Preparation and In Vitro/In Vivo Evaluation of Luteinizing Hormone Releasing Hormone (LHRH)-Loaded Polyhedral and Spherical/Tubular Niosomes

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Received July 15, 1998. Accepted for publication October 6, 1998.

Abstract □ Niosomes are vesicles formed by the self-assembly of nonionic surfactants in aqueous dispersions. They can entrap drugs and have been used experimentally as sustained drug delivery systems. Apart from conventional spherical niosomes, various types of vesicle ultrastructures can be formed by varying the composition of the vesicle membrane. Hexadecyl diglycerol ether ($C_{16}G_2$), cholesterol, and poly-24-oxyethylene cholesteryl ether (Solulan C24) in the ratio 91:0:9 gave polyhedral niosomes, whereas spherical and tubular niosomes are produced at a composition ratio of 49:49:2. The mean size of both polyhedral and spherical/tubular niosomes were within the range of 6 to 9 μm. Both types of vesicle were visualized by cryo-scanning electron microscopy. The properties of the two forms of niosomes were studied using luteinizing hormone releasing hormone (LHRH) as a model peptide. Analysis by high-performance liquid chromatography demonstrated high entrapment of LHRH acetate in polyhedral niosomes when prepared by remote loading methods using pH or $(NH_4)_2SO_4$ gradients; in contrast, only low entrapment was achieved by passive loading methods (direct hydration at pH 7.4 or pH 3.0, dehydration–rehydration, and reversed-phase evaporation). In vitro studies demonstrated that both polyhedral and spherical/tubular niosomes were more stable in 5% rat skeletal muscle homogenate than in rat plasma. Also, polyhedral niosomes released more radiolabeled LHRH ([^{125}I]LHRH) than spherical/tubular niosomes in both muscle homogenate and plasma. In clearance experiments in the rat, following intramuscular injection, both polyhedral and spherical/tubular niosomes gradually released [^{125}I]LHRH into the blood, but some radioactivity remained at the injection site for 25 and 49 h, respectively. In contrast, [^{125}I]LHRH in phosphate buffered saline was completely cleared from the injection site at 2 h. The release of drug is sustained by both niosome formulations, but spherical/tubular niosomes possess more stable membranes than polyhedral niosomes due to the presence of cholesterol.

Introduction

Niosomes, analogues of liposomes, are nonphospholipid vesicles formed by the self-assembly of nonionic surfactants in aqueous dispersion and have been used experimentally as drug carriers.[1,2] Apart from conventional spherical vesicles, a range of niosome structures can be formed by varying the membrane composition of the vesicles. The properties of polyhedral, and spherical/tubular niosomes, formed by hexadecyl diglycerol ether ($C_{16}G_2$) cholesterol: poly-24-oxyethylene cholesteryl ether (Solulan C24) with molar ratios of 91:0:9 and 49:49:2, respectively, have been compared previously in relation to differences in ultrastructural morphology and membrane composition.[3,4] The irregular faceted shape of polyhedral niosomes results in a dispersion with a higher viscosity than that formed by their spherical/tubular counterparts.[3] Although polyhedral niosomes are less osmotically responsive in terms of size change in the presence of osmotic gradients, due to their rigid gel state membrane[5] and low curvature, they possess low cholesterol membranes that are very permeable to hydrophilic solutes such as carboxyfluorescein (CF).[4] However, we have found that the co-entrapment of sodium chloride with CF in polyhedral niosomes resulted in a very low CF release rate, regardless of the osmotic conditions.[4] It is believed that the dehydration of the polyoxyethylene chains of Solulan C24 in the membranes of polyhedral niosomes is responsible for this property.

In the present investigation we have studied the morphology of polyhedral and spherical/tubular niosomes using cryo-scanning electron microscopy. Luteinizing hormone releasing hormone (LHRH) was selected as a model peptide drug to investigate the possibility of using polyhedral and spherical/tubular niosomes and polyhedral niosomes prepared in the presence of NaCl as sustained drug delivery systems. A variety of preparation techniques were studied in an attempt to obtain a high level of LHRH entrapment in the vesicles.

