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Effect of liposome size on peritoneal retention and organ distribution after intraperitoneal injection in mice

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ABSTRACT

Peritoneal carcinomatosis is a serious concern when treating digestive or ovarian tumors. Treatment with systemic chemotherapy suffers from poor penetration of cytotoxic agents into the peritoneal cavity and is not quite effective. Local delivery of drugs, especially as controlled-release delivery systems like liposomes, could provide sustained and higher drug levels and reduce systemic toxicity.

In order to investigate the effect of liposome size on peritoneal retention, liposomes composed of distearoylphosphatidylcholine and cholesterol (DSPC/CHOL, molar ratio 2:1) were prepared at four sizes of 100, 400, 1000 and 3000 nm. Subsequently, these liposomes were labeled with ^{99m}Tc complex of hexamethylpropyleneamineoxime (^{99m}Tc-HMPAO) and injected into mouse peritoneum. Then, mice were sacrificed at eight different time points and the percentage of injected radiolabel in the peritoneal cavity and the organ distribution in terms of percentage injected dose/gram tissue (%ID/g) were obtained.

Results showed that the free label (^{99m}Tc-HMPAO) was cleared very rapidly from the cavity so that after 5 min and 7 h only $6.89 \pm 2.51\%$ and $0.91 \pm 0.51\%$ of the injected dose was recovered, respectively. However, for the liposomal formulations, this recovery value ranged from $8.47 \pm 1.62\%$ to $29.99 \pm 12.06\%$ at 7 h. Peritoneal retention of the vesicles was increased with their size, and the highest retention rate was obtained with 1000 nm liposomes with an AUC value 15.51 times that of ^{99m}Tc-HMPAO. In blood, as expected, 100 nm liposomes showed much higher levels because of their greater stability. Their greater blood concentration also caused increased levels in the heart and kidneys, although their organ to blood AUC ratio was the lowest.

Overall, among the different sized neutral liposomes investigated, the 1000 nm vesicles seemed to be the most optimal, achieving a greater peritoneal level and retention.

1. Introduction

Peritoneal carcinomatosis refers to a wide variety of tumors that present with extensive peritoneal involvement with or without parenchymal inclusion of solid organs such as liver, spleen and lymph nodes. This condition has a poor prognosis and is a serious concern for the treatment of gastrointestinal or ovarian tumors (Debree et al., 2002; Tamura et al., 2002; Verwaal et al., 2003). The use of systemic chemotherapy has not been shown to be effective due to the poor penetration of cytotoxic agents into the peritoneal cavity. There has been long-standing interest in the local delivery of drugs into the peritoneum, which can provide high drug concentrations at the site of tumor for an extended period of time, leading to higher efficacy and lower toxicity. However, most cytotoxic agents currently employed in the treatment of peritoneal carcinomatosis are quickly absorbed from the peritoneum leading to a rapid decline in the drug concentration at the target area on the one hand, and the presence of the drug in systemic circulation and toxicity on the other hand (Pourgholami et al., 2005). Controlled release technologies that have evolved over the past few decades could lend themselves well to this problem, as they can be designed to release their drug dose gradually over time (Kohane et al., 2006).

After their original description by Bangham et al. in the 1960s, liposomes have been widely studied as potential carriers for

Abbreviations: DSPC, distearoylphosphatidylcholine; DMPC, dimiristoylphosphatidylcholine; DMPG, dimiristoylphosphatidylglycerol; CHOL, cholesterol; DOX, doxorubicin; L-NDDP, cis-bis-neodecanoato (trans-R, R-1, 2-diaminocyclohexane)-platinum II entrapped in multilamellar vesicles; HMPAO, hexamethylpropyleneamineoxime; MLV, multilamellar vesicles.

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hydrophilic and hydrophobic drugs and diagnostic agents (Phillips, 1999). They can sometimes modify the therapeutic profile of selected anti-tumor drugs in a very favorable manner and as drug carriers, they are likely to be retained in the peritoneum for longer periods than most free drugs (Daoud, 1994).

Sadzuka et al. (1997) reported that 1 h after intraperitoneal injection of doxorubicin solution, only 3.1% of the administered amount remained in the abdominal cavity while 54.3% of the injected dose of plain or PEGylated DMPC/CHOL/DMPG/DOX liposomes (100:100:60:18 mol) was still detectable.

