Cryopreservation Enables Long-Term Storage of 9-(2-Phosphonylmethoxyethyl)Adenine Prodrug-Loaded Reconstituted Lactosylated High-Density Lipoprotein

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INTRODUCTION

Chronic hepatitis B is caused by an infection of parenchymal liver cells with hepatitis B virus (HBV). The nucleoside analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA) is a promising candidate drug for the treatment of HBV infections. PMEA effectively inhibits the replication HBV in cell culture (1,2), and the orally available bis(POM) derivative has entered clinical trials (3). Although initial results are promising, the disposition of PMEA remains a matter of concern. Most of the drug is cleared by the kidneys, whereas only a small fraction taken up by parenchymal liver cells (4). To enhance the effectiveness of PMEA against HBV, we developed a carrier-based strategy for selective delivery of PMEA to parenchymal liver cells. PMEA was conjugated to lithocholic acid-3α-oleate (LO), yielding the lipophilic prodrug PMEA-LO (5). The prodrug was incorporated into reconstituted lactosylated high-density lipoprotein (LacNeoHDL), a lipidic carrier that is selectively taken up by parenchymal liver cells via the asialoglycoprotein receptor (6,7). LacNeoHDL-associated PMEA-LO was delivered rapidly to parenchymal liver cells (8). Once internalized, PMEA was released from the PMEA-LO prodrug and phosphorylated to its active metabolite (8). The selective delivery of PMEA to parenchymal liver cells is expected to result in an enhanced therapeutic efficacy against HBV.

An important prerequisite for clinical application is that PMEA-LO-loaded LacNeoHDL remains stable during storage and transport. An attractive option for storage of PMEA-LO-loaded LacNeoHDL is cryopreservation. Freezing may, however, seriously damage the particles. Studies on cryopreservation of lipoproteins focus on low-density lipoprotein (LDL). Freezing and thawing of LDL results in aggregation of the particles (9,10). The aggregation leads to non-specific binding of LDL to cultured human fibroblasts (9), and to increased plasma clearance and enhanced spleen uptake, suggesting phagocytosis by cells of the reticulo-endothelial system (11). However, cryoprotectants like sucrose protect LDL from freezing-induced damage (9,12).

In this study we investigated the effect of cryopreservation of PMEA-LO-loaded LacNeoHDL on the stability of the PMEA-LO prodrug and on the physical and biological properties of the prodrug-loaded particles. As sucrose is beneficial for LDL during freezing, we examined the ability of sucrose to stabilize the prodrug-loaded carrier during cryopreservation

MATERIALS AND METHODS

Reagents

Sucrose was from BDH Laboratory Supplies (Poole, UK). Emulsifier Safe™, Hionic Fluor™, Monophase S™, and Soluene-350™ were from Packard (Downers Grove, IL). Asialofetuin was prepared as described earlier (13). All other reagents were of analytical grade.

Preparation and Cryopreservation of (^{3}H)PMEA-LO-loaded LacNeoHDL

(^{3}H)PMEA-LO-loaded LacNeoHDL was prepared, and its composition determined, as described earlier (5,8). The particles were dissolved in phosphate-buffered saline (PBS: 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl), containing 1 mM EDTA. Aliquots of 0.1–1.0 ml (0.2 mg of protein/ml) were frozen by placing them at –80°C. When indicated, (^{3}H)PMEA-LO-loaded LacNeoHDL was frozen in the presence of 10% (w/v) sucrose by adding, shortly for freezing, 50% (w/v) sucrose in PBS +1 mM EDTA. The frozen aliquots were stored at –80°C. Before experiments, frozen aliquots were thawed at 37°C for 3 minutes.

Determination of the Hydrolysis of (^{3}H)PMEA-LO in (^{3}H)PMEA-LO-loaded LacNeoHDL

The hydrolysis of LacNeoHDL-associated (^{3}H)PMEA-LO was determined by subjecting 50 μl samples to gel permeation chromatography using a Superose 6 PC column (3.2 × 300 mm; molecular weight fractionation range 5×10^3–5×10^6; SMART system, Pharmacia). The coumn was eluted with PBS at 50 μl/min. Fractions of 100 μl were assayed for radioactivity. Particle-associated (^{3}H)PMEA-LO and free (^{3}H)-PMEA eluted at 1.2–1.7 ml and 2.0–2.4 ml, respectively (5). The amounts of carrier-associated (^{3}H)PMEA-LO were calculated as a percentage of the recovered radioactivity.

