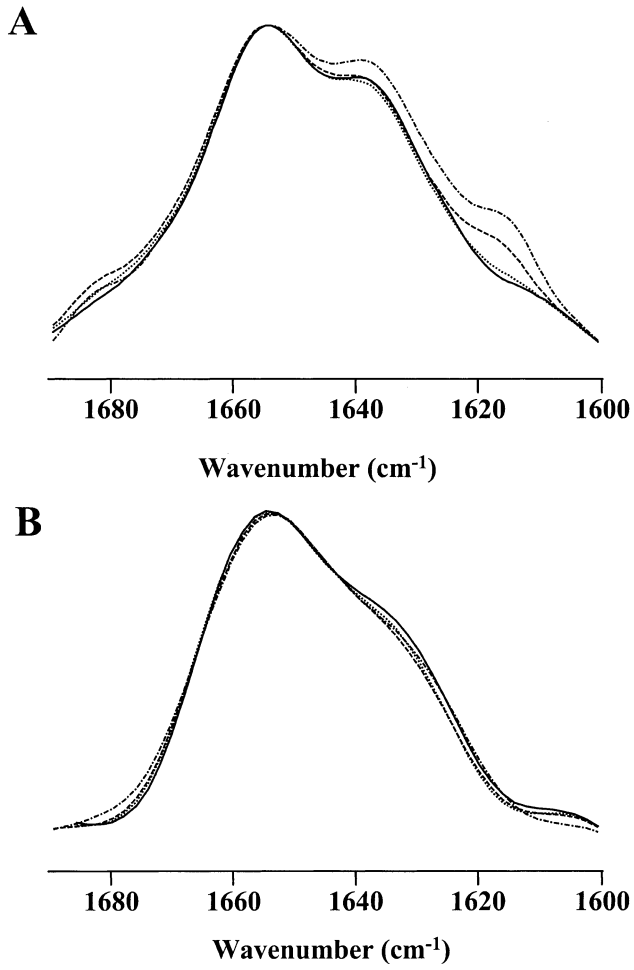

Fig. 3. Deconvolved infrared spectra in the amide I′ region for the nanoErythrosomes in the absence (A) and in the presence of polyethyleneglycols (molecular weight of 2000 g mol^{-1}) (B). The spectra have been recorded at 20 °C (—), 40 °C (· · ·), 50 °C (---) and back to 20 °C (-·-·-·).

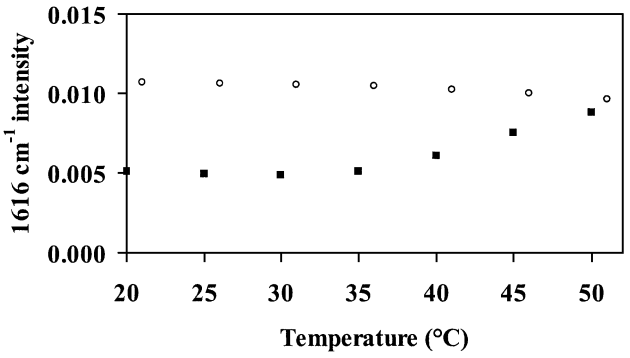

Fig. 4. Normalized intensity of the 1616 cm^{-1} bad as a function of temperature for the pure nanoErythrosomes. The data points represented by filled symbols have been obtained by increasing the temperature from 20 to 50 °C while the hollow points have been obtained by decreasing the temperature down to 20 °C.

the α-helical conformation although the spectral contribution from the unordered conformation cannot be completely ruled out at this frequency [35–37]. In addition, the 1637 cm^{-1} feature is highly characteristic of amide groups involved in the extended β-sheet structure while the low-frequency amide I band at 1616 cm^{-1} has been associated with the presence of intermolecular β-sheet networks, as discussed above. These spectra clearly indicate the increase of the intensity of the band at 1616 cm^{-1} at temperatures above about 37 °C, attributed to the formation of hydrogen-bonded extended structures and therefore to protein aggregation. The normalized intensity of this band (which represents the intensity of the 1616 cm^{-1} component divided by the total area of the amide I band) is plotted as a function of temperature in Fig. 4. These results clearly indicate that the protein thermal denaturation is not reversible and that the intensity of the band stays nearly constant when the temperature is brought down from 50 to 20 °C.