Other studies of radiolabeled liposomes have also shown a very rapid absorption of the free radiolabel versus relatively prolonged retention times of radiolabeled liposomes in mouse peritoneum (Syrigos et al., 2003; Chen et al., 2007). A phase I clinical study on a liposomal formulation (MLV) of a water-insoluble platinum analog (L-NDDP) measuring 1–3 μm in diameter demonstrated prolonged retention in the peritoneal cavity and limited systemic absorption, thereby yielding a high peritoneal/plasmatic ratio (Verschraegen et al., 2003). Use of the aggregating properties of avidin and biotin to retain biotinylated liposomes in the peritoneal cavity for a longer period of time has also been reported (Zavaleta et al., 2007). Furthermore, an increase of the local therapeutic effect of cisplatin when co-administered with low doses of MLVs of valinomycin (Daoud, 1994) or a decrease of toxicity of paclitaxel (Taxol) in the abdominal cavity when formulated in liposomes have also been described (Sharma et al., 1996).

Several studies have reported the marked influence of liposome size on pharmacokinetic profile and tissue distribution of carrier and encapsulated drug after intravenous or subcutaneous administration (Allen et al., 1993; Drummond et al., 1999; Nagayasu et al., 1999; Oussoren and Storm, 2001), but research clarifying the effect of liposome size on peritoneal retention and organ distribution is very limited.

Hirano and Hunt (1985) investigated the peritoneal retention of ^{14}C-sucrose labeled liposomes of egg lecithin, phosphatidic acid, cholesterol and α-tocopherol in the molar ratios of 4:1:5:0.1 prepared at four different sizes of liposomes (48, 170, 460 and 720 nm) in rats. They saw no size effect on absorption from the peritoneal cavity, thereby arguing that an effect would only be expected if the size were to be increased to the point that the entrance of vesicles into lymphatic capillaries became restricted.

Sadzuka et al. (2000) reported that for doxorubicin liposomes containing DSPC/CHOL/DMPG/DOX (100:100:60:16 μmol) with a size of 150, 600 or 4000 nm, the clearance from the peritoneal cavity of small and medium vesicles was similar but large liposomes remained longer in place, showing 4 and 8.4 fold the amount of small vesicles in the cavity at 8 and 24 h after injection, respectively. In this study, the zeta potential of all liposomes was around −35 mV and the negative charge of liposomes might have affected their distribution. Furthermore, sampling was limited to four time points in this study.

Studies examining intravenously injected liposomes demonstrate a diameter of about 100 nm as the optimal size to prolong their blood circulation time. Increasing liposome size augments their rate of disappearance from blood because of their increased uptake by the mononuclear phagocytic system (Nagayasu et al., 1999). Macrophages are also present in the peritoneal cavity (Carr, 1973). Thus, after intraperitoneal administration, ingestion of vesicles by the peritoneal macrophages, particularly for the larger ones, could have a negative effect on their local retention.

Due to the lack of sufficient research about the effect of liposome size on peritoneal retention, in the present study we investigated the effect of four different sizes of DSPC/CHOL liposomes in regard to their cavity retention and organ distribution. Importantly, all liposomes used had a nearly neutral charge and their sizes ranged from 100 to 3000 nm.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylcholine (DSPC) was purchased from Lipoid GmbH (Germany) and cholesterol (CHOL) from Northern Lipids Inc. (Vancouver, Canada). Nuclepore® polycarbonate membranes with pore sizes of 100, 400, 1000 and 3000 nm and a diameter of 25 mm were used with a water-jacketed 10 mL Lipex extruder (Northern Lipids Inc., Vancouver, Canada) and were provided by Whatman Int. Ltd. (Maidstone, England). Chloroform, methanol, glutathione (GSH), ammonium thiocyanate, $FeCl_3 \cdot 6H_2O$, potassium chloride, $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4 were purchased from Merck (Darmstadt, Germany). Sodium chloride was obtained from Riedel-de Haën (Germany). Sephadex G-25 was provided by Pharmacia (Uppsala, Sweden). HMPAO kits containing 0.5 mg HMPAO and 5 μg $SnCl_2 \cdot 2H_2O$ were prepared in-house as described earlier (Mirahmadi et al., 2008). ^{99m}Tc was eluted from a ^{99}Mo/^{99m}Tc generator.