Experimental Section

Materials—Hexadecyl diglycerol ether ($C_{16}G_2$) was a gift from L'Oreal (Paris, France), and poly-24-oxyethylene cholesteryl ether (Solulan C24) was donated by Ellis and Everald Anstead International (Essex, UK). Luteinizing hormone releasing hormone (LHRH) acetate salt, radiolabeled LHRH ([^{125}I]LHRH), and cholesterol were obtained from Sigma Chemical Company (Poole, UK). Sodium chloride, diethyl ether, and ammonium sulfate were supplied by BDH Laboratory Supplies (Poole, UK). Chloroform (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, UK). The water source was from an ultrahigh quality reverse osmosis water purifier (Elgastat UHQPS, Elga Ltd., Bucks, UK).

Methods—*Preparation of LHRH Acetate-Loaded Polyhedral Niosomes*—Six methods of preparation of LHRH loaded polyhedral niosomes, formed from $C_{16}G_2$:Solulan C24 (91:9) (60 μmol) and LHRH acetate (2 mg/mL), were investigated ($n = 3$).

Method A was direct hydration (hand-shaking) at pH 7.4. Dried surfactant film was hydrated with LHRH acetate prepared in pH 7.4 phosphate buffered saline (PBS) by mechanical shaking at 55 °C for 30 min.

Method B was direct hydration at pH 3.0. Dried surfactant film was hydrated with LHRH acetate prepared in pH 3.0 PBS at 55 °C for 30 min.

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10.1021/js980286u CCC: $18.00 *Published on Web 12/05/1998*

Method C was dehydration−rehydration of vesicles (DRV). Surfactant film was hydrated with 5 mL of water at 55 °C for 30 min before adding of 1 mL of LHRH acetate in water. The niosome dispersion was then freeze-dried overnight and rehydrated with 1 mL of pH 7.4 PBS.

Method D was reversed-phase evaporation of vesicles (REV). Surfactants were dissolved in 10 mL of $CHCl_3$:diethyl ether (1:1). Then, 1 mL of LHRH acetate in pH 7.4 PBS was injected into the dispersion, which was then bath sonicated for 2 min. Finally, the organic solvents were vacuum evaporated.

Method E was remote loading by $(NH_4)_2SO_4$ gradient. Surfactant film was hydrated with 1 mL of 120 mM $(NH_4)_2SO_4$ at 55 °C for 15 min. The dispersion was ultracentrifuged at 4 °C, and the pellets were redispersed with LHRH acetate in pH 7.4 PBS and shaken gently at 60 °C for 15 min.

Method F was remote loading by pH gradient. Dried surfactant film was hydrated with 1 mL of pH 3.0 PBS at 55 °C for 30 min. The dispersion was ultracentrifuged at 4 °C, and the pellets were redispersed with LHRH acetate in pH 7.4 PBS and shaken gently at 60 °C for 15 min.

The dispersions were washed twice with PBS pH 7.4 (or at pH 3.0) by ultracentrifugation at 4 °C. The entrapped LHRH acetate was analyzed by reversed-phase HPLC (μBondapak C18, 3.9 × 150 mm; mobile phase: 0.03 M CH_3COONH_4 :ACN, 70:30, flow rate, 0.6 mL/min, detection wavelength, 279 nm).

For the in vitro/in vivo evaluation, polyhedral and spherical/tubular niosomes prepared from $C_{16}G_2$:cholesterol: Solulan C24 (91:0:9) and (49:49:2), respectively, were loaded with [^{125}I]LHRH (1.67 μCi/mL in pH 7.4 PBS) using the pH gradient method described in Method F. To study the effect of NaCl, phosphate buffer at pH 7.4 prepared in 2 M NaCl was used instead of pH 7.4 PBS, except when the washed niosomes were redispersed prior to injection. Niosomes were sized using a laser diffraction method (MasterSizer X, Malvern Instruments Ltd., Worcestershire, UK).

Cryo-scanning Electron Microscopy—Polyhedral and spherical/tubular niosomes, prepared in water, were plunge frozen in liquid nitrogen slush and transferred to a Philips XL20 scanning electron microscope (SEM), and the surface moisture was removed by sublimation (at −40 °C for 10 min). Samples were gold coated at −180 °C and photographed by SEM.

In Vitro Evaluation—*Animal Husbandry*—Male Wistar rats weighting 200 g (Bantin and Kingman Universal Ltd., Aldbrough, Hull, UK), were given diet (Rat and Mouse No. 1, SDS Ltd, Witham, Essex, UK) and drinking water ad libitum. A temperature of 20−22 °C was maintained with a relative humidity of 45 to 65% and a 12:12 h light:dark cycle (lights on at 07.00 h). Animals were acclimatized for at least 5 days before each experiment and were observed daily for clinical signs of illness.