Physical Characterizaton of (^{3}H)PMEA-LO-loaded LacNeoHDL

The size of (^{3}H)PMEA-LO-loaded LacNeoHDL was determined by gel permeation chromatography using the SMART system described above. Fractions were assayed for radioactivity, and for cholesteryl oleate as described earlier (5). The mean diameter of the particles was calculated as described previously (5). The net negative charge of the pro-

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drug-loaded carrier was determined by gel electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris-hippuric acid buffer + 0.65 mM EDTA). Gel slices were assayed for radioactivity. Bromophenol blue was used as front marker.

Determination of Plasma Clearance and Tissue Distribution of (^{3}H)PMEA-LO-Loaded LacNeoHDL

Male Wistar rats (170–210 g) were used. The animals were handled in compliance with guidelines issued by the Dutch authorities. The rats were anesthetized as described previously (8), and the abdomen was opened. (^{3}H)PMEA-LO-loaded LacNeoHDL, dialyzed against PBS, was injected via the vena penis (2 ml/kg). When indicated, preinjections (2 ml/kg) were administered via the vena cava inferior. Blood samples of 0.2–0.3 ml were taken from the vena cava inferior, collected in heparinized tubes, and the plasma was assayed for radioactivity. Liver lobules were tied off and excised. At the end of the experiment, the remainder of the liver and other tissues were removed. Radioactivity in plasma and tissues was calculated as described earlier (8).

Determination of Radioactivity

All samples were counted in a Packard Tri-Carb 1500 liquid scintillation counter (Packard, Downers Grove, IL). SMART and plasma samples were counted directly in Emulsifier Safe™. Gel slices were first digested overnight with Soluene 350™ and then counted in Hionic Fluor™. Tissue samples were processed using a Packard Tri-Carb 306 sample oxidizer and subsequently counted in Monophase S™.

RESULTS

Stability of (^{3}H)PMEA-LO in (Cryopreserved) (^{3}H)PMEA-LO-Loaded LacNeoHDL

(^{3}H)PMEA-LO-loaded LacNeoHDL was prepared by cosonicating prodrug, lipids, and apoprotein (5,8). The composition (percentage of total weight) was as follows: protein, 30.2 ± 1.2%; lactose, 12.9 ± 1 .2%; phosphatidylcholine, 37.7 ± 0.7%; cholesteryl oleate, 15.9 ± 1.9%; (^{3}H)PMEA-LO, 3.1 ± 0.1% (means ± variation of two preparations). The particles were stored at 4°C or cryopreserved at –80°C, in the presence or absence of 10% (w/v) sucrose. The hydrolysis of carrier-associated (^{3}H)PMEA-LO under these storage conditions was determined by measuring the release of (^{3}H)-PMEA. Figure 1 shows that carrier-associated (^{3}H)PMEA-LO is hydrolyzed during storage at 4°C in solution. Approximately 20% of the initially associated (^{3}H)PMEA-LO was lost from the carrier at day 11. Cryopreservation of (^{3}H)-PMEA-LO-loaded LacNeoHDL completely prevented hydrolysis of (^{3}H)PMEA-LO. After 26 days at –80°C , >98% of initially incorporated (^{3}H)PMEA-LO was still associated with the carrier, irrespective of the presence of sucrose.

Physical Properties of Cryopreserved (^{3}H)PMEA-LO-Loaded LacNeoHDL

Physical properties, like size, and net negative charge are crucial for the biological fate of a drug carrier. The state of aggregation of (^{3}H)PMEA-LO-loaded LacNeoHDL, cryopreserved for 28 days with or without 10% (w/v) sucrose, was examined by high-resolution gel permeation chromatography. Figure 2 shows that cholesteryl oleate and (^{3}H)PMEA-LO in both cryopreserved preparations display a virtually identical elution profile, indicating that the particles remain intact during cryopreservation. The diameters of prodrug-loaded particles cryopreserved with or without sucrose were very similar to that of freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL (11.7 ± 0.2, 11.5 ± 0.2, and 11.0 ± 0.6 nm, respectively; means ± variation of two preparations).

