Effect of Particle Size and Charge on the Disposition of Lipid Carriers After Intratumoral Injection into Tissue-isolated Tumors

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Purpose. Pharmacokinetic properties of various lipid carriers (liposome and emulsions) after intratumoral injection were studied in perfusion experiments using tissue-isolated tumor preparations of Walker 256 carcinosarcoma.

Methods. Four types of lipid carriers, large emulsion (254 nm), small emulsion (85 nm), neutral liposomes (120 nm) and cationic liposomes (125 nm) were prepared. We quantified their recovery from the tumor, leakage from the tumor surface and venous outflow after intratumoral injection into perfused tissue-isolated tumors, and analyzed venous appearance curves based on a pharmacokinetic model.

Results. In contrast to the small emulsion and neutral liposomes, which immediately appeared in the venous outflow perfusate following intratumoral injection, the appearance of the cationic liposomes and the large emulsion was highly restricted, clearly demonstrating that intratumoral clearance of these formulations can be greatly retarded by the cationic charge and large particle size, respectively. The venous appearance rate-time profiles were fitted to equations derived from a two-compartment model by nonlinear regression analysis. When the calculated parameters were compared among these four formulations, the venous appearance rate did not exhibit such a large difference; however, the rate of transfer from the injected site to the compartment which involves clearance by venous outflow was all very different.

Conclusions. The results of this study indicate that the determining factor which alters the pharmacokinetic properties of these lipid carriers after intratumoral injection is not the rate of transfer from the interstitial space to the vascular side but the rate of intratumoral transfer from the injection site to the well-vascularized region.

KEY WORDS: pharmacokinetics; tissue-isolated tumor; liposome; emulsion; intratumoral injection.

INTRODUCTION

Efficient cancer chemotherapy requires a high degree of selective localization of cytotoxic drugs in the tumor. In this context, various drug delivery systems have been proposed and extensively investigated for their potential therapeutic application. Among the various ways of administration, the intratumoral route is a promising approach for solid local tumors to minimize side-effects and maximize cytotoxicity at the tumor site (1–10). However, in most cases, retention of anticancer agents injected intratumorally is considered to be very low because of their low molecular size. Lipid carrier systems, such as liposomes and emulsions, have attracted considerable interest because of their favorable characteristics as a biodegradable drug reservoir.

For the evaluation of the disposition of these lipid carriers in the tumor at the organ level, tissue-isolated tumor preparations are a suitable experimental system. In previous reports, we have described the methodology of experiments and pharmacokinetic analysis for the basic disposition of a model drug (Phenol Red, *Mr;* 354) (11), and the pharmacokinetic behavior of macromolecules (12), plasmid DNA and cationic liposome complexes (13) after intratumoral injection in a perfusion system using the tissue-isolated tumor preparation of Walker 256 carcinosarcoma (14–16).

Despite extensive studies on tumor disposition following systemic administration of various lipid carrier systems and the effects of particle size and charge (17–22), there is little information about drug disposition following their intratumoral injection. In this study, we prepared four lipid carrier formulations consisting of large emulsion (250 nm in diameter), small emulsion (85 nm), neutral liposomes (120 nm) and cationic liposomes (125 nm), and studied the pharmacokinetic behavior of these lipid carriers after intratumoral injection and the effects of particle size and charge on the retention in the tumor.

MATERIALS AND METHODS

Chemicals

Evans Blue (EB) was purchased from Nacalai Tesque, Kyoto, Japan. Bovine serum albumin (BSA) (Fraction V) was obtained from Intergen Co., NY, U.S.A. An O/W emulsion (10%), which is used clinically for parenteral nutrition, was purchased from Otsuka Pharmaceutical Co., Tokushima, Japan (Intralipid®) and diluted with an equal volume of water. Egg phosphatidylcholine (PC) was purchased from Nippon Oil & Fats Co., Hyogo, Japan. 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) was purchased from Avanti Polar-Lipids. Inc., Birmingham, AL. Dimethyldioctadecylammonium bromide (DDAB) was purchased from Sigma Chemical Co., U.S.A. [Cholesteryl-1,2-^{3}H(N)]-Cholesteryl hexadecylether (1905.5GBq/mmol) was purchased from Daiichi Radioisotopes, Tokyo, Japan. All other chemicals were of the finest grade available.