Fig. 3B shows the amide I band obtained for the PEG–nanoErythrosome sample (PEG 2000) as a function of temperature. These spectra reveal bands at 1656 and 1635 cm^{-1}, with relative intensity of 56% and 44%, respectively. These bands are also much broader than those obtained in the absence of polyethyleneglycols. These results first suggest that the presence of polyethyleneglycols does not significantly affect the secondary structure of the nanoErythrosome proteins but induces a slight increase in their disorder. It is also really interesting to note in these spectra that the presence of the polyethyleneglycol molecules at the surface of the nanoErythrosomes completely prevents the aggregation of the proteins since no band characteristic of protein aggregation at a wavenumber of about 1616 cm^{-1} is observed in the spectra with increasing temperature. Similar results have been obtained for polyethyleneglycols with molecular weights of 2000 and 5000 g mol^{-1}. These results are in agreement with the fact that the attachment of PEG molecules to the surface of liposomes has been shown to both strongly reduces the attractive forces and increases the repulsive ones (steric and hydration) between liposomes to each other and to cells.

3.2. NMR spectroscopy and electron microscopy results

Solid-state ^{31}P-NMR spectroscopy is a valuable technique to study the lipid head groups as well as the different phases formed by phospholipid membranes. The ^{31}P-NMR line shapes are very characteristic of the different phases such as the gel and liquid-crystalline lamellar phases, the inverted hexagonal phase and isotropic phases such as small vesicles, micelles and cubic phases [26,27].

We have therefore used ^{31}P-NMR to investigate the structure of the pure nanoErythrosomes as well as the effect of the addition of PEG molecules at the surface of the nanoErythrosomes. The ^{31}P-NMR spectra obtained for the nanoErythrosomes in the absence and in the presence of polyethyleneglycols (PEG 5000) are shown in Fig. 5A,B at 20 and 50 °C, respectively. Three main features are present

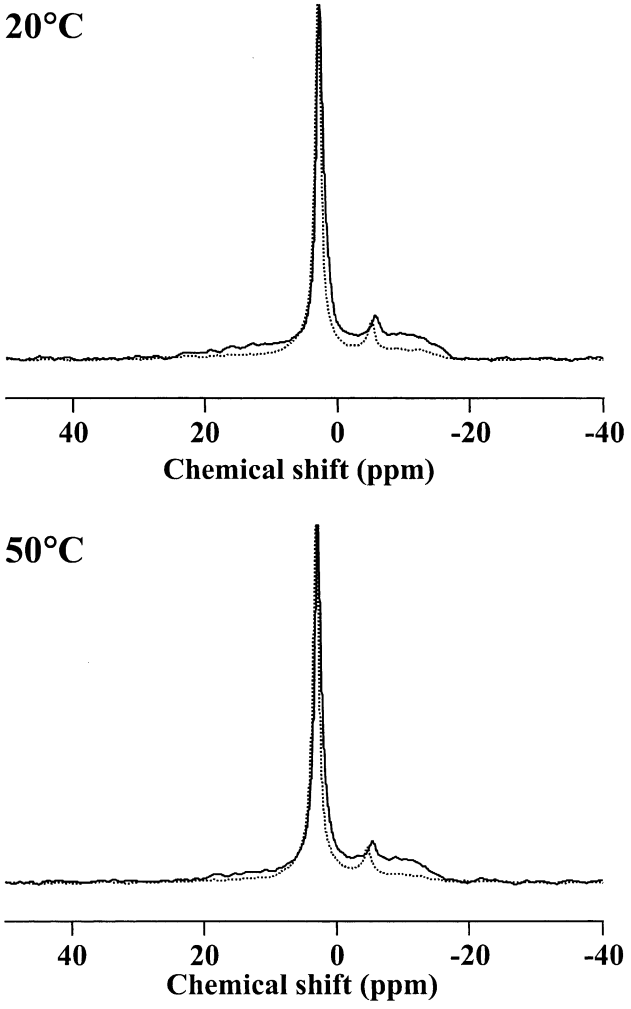

Fig. 5. ^{31}P-NMR spectra obtained at 20 and 50 °C for the nanoErythrosomes in the absence (—) and in the presence (···) of polyethyleneglycols (molecular weight of 5000 g mol^{-1}).