Preparation of Plasma—Rats were killed by the intraperitoneal (ip) injection of pentobarbitone sodium (Sagatal, Rhône Mérieux Ltd., Harlow, Essex, UK). Blood was removed from the abdominal aorta and anticoagulated with lithium heparin, and the plasma was removed after centrifugation and stored at −30 °C.

Preparation of 5% Muscle Homogenate—Rats were sacrificed as already described. Skeletal muscles were removed from the posterior and anterior thighs, weighed, and homogenized with 19 parts of PBS, and aliquots were stored at −30 °C.

Evaluation of Niosomes In Vitro—A 0.1-mL sample of each niosome dispersion was mixed with 0.9 mL of rat plasma or 0.9 mL of 5% muscle homogenate in a glass vial, which was then incubated at 37 °C in a thermostated water bath (Grant Instruments, Cambridge Ltd., Cambridge, UK). Then, 0.1-mL aliquots were collected at 30 min, and 2, 5, and 9 h and mixed with 1.9 mL of PBS before being ultracentrifuged. Radioactivity in 1 mL of supernatant was counted (1275 Minigamma Gamma Counter, LKB Wallac, Turku, Finland), and the count/min in 2 mL was calculated as % LHRH released by comparison with the initial count/min of 0.01 mL of niosome dispersion.

In Vivo Evaluation—Male Wistar rats, 190 to 230 g, were injected intramuscularly (im; 25 GA 5/8 needle; right posterior thigh) with 0.1 mL of [^{125}I]LHRH prepared in pH 7.4 PBS, or spherical/tubular niosomes, polyhedral niosomes, or polyhedral niosomes prepared with a high concentration of NaCl. At 10 min, and 2, 7, 25, 32, and 49 h after injection, animals ($n = 2$) were weighed and killed by ip injection of pentobarbitone sodium, and blood was collected into lithium heparin. The radioactivity remaining at the injection site was determined by removing the injected leg and detaching the thigh muscles. The radioactivity in whole blood (2 mL) and muscle was counted using the gamma counter. The [^{125}I]LHRH in the total blood volume at each time point was calculated, based on the formula of total blood volume being 6 mL/100 g body weight in the mature male rat, and the count of radioactivity in 0.1 mL of [^{125}I]LHRH solution or niosome dispersion.

Results

Cryo-scanning Electron Microscopy—Cryo-scanning electron micrographs confirmed that niosomes formed by varying the amount of $C_{16}G_2$, cholesterol, and Solulan C24 resulted in a variety of vesicle structures (Figure 1). Polyhedral niosomes were formed in the absence of cholesterol whereas conventional spherical vesicles and also tubules were produced in the lipid/surfactant mixtures prepared with an equimolar level of cholesterol, confirming our previous findings.[3,4]

Effects of Preparation Methods on Entrapment Efficacy—Figure 2 shows that only a small amount of LHRH acetate could be entrapped into polyhedral niosomes using passive loading methods (i.e., direct hydration at pH 7.4 and 3.0, and DRV and REV) in comparison with higher levels of entrapment obtained by active loading methods [i.e., $(NH_4)_2SO_4$ gradient and pH gradient]. Calculated on the basis of available LHRH (2 mg), the entrapment levels obtained by passive loading methods were in the range 14−24%. The $(NH_4)_2SO_4$ gradient and pH gradient methods led to entrapments of 44% and 51%, respectively; these figures correspond to ratios of 12.4 and 14.5 nmol of entrapped LHRH/μmol of surfactants, respectively.

In Vitro Stability Study—As shown in Figure 3, polyhedral vesicles release more LHRH in both plasma and muscle homogenate than do spherical/tubular systems. Muscle homogenates are perhaps a better model for im formulations. Both formulations release less LHRH in these homogenates than in plasma.

In Vivo Clearance from Intramuscular Site—All niosomes used in vivo were unsonicated. The mean sizes were in the range of 6 to 9 μm, which was deemed most appropriate for im depot formulation. From Figure 4, it is seen that 99% of [^{125}I]LHRH in PBS was cleared from the site of injection in the first 2 h, whereas 14% and 70% of injected [^{125}I]LHRH prepared in polyhedral and spherical/tubular niosomes, respectively, was still present at the injection site. All the [^{125}I]LHRH in polyhedral niosomes was cleared from the injection site by 25 h, whereas some (8.5%) of the radioactivity in spherical/tubular niosomes could still be detected.