2.2. Liposome preparation

Liposomes composed of DSPC and CHOL at a molar ratio of 2:1 were prepared by the thin film hydration technique (Hope et al., 1985). In this mixture, cholesterol counts for 33.3 mol% of total lipids, thus contributing to the maintenance of membrane bilayer stability (Drummond et al., 1999) as it is stated that incorporation of more than 30 mol% CHOL in liposomes prolongs their circulation times (Moghimi et al., 2001). In brief, lipids were dissolved in a mixture of 10:1 chloroform and methanol. Organic solvents were removed under reduced pressure in a rotary evaporator and the thin layer of lipids was hydrated in PBS pH 7.4 containing 100 mM GSH. Hydration continued for 2 h at 64 °C. Multilamellar vesicles were multiple extruded using the optimal size of polycarbonate membrane filters at 64 °C to obtain liposomes with different sizes (about 100, 400, 1000 and 3000 nm).

Removal of the unencapsulated GSH was performed by overnight dialysis of extruded liposomes at 2–8 °C. Phospholipid content of the vesicles was assayed using the method by Stewart (1980), which showed a yield of more than 85%. Liposome size was determined by dynamic laser light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments, London, England). The average diameter obtained in increasing order of size was as follows: 128, 479, 1258 and 3553 nm with a polydispersity index of less than 0.1. Zeta potentials of liposomes were determined using 90 PLUS particle size analyzer with ZETA PALS system (Brookhaven Corp., Hostville, NY) at 25 °C.

2.3. Labeling of liposomes

Liposomes were labeled with ^{99m}Tc-HMPAO as reported by Phillips et al. (1992) and optimized in our previous study (Mirahmadi et al., 2008).

In brief, with regard to the final lipid amount available according to the Stewart assay method, the required radioactivity up to a maximum of 15 mCi $^{99m}TcO_4^-$ was added to one or two lyophilized HMPAO kits and the volume was adjusted to 1 mL with 0.9% degassed saline. Approximately 5–10 min after preparation of the kits, liposomes were added at the rate of 1 μmol lipid for 0.3 mCi radioactivity. After 25 min incubation at room temperature, 100 or 400 nm liposomes were separated from any free ^{99m}Tc by passage over a 1.1 cm × 23 cm Sephadex G-25 column. Labeling efficiency was checked by determining the activity of the liposomal portion of the eluate compared to the activity of an equivalent volume of intact ^{99m}Tc-liposomes using a dose calibrator.

For 1 and 3 μm vesicles, removal of the free label was done by 10 min of centrifugation at 12,000 rpm. Labeling efficiency was calculated by dividing the radioactivity of the sediment to the total radioactivity of the vial.

Labeling efficiency varied between 80.71 and 88.89%. After removal of the free label, the liposomal portion was collected in a vial and diluted with PBS in order to get around 280 μCi in 1 mL to inject to each mouse.

To compare the biodistribution of liposomes with free label, 9 mCi $^{99m}TcO_4^-$ was added to an HMPAO kit and the volume was adjusted to 1 mL with normal saline. After 5 min, the solution was further diluted to give approximately 280 μCi/mL for injection in each mouse.

2.4. Animal experiments

Animal experiments were performed under the National Institutes of Health Animal Care and Use guidelines and were approved by the animal welfare commission of the Shaheed Beheshti Medical Sciences University. Female NMRI mice weighing between 20 and 35 g were allowed at least 5 days to acclimate to the surroundings and had access to food and water *ad libitum*.

On the day of experiment, 1 mL of the free label or liposomal dispersion was injected into the peritoneum of each mouse with a 27 G × ½ in. (0.4 mm × 12 mm) syringe in the left lower quadrant. Animals (3 for each time point) were euthanized with carbon dioxide 0.5, 1, 2, 4, 7, 24, 30 and 48 h after injection. For control (^{99m}Tc-HMPAO), an additional sampling point was considered 5 min after injection.

After euthanasia, mice were weighed and blood was collected by venal puncture. Ventral skin was completely and carefully removed in order to not damage the parietal peritoneum. After sampling from the skin and thigh muscle, 3 mL normal saline was flushed into the peritoneal cavity and re-collected after gentle massage of the abdomen with the lateral side of the nippers (Maincent et al., 1992; Zavaleta et al., 2007). Then mice were dissected and bladder, spleen, stomach, intestines, liver, kidneys, heart, lungs and carcass were deposited in separate pre-weighed disposable plastic glasses, after drying the internal organs on a 3 mm Whatman filter paper.

Radioactivity of each organ was counted with a gamma well counter after weighing. At least one standard of the injected material was set aside and counted at the same time as the mouse organs to correct for physical decay of ^{99m}Tc. Data were expressed in terms of percent of the injected dose per gram of tissue (%ID/g) and for the fluid collected from the peritoneal cavity in %ID. In addition, the AUC between 0 and 48 h was derived for each tissue by the trapezoidal rule method (Shargel et al., 2004) and the standard deviation of the AUCs was calculated according to Bailer's method (1988).