Table 1
Proportion of the different lipid phases for the nanoErythrosomes in the absence and presence of polyethyleneglycols with molecular weights of 2000 and 5000 g mol^{-1}

Sample	Temperature (°C)	Isotropic peak (2.8 ppm)	Isotropic peak (−5.6 ppm)	Lamellar phase spectrum
Pure nEryt	20	57%	7%	36%
	50	62%	6%	32%
nEryt–PEG 2000	20	77%	8%	15%
	50	82%	7%	11%
nEryt–PEG 5000	20	77%	9%	14%
	50	79%	5%	16%

The error on the proportions of each phase is estimated to ±2%.

in these spectra: an important isotropic peak centered at about 2.8 ppm, a smaller isotropic peak centered at about −5.6 ppm and a broad lamellar phase spectrum. On one hand, the peak at 2.8 ppm can be attributed to the phospholipid head groups in the nanoErythrosome membranes while the small peak at −5.6 ppm is most likely due to the presence of residual nucleotides in the samples. On the other hand, the broad lamellar phase spectrum can either be attributed to large vesicular structures or to the aggregation of small nanoErythrosome structures. In both cases, the tumbling of the large and/or aggregated structures would not be fast enough to average the ^{31}P chemical shift anisotropy [26,27]. A small shift towards higher chemical shift values (by about 1.5 ppm) is also observed for the two isotropic peaks in the presence of polyethyleneglycols. This behavior is in agreement with a deshielding of the lipid phosphate head group by the presence of the hydrophilic polyethyleneglycol molecules at the surface of the nanoErythrosomes.

The comparison of the spectra obtained at 20 and 50 °C indicates that the relative proportions of these different phases do not change significantly as a function of temperature. However, significant differences can be observed between the spectra of the nanoErythrosomes in the absence and in the presence of polyethyleneglycols. To determine the proportion of each phase, spectral simulations have been performed by varying the amounts of isotropic and lamellar spectra and the results of these simulations are presented in Table 1. These results first indicate that the proportion of the lamellar phase spectrum is significantly reduced upon the addition of PEG at the surface of the nanoErythrosomes, with proportions of about 34±2% for the pure nanoErythrosomes and about 14±3% in the presence of polyethyleneglycols with molecular weights of both 2000 and 5000 g mol^{-1}. On the other hand, the proportion of the smaller isotropic peak at about −5.6 ppm is not significantly affected by the presence of polyethyleneglycols.

The decrease in the proportion of the lamellar phase spectrum suggests a decrease in the size and/or aggregation of the nanoErythrosome structures in the presence of polyethyleneglycols. To verify this hypothesis, electron microscopy measurements have been performed on the nanoErythrosomes in the absence and presence of polyethyleneglycols and the results are shown in Fig. 6. These results clearly confirm the presence of aggregated structures for the pure nanoErythrosome system (Fig. 6A). In addition, the results indicate that the addition of PEG drastically reduces the aggregation of the nanoErythrosome structures, as shown in Fig. 6B. This observation is in agreement with the ^{31}P-NMR results, which indicated a significant decrease in the proportion of the lamellar phase spectrum in the presence of polyethyleneglycols. The electron microscopy results also indicate that the average size of the nanoErythrosomes is not significantly affected by the presence of polyethyleneglycols.