Figure 5 shows the level of [^{125}I]LHRH in the blood over the 49-h period after dosing. Unformulated [^{125}I]LHRH disappears from the injection site into the blood very rapidly, with a t_{max} of 12 min post dosing. Niosome preparations sustain the release of drug into the blood and prolong blood levels. Polyhedral niosomes exhibited a t_{max} of 2 h. Spherical/tubular niosomes provide a better sustained release system, with a t_{max} of 7 h post dosing. These results closely parallel the clearance data.

Co-entrapment of NaCl with [^{125}I]LHRH in polyhedral niosomes, which reduces the permeability of the bilayers,[4] was found not to change either the release pattern of the drug from the injection site (Figure 4), or the level of [^{125}I]-LHRH in the blood (Figure 5).

Discussion

We have previously reported the entrapment of hydrophilic solutes in $C_{16}G_2$ niosomes and described the morphology of these vesicle using fluorescence light microscopy and confocal laser scanning microscopy.[6,7] In this paper,

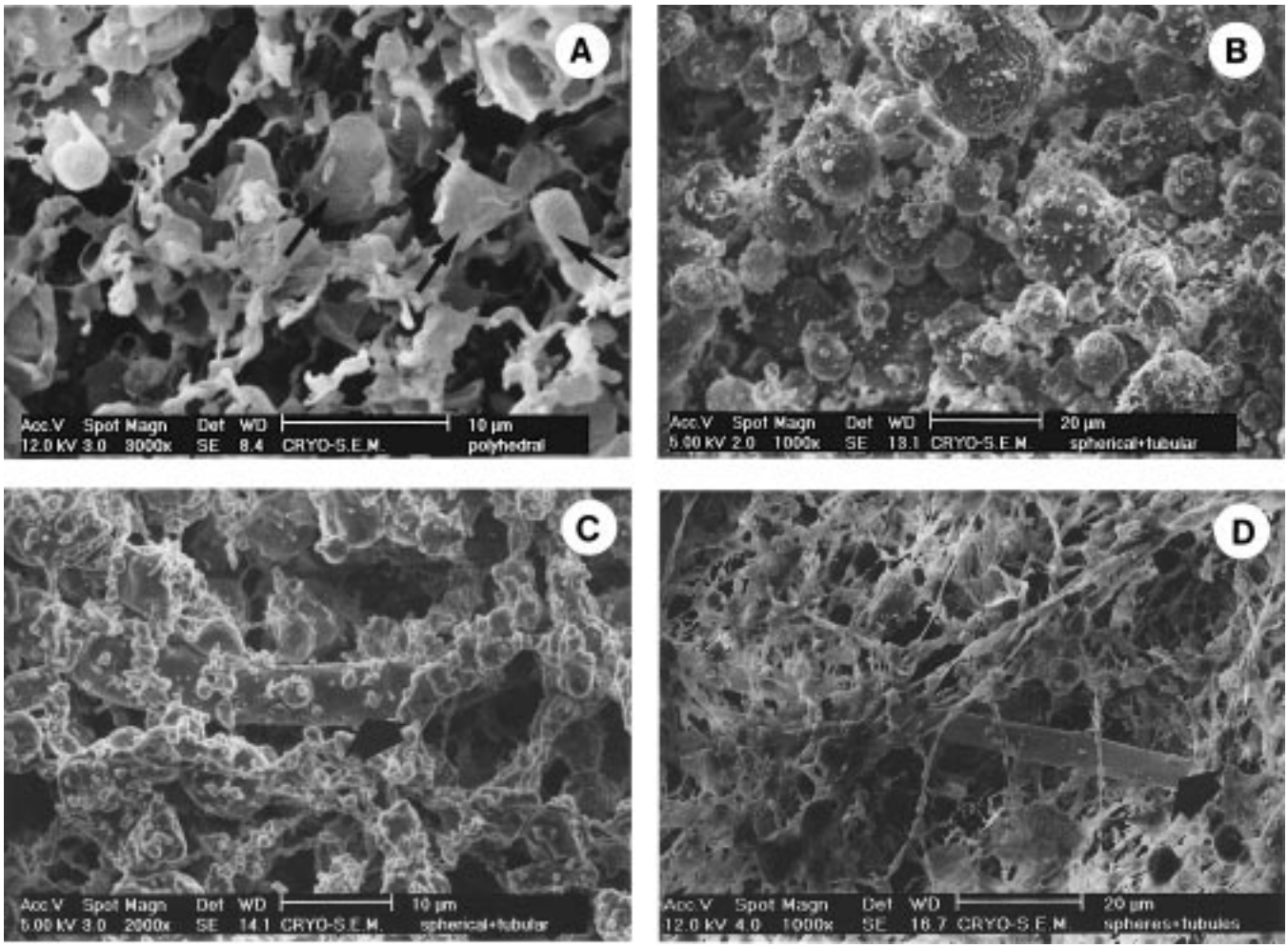

Figure 1—Cryo-scanning electron micrographs of (A) polyhedral niosomes (→) formed by $C_{16}G_2$:Solulan C24 (91:9), (B) spherical niosomes formed by $C_{16}G_2$:cholesterol:Solulan C24 (49:49:2), and (C) and (D) tubular structures (→) identified in the spherical niosome preparations of $C_{16}G_2$:cholesterol:Solulan C24 (49:49:2).