2.5. Statistical analysis

Statistical comparisons of the AUCs were performed by ANOVA with Tukey post-test using GraphPad Instat software. Statistical significance was established at $p < 0.05$.

2.6. In vitro stability of liposomes

The stability of purified radiolabeled liposomes was evaluated by incubation in human plasma at 37 °C. Samples were taken after 0.5, 2, 4, 24 and 48 h of incubation and the percentage of radioactivity of the liposomal portion was calculated. For 100 and 400 nm liposomes, separation from the free label was obtained by passage through a 1.5 cm × 7 cm Sephadex G-25 column. Then, count of the liposomal portion of the eluate was divided by the activity of an equivalent volume of intact liposomes. For 1000 and 3000 nm vesicles, 1 mL of the mixture was removed and centrifuged at 5000 rpm for 30 min. Then, the supernatant was carefully separated and the radioactivity counted in the liposomal portion (the sediment) was divided by the sum of the counts of the two portions.

3. Results

3.1. In vitro stability studies

The in vitro stability of liposomes after incubation in human plasma at 37 °C is shown in Table 1. As shown ^{99m}Tc-HMPAO was not released from 100 nm vesicles over a period of 48 h incubation, while about 6.36% and 8.63% of the radiolabel dissociated from the 400 nm ones after 24 and 48 h, respectively. These values increased to 14.41% and 17.9% for 1000 nm liposomes and to 22.15% to 25.96% for 3000 nm vesicles, respectively. This is in accordance with previous studies that showed that a decrease in liposome size reduces extent of protein or opsonin adsorption (Harashima et al., 1995; Moghimi et al., 2001). Also in vivo studies have shown that clearance rates increase progressively with size increase of neutral or anionic liposomes, indicating that surface curvature changes may affect the extent and/or type of protein or opsonin adsorption (Moghimi et al., 2001).

3.2. In vivo studies

Results obtained after intraperitoneal injection of ^{99m}Tc-HMPAO and ^{99m}Tc-HMPAO radiolabeled liposomes with an approximate size of 100, 400, 1000 and 3000 nm to mice are described below. All statistical comparisons refer to AUCs.

3.2.1. Peritoneal retention

The radioactivity–time curves from peritoneal cavity data after intraperitoneal (ip) injection of labeled liposomes and free label, as well as the relevant AUC values, are shown in Fig. 1 and Table 2. Table 3 summarizes the relative ratio of AUCs (RR-AUCs) for liposomes versus ^{99m}Tc-HMPAO in various organs, showing more clearly the different biodistribution and concentration patterns of vesicles compared to control in organs tested. As shown in Fig. 1, all liposomal formulations exhibited a greater peritoneal level compared to the free label ($p < 0.001$) with an AUC ranging from 6.34 to 15.51 fold that of ^{99m}Tc-HMPAO for the 100 nm and 1000 nm liposomes, respectively (Table 3). Increasing liposome size from 100 to 400 nm produced a higher retention ($p < 0.05$). Vesicles that were 1000 nm in size had the greatest cavity retention with a much slower decrease in concentration from 4 h

Table 1
% Remained label in liposomes after incubation in human plasma at 37 °C (mean ± S.D., $n = 3$).

Incubation time (h)	DSPC/CHOL, 100 nm	DSPC/CHOL, 400 nm	DSPC/CHOL, 1 μm	DSPC/CHOL, 3 μm
0.5	76.03 ± 4.34	80.71 ± 1.90	90.93 ± 0.05	83.04 ± 0.54
2	77.97 ± 0.33	81.90 ± 4.31	89.74 ± 0.08	81.03 ± 0.36
4	75.23 ± 1.29	79.73 ± 1.44	88.82 ± 0.38	77.23 ± 0.24
24	80.1 ± 2.93	74.35 ± 1.05	76.52 ± 0.13	60.89 ± 0.26
48	81.19 ± 2.44	72.08 ± 2.65	73.03 ± 1.38	57.08 ± 3.29

Table 2
Area under the %ID/g versus time curves for different sized liposomes and ^{99m}Tc-HMPAO after ip injection to mice (AUC ± S.D.).