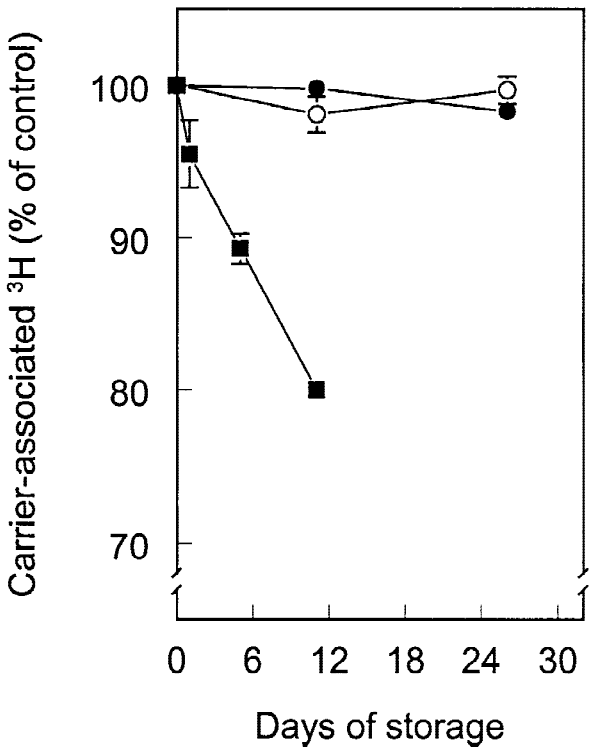

Fig. 1. Hydrolysis of carrier-associated (^{3}H)PMEA-LO during storage at 4°C or –80°C. (^{3}H)PMEA-LO-loaded LacNeoHDL was stored at 4°C (■) or cryopreserved in the presence (●) or absence (○) of 10% (w/v) sucrose. At the indicated days, aliquots were subjected to gel permeation chromatography to separate carrier-associated (^{3}H)-PMEA-LO from free (^{3}H)PMEA (column recoveries >78%). The amounts of carrier-associated (^{3}H)PMEA-LO in the stored aliquots are expressed as a percentage of the amount of carrier-associated (^{3}H)PMEA-LO in freshly prepared preparations (97.0 ± 0.4% of total drug in preparation). Values represent means ± variation of two experiments.

Cryopreserved (^{3}H)PMEA-LO-loaded LacNeoHDL was also subjected to agarose gel electrophoresis (Fig. 3). The electrophoretic mobilities of (^{3}H)PMEA-LO in preparations cryopreserved for 28 days with or without sucrose were very similar to that of freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL: 0.40 ± 0.02, 0.41 ± 0.01, and 0.36 ± 0.01, respectively (means ± variation of two experiments). Staining of the gels with Sudan Black and Coomassie Brilliant Blue revealed that the lipid and protein moieties in the preparations had the same electrophoretic mobility as the prodrug (data not shown). The results from the gel filtration and electrophoretic analyses clearly indicate that (^{3}H)PMEA-LO-loaded Lac-NeoHDL remains stable during cryopreservation, even in the absence of a cryoprotectant.