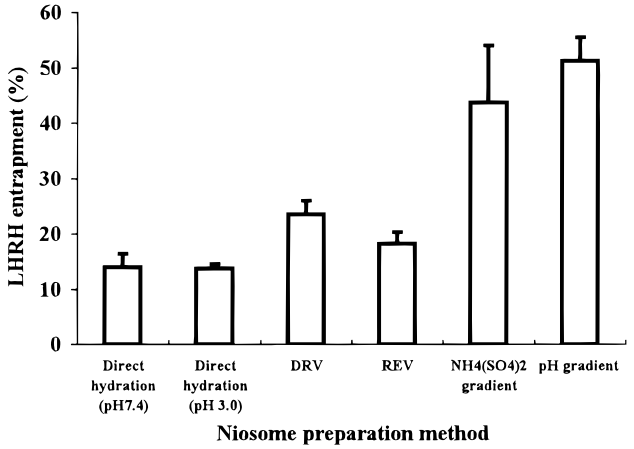

Figure 2—Percentage entrapment of LHRH acetate into polyhedral niosomes by six different preparation methods. Data are expressed as means and SD; $n = 3$, except for direct hydration at pH 3.0 and pH gradient entrapment, where $n = 2$ (DRV = dehydration–rehydration vesicles; REV = reversed-phase evaporation vesicles).

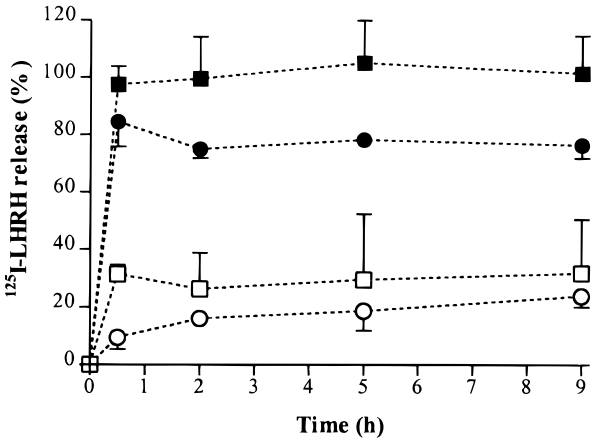

Figure 3—Release profile of [^{125}I]LHRH from polyhedral niosomes over a 9-h period in plasma (■) and in 5% muscle homogenate (□), and from spherical/tubular niosomes in plasma (●) and in 5% muscle homogenate (○). Data are expressed as means and SD; $n = 2$ for each niosome preparation at each time point.

the morphology of the vesicles has been examined using cryo-SEM. Polyhedral niosomes formed from $C_{16}G_2$ and Solulan C24 without cholesterol are asymmetric in nature, and different in their morphology from vesicles formed by these surfactants with 45 mol % cholesterol. The absence of cholesterol causes the membranes of $C_{16}G_2$ to form vesicle membranes with a lower curvature and fluidity.[4,7]