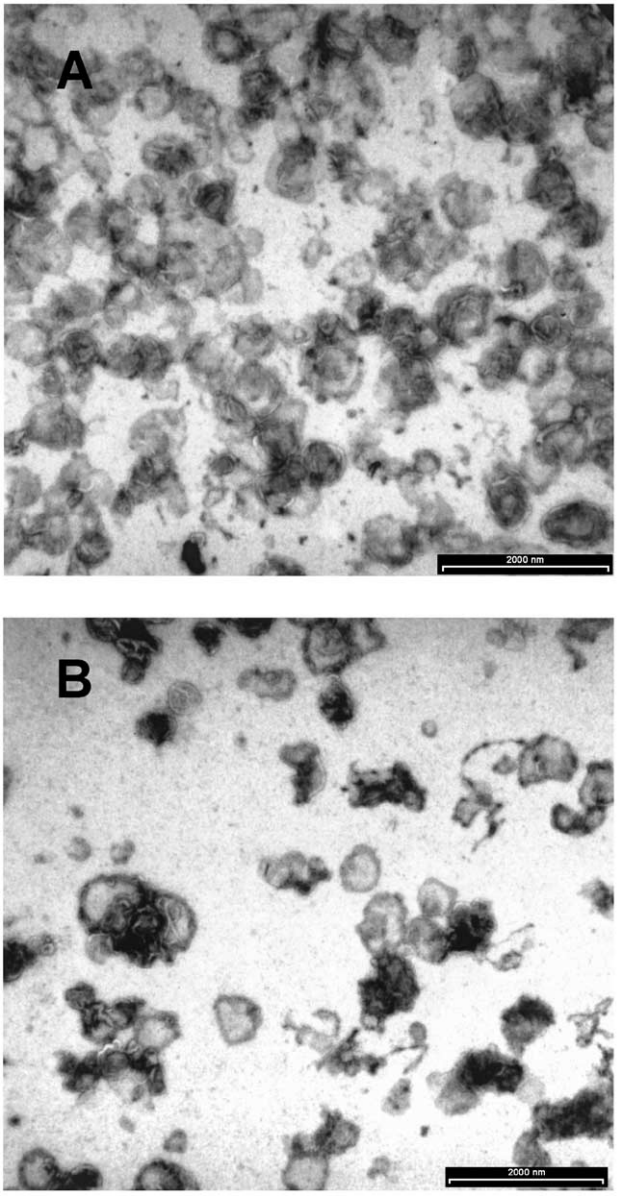

Fig. 6. Electron micrographs of negatively stained suspensions of nanoErythrosomes in the absence (A) and the presence (B) of polyethyleneglycols. Bar = 2 μm.

4. Conclusions

The results obtained in the present study using a combination of infrared spectroscopy, ^{31}P-NMR spectroscopy and electron microscopy indicate that the grafting of polyethyleneglycol molecules at the surface of nanoErythrosomes significantly changes their aggregation properties. More specifically, a smaller proportion of lamellar phase has been observed in the ^{31}P-NMR spectra of the nanoErythrosomes in the presence of polyethyleneglycols, suggesting a decrease in the aggregation of the nanoErythrosome structures. These results have been confirmed from the electron micrographs of the nanoErythrosomes in the absence and presence of polyethyleneglycols. On the other hand, no evidence of protein aggregation has been observed by heating the PEG containing nanoErythrosomes at temperatures up to 50 °C while significant and irreversible protein aggregation occurs in the pure nanoErythrosome samples when these samples are heated at temperatures above about 37 °C. Finally, the presence of polyethyleneglycols slightly decreases the order of the lipid acyl chains and proteins in the nanoErythrosomes and decreases the water accessibility to the lipid interfacial region.