Organ	^{99m}Tc-HMPAO	100 nm	400 nm	1000 nm	3000 nm
Blood	57.10 ± 10.15	460.11*** ± 50.22	59.85 ± 10.90	56.85 ± 5.29	45.64 ± 8.42
Peritoneal rinsing fluid	51.65 ± 9.32	327.34*** ± 21.82	500.54*** ± 49.98	801.06*** ± 94.00	433.93*** ± 31.15
Spleen	63.31 ± 5.42	1390.85*** ± 144.75	3265.50*** ± 446.36	1133.78*** ± 69.02	995.31** ± 90.10
Liver	194.01 ± 15.41	707.34*** ± 65.11	770.03*** ± 87.58	392.85** ± 22.25	265.35 ± 24.03
Intestines	197.45*** ± 30.72	111.96 ± 12.35	49.09 ± 9.38	55.04 ± 5.7	104.29 ± 7.88
Stomach	101.39 ± 19.57	75.64 ± 7.00	112.01 ± 18.34	154.17 ± 16.7	557.68*** ± 47.12
Kidneys	110.24 ± 4.09	307.32*** ± 25.74	102.15 ± 9.78	120.56 ± 8.73	119.19 ± 9.19
Skin	17.50 ± 3.52	52.15*** ± 12.92	7.36 ± 0.73	13.48 ± 1.34	10.60 ± 1.12
Heart	37.09 ± 2.23	49.50** ± 4.79	12.11*** ± 1.39	11.46*** ± 0.75	18.18*** ± 2.33
Lungs	76.92 ± 12.61	138.16 ± 15.6	119.87 ± 39.87	107.47 ± 28.1	51.02 ± 6.96

** $p < 0.01$
*** $p < 0.001$ compared to the control (^{99m}Tc-HMPAO)

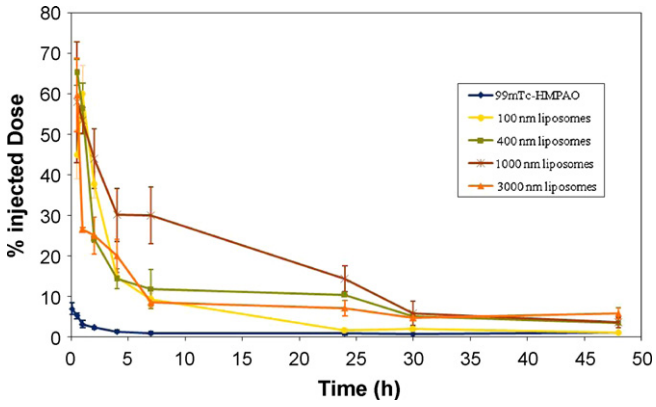

Fig. 1. Detectable radioactivity (in terms of %ID) versus time in the peritoneal lavage fluid collected after flushing the cavity with normal saline (mean ± S.E.).

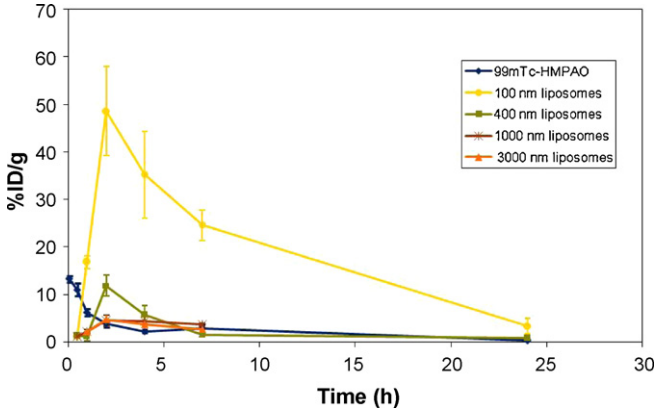

Fig. 2. Percent of injected radioactive dose recovered per gram blood at different times (mean ± S.E.).

(30.07 ± 11.27 %ID) to 24 h (14.35 ± 5.47 %ID) compared to all others ($p < 0.001$).

The peritoneal to blood AUC ratio was also calculated and is illustrated in Table 4. The vesicles that were 1000 nm in size had the highest peritoneal to blood ratio. By comparison, the 100 nm liposome ratio was even less than free label, which was due to the high blood concentration and reduced peritoneal retention of this vesicle size. Further increasing liposome size to 3000 nm decreased the peritoneal level, producing a profile not significantly different from the 100 and 400 nm vesicles ($p > 0.05$).

Table 3
Relative ratio of AUCs for different sized liposomes versus ^{99m}Tc-HMPAO.