To study the possible use of such systems as sustained-release vehicles, LHRH acetate was selected as a model peptide. The binding of peptide to lipids can be enhanced by modifying the electrical charge or the hydrophobicity of the peptide.[8] However, LHRH is very hydrophilic and becomes a univalent cation at physiological pH. An increase in lipid–peptide interaction via electrostatic binding was

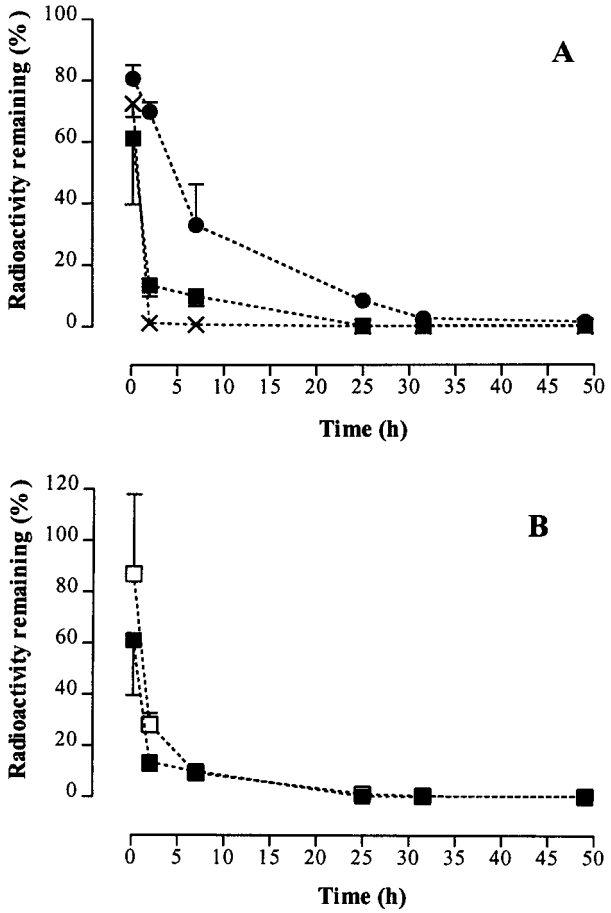

Figure 4—The amount of [^{125}I]LHRH as percent remaining at the injection site. (A) The [^{125}I]LHRH prepared in PBS at pH 7.4 (X), in polyhedral niosomes (■), and in spherical/tubular niosomes (●). (B) The [^{125}I]LHRH prepared in polyhedral niosomes (■), as presented in Figure 4A, and in polyhedral niosomes with NaCl (□). Data are expressed as means and SD; $n = 2$ for each niosome preparation at each time point.

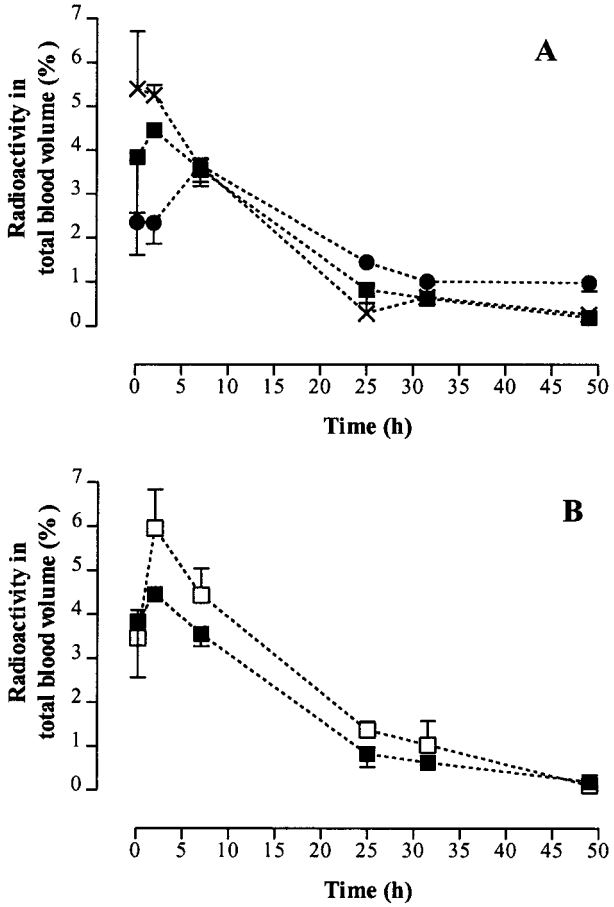

Figure 5—The amount of [^{125}I]LHRH as percent detected in blood after injection. The amount of radioactivity in the total blood volume was estimated and expressed as a percentage of the dose of radioactivity administered. (A) The [^{125}I]LHRH prepared in PBS at pH 7.4 (X), in polyhedral niosomes (■), and in spherical/tubular niosomes (●). (B) The [^{125}I]LHRH prepared in polyhedral niosomes (■), as presented in Figure 5A, and in polyhedral niosomes with NaCl (□). Data are expressed as means and SD; $n = 2$ for each niosome preparation at each time point.