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References

[1] A. Lejeune, M. Moorjani, C. Gicquaud, J. Lacroix, P. Poyet, R.C. -Gaudreault, Anticancer Res. 14 (1994) 915–920.
[2] M. Moorjani, A. Lejeune, C. Gicquaud, J. Lacroix, P. Poyet, R.C. -Gaudreault, Anticancer Res. 16 (1996) 2831–2836.
[3] A. Lejeune, P. Poyet, R.C. -Gaudreault, C. Gicquaud, Anticancer Res. 17 (1997) 3599–3604.
[4] J. Désilets, A. Lejeune, J. Mercer, C. Gicquaud, Anticancer Res. 21 (2001) 1741–1747.
[5] F. Yuan, M. Leunig, S.K. Huang, D.A. Berk, D. Papahadjopoulos, R.K. Jain, Cancer Res. 54 (1994) 3352–3356.
[6] D.D. Lasic, F.J. Martin, A. Gabizon, S.K. Huang, D. Papahadjopoulos, Biochim. Biophys. Acta 1070 (1991) 187–192.
[7] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Biochim. Biophys. Acta 1066 (1991) 29–36.
[8] M.C. Woodle, K.K. Matthay, M.S. Newman, J.E. Hidayat, L.R. Collins, C. Redemann, F.J. Martin, D. Papahadjopoulos, Biochim. Biophys. Acta 1105 (1992) 193–200.
[9] M.C. Woodle, D.D. Lasic, Biochim. Biophys. Acta 1113 (1992) 171–199.
[10] M.C. Woodle, G. Storm, M.S. Newman, J.J. Jekot, L.R. Collins, F.J. Martin, F.C. Szoka Jr., Pharm. Res. 9 (1992) 260–265.
[11] M.C. Woodle, Chem. Phys. Lipids 64 (1993) 249–262.
[12] M.C. Woodle, M.S. Newman, J.A. Cohen, J. Drug Target. 2 (1994) 397–403.
[13] M.C. Woodle, Adv. Drug Deliv. Rev. 32 (1998) 139–152.
[14] G.E. Francis, C. Delgado, D. Fisher, F. Malik, A.K. Agrawal, J. Drug Target. 3 (1996) 321–340.
[15] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11460–11464.
[16] S.K. Huang, E. Mayhew, S. Gilani, D.D. Lasic, F.J. Martin, D. Papahadjopoulos, Cancer Res. 52 (1992) 6774–6781.
[17] K.R. Reddy, Ann. Pharmacother. 34 (2000) 915–923.
[18] M.H. Gaber, N.Z. Wu, K. Hong, S.K. Huang, M.W. Dewhirst, D. Papahadjopoulos, Int. J. Radiat. Oncol. Biol. Phys. 36 (1996) 1177–1187.
[19] D.D. Lasic, Nature 380 (1996) 561–562.
[20] S.K. Huang, F.J. Martin, G. Jay, J. Vogel, D. Papahadjopoulos, D.S. Friend, Am. J. Pathol. 143 (1993) 10–14.
[21] H.L. Casal, H.H. Mantsch, Biochim. Biophys. Acta 779 (1984) 381–401.
[22] I.M. Asher, I.W. Levin, Biochim. Biophys. Acta 468 (1977) 63–72.
[23] W.K. Surewicz, J.J. Leddy, H.H. Mantsch, Biochemistry 29 (1990) 8106–8111.
[24] A. Muga, H.H. Mantsch, W.K. Surewicz, Biochemistry 30 (1991) 7219–7224.
[25] M.-J. Paquet, M. Laviolette, M. Pézolet, M. Auger, Biophys. J. (2001) 305–312.
[26] J. Seelig, Biochim. Biophys. Acta 515 (1978) 105–140.
[27] I.C.P. Smith, I.H. Ekiel, in: D. Gorenstein (Ed.), Phosphorus-31 NMR. Principles and Applications, Academic Press, London, 1984, pp. 447–475.
[28] F.M. Veronese, P. Caliceti, A. Pastorino, O. Schiavon, L. Sartore, L. Banci, L. Monsu Scolaro, J. Control. Release 10 (1989) 145–154.
[29] L.J. Karr, D.L. Donnelly, A. Kozlowski, J.M. Harris, Methods Enzymol. 228 (1994) 377–390.
[30] P.R. Griffiths, G.L. Pariente, Trends Anal. Chem. 5 (1986) 209–215.
[31] J.K. Kauppinen, D.J. Moffatt, H.H. Mantsch, Appl. Spectrosc. 35 (1981) 271–276.
[32] A. Blume, W. Hübner, G. Messner, Biochemistry 27 (1988) 8239–8249.
[33] W. Hübner, H.H. Mantsch, F. Paltauf, H. Hauser, Biochemistry 33 (1994) 320–326.
[34] O. Tirosh, Y. Barenholz, J. Katzhendler, A. Priev, Biophys. J. 74 (1998) 1371–1379.
[35] D.M. Byler, H. Susi, Biopolymers 25 (1986) 469–487.
[36] W.K. Surewicz, H.H. Mantsch, Biochim. Biophys. Acta 952 (1988) 115–130.
[37] E. Goormaghtigh, V. Cabiaux, J.-M. Ruysschaert, in: H.J. Hilderson, G.B. Ralston (Eds.), Subcellular Biochemistry, vol. 23, Plenum, New York, 1994, pp. 329–362.