Organ	100 nm	400 nm	1000 nm	3000 nm
Blood	8.06	1.05	1.00	0.80
Peritoneal rinsing fluid	6.34	9.69	15.51	8.40
Spleen	21.97	51.58	17.91	15.72
Liver	3.65	3.97	2.02	1.37
Intestines	0.57	0.25	0.28	0.53
Stomach	0.75	1.10	1.52	5.50
Kidneys	2.79	0.93	1.09	1.08
Skin	2.98	0.42	0.77	0.61
Heart	1.33	0.33	0.31	0.49
Lungs	1.80	1.56	1.40	0.66

Table 4
Organ to blood AUC ratio for each one of different liposomes and ^{99m}Tc-HMPAO.

Organ	^{99m}Tc-HMPAO	100 nm	400 nm	1000 nm	3000 nm
Blood	1.00	1.00	1.00	1.00	1.00
Peritoneal rinsing fluid	0.90	0.71	8.36	14.09	9.51
Spleen	1.11	3.02	54.56	19.94	21.81
Liver	3.40	1.54	12.87	6.91	5.81
Intestines	3.46	0.24	0.82	0.97	2.29
Stomach	1.78	0.16	1.87	2.71	12.22
Kidneys	1.93	0.67	1.71	2.12	2.61
Skin	0.31	0.11	0.12	0.24	0.23
Heart	0.65	0.11	0.20	0.20	0.40
Lungs	1.35	0.30	2.00	1.89	1.12

3.2.2. Level in blood

As seen in Fig. 2, ^{99m}Tc-HMPAO cleared rapidly from the peritoneal cavity in such a way that the absorption phase could not be detected in blood or other organs. This rapid clearance was perhaps because of the intraperitoneal route of delivery, which is the third most rapid absorption pathway after intravenous and respiratory routes (Gad and Chengelis, 1992). In all liposomal formulations, the peak blood level was reached at 2 h with a maximum concentration of 48.59 ± 16.29 %ID/g for 100 nm vesicles versus 11.90 ± 3.89 %ID/g for 400 nm liposomes, and less than 5 %ID/g for the 1000 and 3000 nm vesicles. The 100 nm liposomes showed, as expected, the highest blood level, which was detectable until 24 h ($p < 0.001$ compared to all other tested samples) with an AUC about 8.06 times that of the free label (Tables 2 and 3). The three other formulas had a much lower count that decreased further with increasing size, although statistical analysis of the AUCs demonstrated no significant differences among these other sizes and the free label.

3.2.3. Distribution in other organs

The biodistribution of different sized liposomes as well as unencapsulated ^{99m}Tc-HMPAO are presented by the AUC values for each organ and are shown in Table 2 and Figs. 3 and 4. To better characterize the tissue distribution of tested samples, organ to blood AUC ratios are compared in Table 4. This ratio represents the distribu-

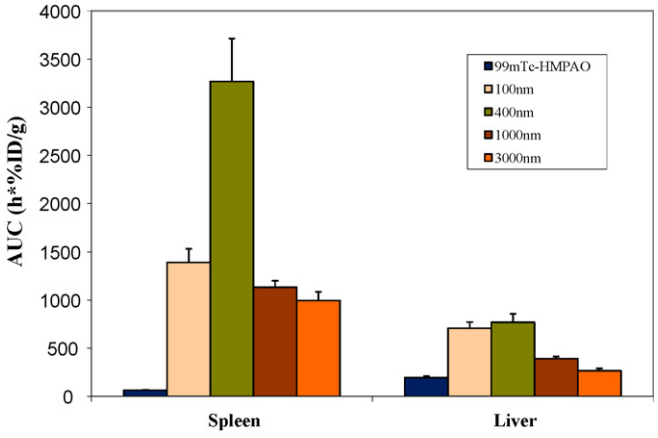

Fig. 3. AUC + S.D. of %ID/g versus time curves for different liposomes and the free label in the spleen and liver.

tion of intraperitoneally administered products from the systemic circulation to each organ.

As shown in Fig. 3 and Table 4, 400 nm liposomes displayed a markedly higher level per gram of spleen compared to other sized liposomes ($p < 0.001$). In regard to liver, distribution of 100 and 400 nm vesicles to this tissue were comparable and significantly higher than other sizes ($p < 0.001$). However, the organ to blood AUC ratio (Table 4) demonstrates that 100 nm liposomes had the lowest ratio in these two organs among all vesicles examined. This result can be attributed to the 100 nm vesicles greater stability, which is due to their optimal size for circulation in blood (Nagayasu et al., 1999) and lower uptake by the macrophages. For 1000 and 3000 nm liposomes, greater peritoneal, abdominal and perhaps lymph node retention caused a reduced level in the liver and spleen compared to smaller liposomes ($p < 0.001$ for the liver), but the levels were higher than the free label.