Animals and Tumors

Female SPF Wistar rats weighing 100 to 130 g were obtained from the Shizuoka Agricultural Association for Laboratory Animals, Shizuoka, Japan. The Walker 256 carcinoma was kindly supplied by Shionogi & Co., Osaka, Japan, and was maintained by subcutaneously inoculating rats every 2 weeks.

Preparation of Emulsions and Liposomes

A large emulsion formulation (5% w/w) was prepared by diluting Intralipid® (10%) with an equal volume of water. Its lipid composition is PC: soybean oil = 0.12:1 (w/w). The small emulsion formulation (5% w/w) was prepared with PC and

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ABBREVIATIONS: EB, Evans blue; BSA, bovine serum albumin; EB/BSA, bovine serum albumin labeled with Evans blue; VRS, vascular reference substance; MMC, mitomycin C; MMC-D, Mitomycin C conjugated with dextran.

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soybean oil (1:1, w/w) by sonication (Otake Works, Output 200W, 60 min). Liposomes (5% w/w) were prepared by thin lipid film hydration with HEPES buffer (pH 7.4). Neutral liposomes were prepared with Egg PC and DOPE (1:1, mol/mol). Cationic liposomes were prepared with Egg PC, DOPE and DDAB (2:1:1, mol/mol). Multilamellar vesicles were formed by vortex mixing the lipid dispersions at room temperature for 5 min. Liposomes with homogenous size distribution were obtained by extrusion of the vortex mixed preparation through double polycarbonate membranes of 0.2-(5 times) or 0.1-μm (10 times) pore size (Nucleopore), using an extruder device from Lipex Biomembranes (Vancouver, BC, Canada). When high-phase-transition phospholipids were used, the extruder device was preheated to 45°C.

Liposome size was determined by dynamic light scattering (Super Dynamic Light Scattering Photometer, LS-900, Otsuka Electronics Co., Ltd., Osaka, Japan).

Emulsions and liposomes were radiolabelled with ^{3}H-Cholesteryl hexadecylether and stored at 4°C and room temperature, respectively, and tested within 3 days of preparation.

Preparation of Tissue-isolated Tumors

Ovarian tissue-isolated tumors were prepared as previously reported (11). Briefly, minced Walker 256 carcinosarcoma was inoculated into the adipose tissue around the ovary and the inoculated adipose tissue was then wrapped with Sealon film to separate it from other tissue. The tumor-inoculated tissue was placed between the cutaneous tissue and muscle wall in the abdomen. Then, 10–14 days after tumor inoculation, when the tumor had grown to 10–15 mm in diameter, it was used for the perfusion experiment.

Perfusion of Tissue-isolated Tumors

The tissue-isolated tumor was perfused as previously reported (11,14,15). Briefly, the inferior vena cava and aorta were cannulated with vinyl tubes and perfused at a rate kept constant by arterial pressure (40–70 mmHg). During the first 25 min at a constant perfusion rate, the perfusion pressure continued to decrease while the tumor was cleared of erythrocytes, as confirmed by direct observation of the tumor periphery and venous outflow, and a constant perfusion pressure was maintained.

To obtain basic physiological information for each tumor preparation, indicator dilution experiments were carried out as previously reported (11,14) prior to the intratumoral injection experiments. Briefly, EB/BSA, a vascular reference substance (VRS), was injected from the arterial side of the tumor using a six-position valve injector as a pulse function. After measuring the concentration of the outflow sample, dilution curves were analyzed using moment theory (24). Statistical moment parameters were calculated to estimate the intravascular volume of each tumor preparation.