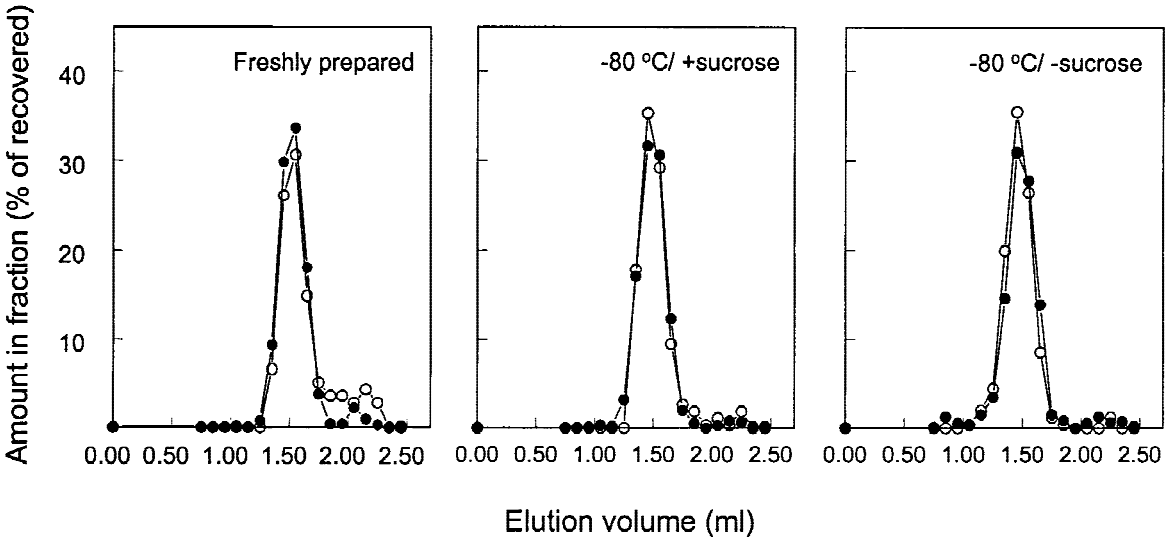

Fig. 2. Analysis of (cryopreserved) (^{3}H)PMEA-LO-loaded LacNeoHDL by gel permeation chromatography. Freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL (left panel), (^{3}H)PMEA-LO-loaded LacNeoHDL cryopreserved for 28 days with 10% (w/v) sucrose (center panel), and (^{3}H)PMEA-LO-loaded LacNeoHDL cryopreserved for 28 days without sucrose (right panel) were subjected to high-resolution gel permeation chromatography. Fractions of 100 μl were collected and assayed for cholesteryl oleate (○) and radioactivity (●). The results are expressed as a percentage the recovered amounts of cholesteryl oleate and radioactivity (recoveries >75%).

Plasma Clearance and Tissue Distribution of Cryopreserved (^{3}H)PMEA-LO-Loaded LacNeoHDL

To determine the effect of cryopreservation on the biological fate of PMEA-LO-loaded LacNeoHDL, rats were injected with (^{3}H)PMEA-LO-loaded LacNeoHDL that had been cryopreserved for 28 days with or without 10% (w/v) sucrose. For comparison, rats were also injected with freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL. After injection, radioactivity was rapidly cleared from the circulation (Fig. 4, A–C; control). The cleared radioactivity accumulated in the liver (Fig. 4, D–F; control). At 30 minutes after injection of freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL, preparations cryopreserved with sucrose, or preparations cryopreserved without sucrose, the liver contained 68.9 ± 7.7%, 63.9 ± 1.1%, or 65.4 ± 1.6% of the dose, respectively. To ascertain the role of the asialoglycoprotein receptor, asialofetuin was preinjected as a specific competitor (14). Preinjection with asialofetuin substantially (>75%) reduced liver uptake (Fig. 4), which indicates that cryopreserved PMEA-

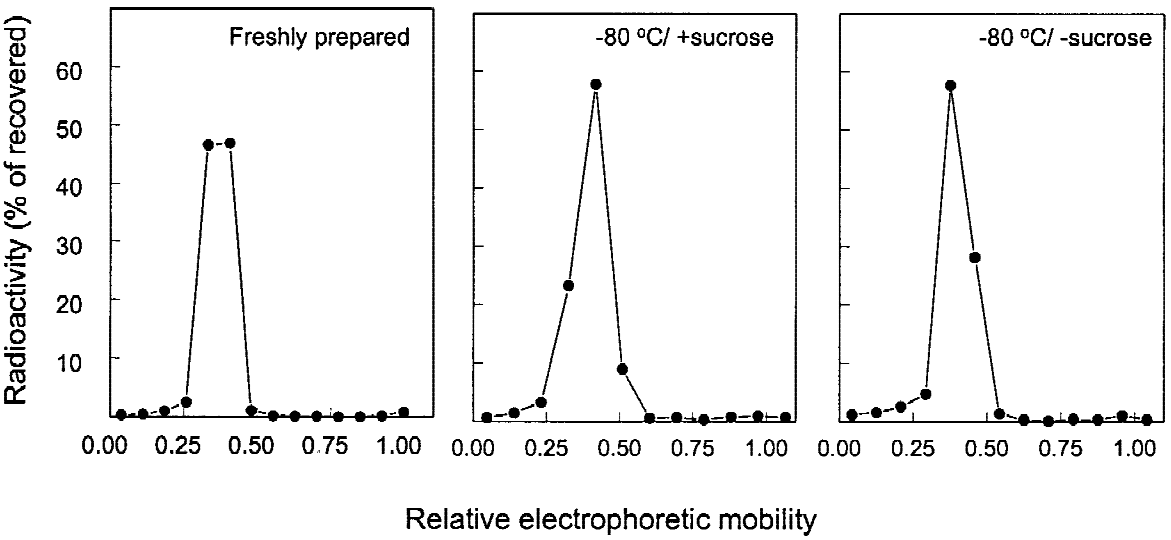

Fig. 3. Analysis of (cryopreserved) (^{3}H)PMEA-LO-loaded LacNeoHDL by gel electrophoresis. Freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL (left panel), (^{3}H)PMEA-LO-loaded LacNeoHDL cryopreserved for 28 days with 10% (w/v) sucrose (center panel), and (^{3}H)PMEA-LO-loaded LacNeoHDL cryopreserved for 28 days without sucrose (right panel) were subjected to electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris-Hippuric buffer). The gel was cut in 0.5-cm slices that were assayed for radioactivity. The radioactivity in each slice is given as a percentage of total recovered radioactivity (recoveries >80%). Migration is given relative to the migration of Bromophenol blue.