Consistent with their much higher blood level, 100 nm vesicles showed the greatest concentration in kidneys compared to all other tested formulas (Table 2 and Fig. 4) ($p < 0.001$), but their kidney to blood AUC ratio was the lowest among all sizes examined (Table 4). The three other sizes had a similar profile and showed no significant difference in AUCs among each other and with the control ($p > 0.05$).

In the intestines (Table 2 and Fig. 4), ^{99m}Tc-HMPAO presented the highest concentration because of its hepatobiliary elimination pathway (along with urinary excretion) (Neirinckx et al., 1987) ($p < 0.001$ relative to others), and the relative ratio of AUCs for different liposomes versus the free label was always less than 1 (Table 3). The 100 nm liposomes, due to their higher blood concentration, and the 3000 nm vesicles resulted in considerable intestinal levels

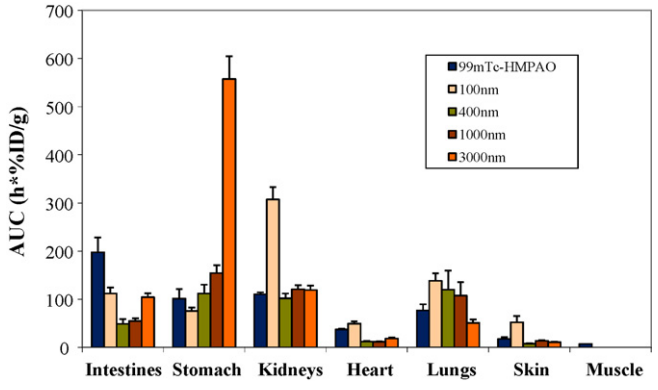

Fig. 4. AUC + S.D. of %ID/g versus time curves for different liposomes and the free label in various organs.

($p < 0.01$ compared to 400 and 1000 nm). Despite these similar findings, the AUC ratios to blood (Table 4) demonstrated the smallest value for 100 nm liposomes (0.24), while that for 3000 nm vesicles (2.29) was nearer to the ratio for the free label (3.46). The 400 and 1000 nm vesicles had a similar profile that was not significantly different from each other ($p > 0.05$).

In the stomach (Table 2 and Fig. 4), 3000 nm liposomes showed the very highest level among all sizes tested ($p < 0.001$). It seems that although these liposomes were not greatly enriched in the peritoneal lavage fluid, they tended to sediment upon abdominal organs, especially the stomach and intestines ($p < 0.05$ compared to 400 and 1000 nm liposomes).

Profiles of the three other sizes and the free label in the stomach were similar, although 1000 nm vesicles demonstrated a slightly greater level ($p < 0.05$ compared to 100 nm vesicles). Consideration of the relative ratio of AUCs for different sizes of liposomes versus ^{99m}Tc-HMPAO and blood (Tables 3 and 4) illustrates clearly that increasing liposome size, perhaps because of a greater retention in the peritoneal cavity and a longer exposure to the abdominal organs, causes corresponding increases in radioactive counts of the stomach and the intestines to a lesser extent. The direct penetration of intraperitoneally administered liposomes into abdominal organs has been reported before (Syrigos et al., 2003).

Low radioactivity was detected in the heart and lungs (Table 2 and Fig. 4). Due to their high blood level, 100 nm liposomes concentrated more in the heart and lungs exhibiting a respective significant ($p < 0.01$) to very significant ($p < 0.001$) difference compared to the free label and other vesicles in the heart, although their organ to blood AUC ratio was the smallest (Table 4). In lungs, the AUC decreased with increasing liposome size but the difference was not statistically significant except between 3000 nm vesicles and 100 nm ($p < 0.01$) or 400 nm liposomes ($p < 0.05$). Perhaps a greater retention in the abdominal space or in the lymph nodes, a lower blood level and relative instability are some of the reasons for the reduced presence of 3000 nm liposomes in the lungs.

Radioactive counts in the skin were lower than other organs. Due to their high blood concentration, 100 nm vesicles had a greater level ($p < 0.001$) but again a small skin to blood AUC ratio (Table 4). Other formulas showed much smaller levels that were not significantly different from each other or the free label. None of the liposomal formulations could be detected in muscle.