described by Schäfer et al.[9] by the addition of phosphatidic acid, an anionic lipid, into the membranes of phosphatidyl choline vesicles. We obtained a very low entrapment of LHRH by passive loading methods (Figure 2), with the highest entrapment being achieved by the remote loading method (so-called "active" loading), which makes use of either the difference in pH, or in the amount of $(NH_4)_2SO_4$, between the inside and the outside of the vesicle. It is also of interest that LHRH (a decapeptide with a MW of 1182 Da) can be introduced through the vesicle membrane using a pH gradient[10] or an $(NH_4)_2SO_4$ gradient,[11] as has been demonstrated for other small molecules such as doxorubicin (MW of 543.5 Da). In our studies, the highest amount of drug to be entrapped using the pH gradient method was 14.5 nmol of LHRH/μmol of surfactants. This level appears low in comparison with values achieved for other drugs such as doxorubicin.[12] However we used a low concentration of LHRH (1 mM surfactants: 0.028 mM LHRH) and the amount of LHRH encapsulated was around 50% of the available drug.

The stability of parenteral drug delivery systems can be evaluated by measuring drug release during incubation in serum or plasma,[13,14] and muscle homogenate.[15] Both polyhedral and spherical vesicles are more stable in a 5% muscle homogenate than in plasma (Figure 3). Zuidema et al.[16] showed that plasma had a higher potential than muscle components to induce solute leakage from liposomes. Figure 3 also demonstrates that spherical/tubular niosomes released less [^{125}I]LHRH than polyhedral niosomes and these results compare with our earlier findings using CF as a marker.[4]

The mean size of both the polyhedral and the spherical/tubular niosomes were within the range of 6 to 9 μm. Zuidema et al. suggested that small liposomes (<200 nm) can be cleared from the im injection site by the lymphatic system,[16] whereas larger vesicles (4.9 μm) have been found to have a longer retention time at the site of injection.[17] It was thought that polyhedral niosomes, having a higher viscosity than spherical/tubular niosomes,[3] might remain at the site of injection for a longer period than spherical vesicles. However, probably due to differences in membrane permeability, this effect is masked as spherical/tubular vesicles were able to more effectively prolong the blood level of the peptide. These results compare therefore with the earlier in vitro stability studies.

Earlier we reported that the co-entrapment of NaCl with drug reduced the membrane permeability of polyhedral niosomes.[4] However, only a slight improvement in the release profile of LHRH-loaded polyhedral niosomes was observed in vivo (Figure 4B). Although liposomes may remain at the site of injection for <1 week,[18] their ability to retain the solute is an important factor in their use as a sustained-release system. Such an ability is related to the composition of the membranes, their lamellarity,[19] and the presence of cholesterol in the membranes.[14] Polyhedral niosomes formed without cholesterol, although being in the

gel phase, are therefore more leaky than their spherical/tubular counterparts.

LHRH is fairly stable in aqueous solution over a wide range of pH and temperature.[20] The blood levels of [^{125}I]-LHRH detected in this study were low, indicating that the peptide rapidly becomes accessible to protease enzymes on its release from the niosomes.

Conclusions

Niosomes can be used as sustained drug delivery systems that remain at the injection site over a period of around 48 h and slowly release drug into the blood. The shape of the vesicle, which gives a high viscosity to concentrated polyhedral niosome suspensions, is not as important as membrane permeability. Spherical/tubular niosomes that possess more stable membranes than polyhedral niosomes are more effective intramuscular depots.

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Acknowledgments

We thank L'Oreal, France, for providing $C_{16}G_2$. We appreciate the cooperation of Mr. David McCarthy, School of Pharmacy, for assistance with the cryo-SEM investigations. P.A. was supported by the Government Pharmaceutical Organization, Thailand.

JS980286U