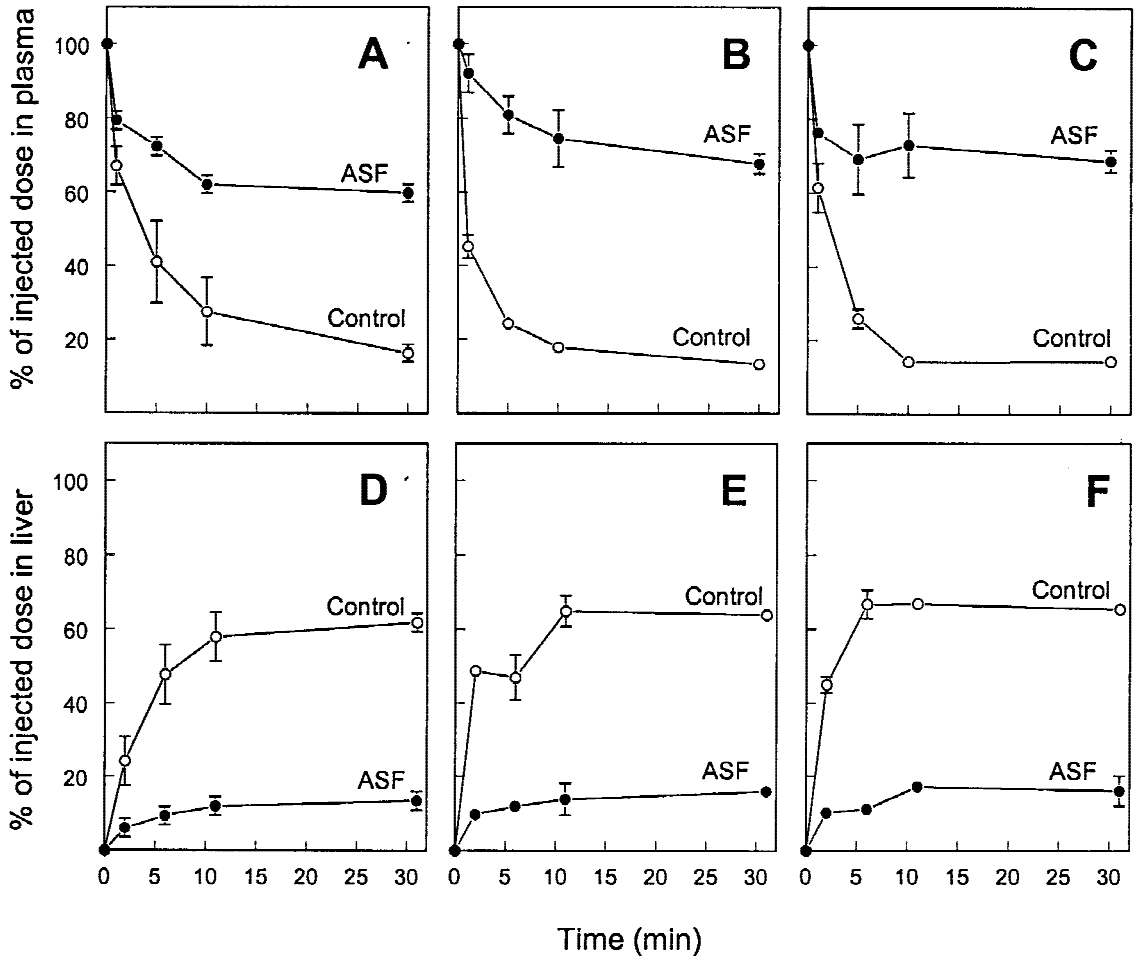

Fig. 4. Plasma clearance and liver association of (cryopreserved) (^{3}H)PMEA-LO-loaded LacNeoHDL; effects of asialofetuin. Rats were intravenously injected with freshly prepared (^{3}H)PMEA-LO-loaded LacNeoHDL (A+D), or with (^{3}H)-PMEA-LO-loaded LacNeoHDL cryopreserved for 28 days with (B+E) or without (C+F) 10% (w/v) sucrose. All animals received 6–10 μg of (^{3}H)PMEA/kg body weight. One min prior to injection, the animals were preinjected with 50 mg of asialofetuin/kg body weight (ASF, ●) or with PBS (Control, ○). At the indicated times, the amounts of radioactivity in plasma (A–C) and liver (D–F) were determined. Values represent the means ± SEM of three rats (freshly prepared preparation) or the means ± variation of two rats (cryopreserved preparations).