4. Discussion

The peritoneal cavity has a relatively large surface area. Its major surface lies over the stomach, small and large intestines, the retroperitoneal muscle and ventral abdominal muscle. It is well perfused with blood capillaries and provides an excellent site for drug exchange with plasma (Flessner et al., 1984). The structures that account for most of the surface area of the peritoneum, including the visceral peritoneum, omentum, and mesentery, drain into the portal circulation. Only the parietal peritoneum drains directly into the systemic circulation, and although a rich network of diaphragmatic lymphatics drains the peritoneal cavity, for drugs with a molecular weight of less than 1000 Da the flow in the lymphatic channels is much less than the portal flow, as such, probably lymphatic system quantitatively does not constitute an important route of drug absorption (Howell, 2008). Thus, ^{99m}Tc-HMPAO, a mixture of dominant lipophilic plus hydrophilic complexes (Mirahmadi et al., 2008) of less than 500 Da leaves the injection site so rapidly that the absorption phase to the blood could not be detected in spite of the additional sampling time point 5 min after injection of the free label (Fig. 2).

Particles larger than 20 nm (Moghimi, 2003) or compounds greater than 20 kDa are drained through the lymphatic ducts, with

a mean opening (stoma) diameter of 3.6 ± 2.0 μm on a mouse subdiaphragm surface (Tsai et al., 2007). Thus, in the present study, it is expected that all liposomal formulations reach the bloodstream by the way of lymphatics and, due to their different and very localized drainage system, liposomes remain in the cavity for a longer time and in a much higher concentration compared to the control; although even the largest sizes could not be retained indefinitely at the injection site. Perhaps, though, increasing the size of liposomes limits their motility, increases their local sedimentation and slows their drainage. From another point of view, it is possible that increasing the size of liposomes affects their integrity, stability and uptake by peritoneal macrophages.

In the present study all liposomal formulations showed a much higher peritoneal level compared to the free label which was in consistent with previous reports (Sadzuka et al., 2000; Syrigos et al., 2003; Chen et al., 2007). Furthermore, increasing the size of vesicles from 100 to 400 nm, and particularly to 1000 nm, significantly augmented the retention in the peritoneum. For example, 7 h after injection only 0.91 ± 0.51 %ID of the control was collected from the peritoneal cavity, while for the liposomes with increasing order of size, the detectable percentages were 9.17 ± 2.65, 11.84 ± 8.35, 29.99 ± 12.06 and 8.47 ± 1.62 %ID, respectively. The reduced presence and AUC of 3000 nm liposomes in the cavity is surprising but their very high concentration in the stomach and their considerable level in the intestines suggest that these heavier vesicles precipitate upon abdominal organs without being readily extracted during peritoneal washing.

Compared to other sized liposomes and free label, 400 nm vesicles revealed a distinctly higher distribution to the spleen. This might be the result of their relatively rapid clearance from the peritoneal cavity through the lymph, and their instability in blood circulation owing to their enlarged size, which makes them prone to be captured by the mononuclear phagocytic system.

Our results did not corroborate previous reports with regard to the effect of liposome size on the peritoneal retention. Hirano and Hunt (1985) did not find a significant difference in the absorption of vesicles sized in the range of 48–720 nm from the peritoneal cavity. In their study, liposomes were composed of egg lecithin, phosphatidic acid, cholesterol and α-tocopherol in the molar ratios of 4:1:5:0.1. Phosphatidic acid has two negative charges per molecule and could contribute to destabilization of vesicles (Drummond et al., 1999). Furthermore, a significant higher uptake for negatively charged liposomes (notably bearing phosphatidic acid) compared to neutral or positive liposomes by peritoneal macrophages has been reported (Aramaki et al., 1995). Thus, it is likely that vesicles instability have affected the peritoneal retention of these four sized liposomes.

In the study of Sadzuka et al. (2000), liposomes were again negatively charged (bearing more than 20 mol% DMPG) and no difference was detected between the peritoneal clearance of 150 and 600 nm vesicles while 4000 nm ones remained in higher levels in the cavity. It should be mentioned that liposomes prepared in our study were relatively neutral with zeta potentials around −9 mV.

In conclusion, all liposomal formulations tested in this study displayed a much higher peritoneal level than the free label. Among the four sizes tested (100, 400, 1000 and 3000 nm), the greatest peritoneal concentration was obtained with 1000 nm liposomes, suggesting this vesicle size is the better choice for further studies. In contrast, the 3000 nm sized vesicles exhibited a relatively low stability and tended to sediment upon abdominal organs and especially the stomach.

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