LO-loaded LacNeoHDL retains its integrity in the circulation and is taken up via asialoglycoprotein receptors. Furthermore, extrahepatic uptake of (^{3}H)PMEA-LO-loaded LacNeoHDL was not appreciably altered by cryopreservation (not shown).

DISCUSSION

The aim of the present study was to evaluate the physicochemical and biological properties of PMEA-LO-loaded LacNeoHDL under different storage conditions. During storage in solution at 4°C, carrier-associated PMEA-LO was hydrolyzed and lost from the carrier. Cryopreservation at −80°C completely prevented hydrolysis of PMEA-LO, irrespective of the presence of sucrose. Physical properties, like size and net negative charge, are crucial for the biological fate of a drug carrier. The size of PMEA-LO-loaded LacNeoHDL cryopreserved with sucrose as cryoprotectant was similar to that of freshly prepared particles. Interestingly, the size of PMEA-LO-loaded LacNeoHDL cryopreserved without sucrose was also similar, indicating that cryopreservation does not induce aggregation of the prodrug-loaded particles. Cryopreservation, both with and without sucrose, had also no effect on the net negative charge of PMEA-LO-loaded LacNeoHDL. Thus, PMEA-LO-loaded LacNeoHDL can be cryopreserved without altering its physical properties, even in the absence of a cryoprotectant.

The plasma clearance and tissue distribution of cryopreserved PMEA-LO-loaded LacNeoHDL were very similar to those of freshly prepared particles, and the presence of sucrose during cryopreservation had no appreciable effect. All preparations were rapidly cleared from the circulation and primarily taken up by the liver. Preinjection with asialofetuin, a specific inhibitor of uptake via the asialoglycoprotein receptor (14), substantially reduced the liver uptake, indicating that this receptor is responsible for the liver uptake. Spleen uptake was negligible, which rules out uptake by cells of the reticuloendothelial system. We conclude that PMEA-LO-loaded LacNeoHDL can be cryopreserved without affecting its biological fate, even without a cryoprotectant.

The present data were collected after 28 days of storage at −80°C. It is expected that LacNeoHDL can be cryopreserved for much longer periods. The critical step during cryopreservation is freezing and thawing, and PMEA-LO-loaded LacNeoHDL is not affected by this step. Furthermore, biological material and reagents are well preserved at −80°C. Indeed, we found that PMEA-LO-loaded LacNeoHDL that had been stored for over 1 year at −80°C had retained is therapeutic activity against HBV-infected hepatoma cells.

The exact mechanism by which PMEA-LO-loaded LacNeoHDL is stabilized during freezing is not entirely understood. Freezing and thawing of LDL has detrimental effects on the particle, which are prevented by adding sucrose during freezing (9–12). However, freezing and thawing of PMEA-LO-loaded LacNeoHDL has no appreciable effects on its physical and biological properties. The protein moiety of the carrier is highly lactosylated, which might protect the particles during freezing. However, native HDL is also not affected by cryopreservation without sucrose (data not shown), which rules out a cryoprotective role of the lactose residues. The major HDL apoprotein, apo A-I, does not undergo post-translational glycosylation (15). Apoprotein-associated sugars thus seem not to play a protective role. Apparently, PMEA-LO-loaded LacNeoHDL and HDL are intrinsically stable during freezing, in contrast to LDL that rapidly aggregates upon freezing. Possibly, apo A-I and the LDL apoprotein apo B-100 are not equally susceptible to damage by freezing. Apo B-100 is a very large protein (514 KD) that extends over the surface of LDL, whereas apo AI is much smaller (28 kDa) and more compact (15).

In conclusion, we show that PMEA-LO-loaded LacNeoHDL can be cryopreserved at −80°C without altering its physical and biological properties. Hydrolysis of PMEA-LO, which occurs during storage in solution at 4°C, was not observed at −80°C. It was further found that the presence of a cryoprotectant is not required. Our present results indicate that cryopreservation facilitates the logistics of the application of PMEA-LO-loaded LacNeoHDL as therapeutic agent for chronic hepatitis B.

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