After additional perfusion for 15 min, radiolabelled lipid carriers were directly injected into the perfused tumors.

Intratumoral Injection Experiments

^{3}H-labelled lipid carriers (0.5–1 μCi/injected dose in 100 μl distilled water) were injected over 30 s into the center of the tumor. The injection site was covered with Sealon film (about 1 cm in diameter) and the injection needle was left in the tumor during the perfusion experiment to prevent fluid leakage from the injection site. The venous effluent was collected into previously tared test-tubes for 120 min, at appropriate time intervals (at first 1 min, subsequently 2–6 min). The fluid leaking from the tumor surface was also collected in a Petri dish. The amount of fluid in each sample was calculated from the gain in weight of the tube, assuming the density of the outflow perfusate to be 1.0 g/cm^3. To measure the level of ^{3}H-labelled lipid carriers, tumors were homogenized and tissue samples (approximately 0.1 g) were placed into counting vials and 0.7 ml Soluen-350 (Packard, Downers Grove, IL) was added. The mixture was heated overnight at 50°C, cooled to room temperature, and 0.2 ml 2N hydrogen chloride was added. The radioactivity in the tissue sample and outflow was determined in a liquid scintillation counter after adding 5 ml scintillation fluid.

Pharmacokinetic Analysis of Outflow Pattern

The venous curve for the appearance of injected material was analyzed using the pharmacokinetic model reported in detail (Fig. 1) (11,13). In this model, the tumor tissue is assumed to be composed of two compartments, one well-perfused and the other poorly-perfused. The material in the well-perfused region is assumed to be quickly cleared from the vascular side and that in the poorly-perfused region is assumed to be transferred to the well-perfused region or to leak out. The poorly-perfused region is assumed to have little blood supply. The well-perfused region, in contrast, is assumed to consist of vascular space and its surrounding area, which is in equilibrium with the vascular space. Based on this model, the following equations were derived to describe the change in the amount of the injected material in these two regions with time.

$$\frac{dX_1}{dt} = k_1X_2 - k_2X_1 \quad (1)$$

$$\frac{dX_2}{dt} = -(k_1 + k_3)X_2 \quad (2)$$

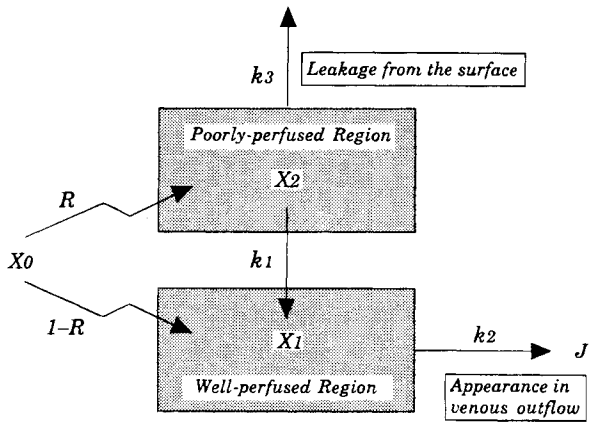

Fig. 1. Pharmacokinetic model for the analysis of carrier disposition after intratumoral injection. k_1, rate constant of transfer from the poorly-perfused region to the well-perfused region; k_2, venous appearance rate constant; k_3, the rate constant of leakage from the surface; R, dosing ratio into the poorly-perfused region; X_0, injected dose; X_1, amount of carriers in the well-perfused region; X_2, amount of carriers in the poorly-perfused region; J, appearance rate in venous outflow.

where X_1 and X_2 are the amounts of material in the well- and poorly-perfused regions (% dose), respectively; k_1 is the rate constant of transfer from the poorly-perfused region to the well-perfused region (/min); k_2 is the venous appearance rate constant (/min); and k_3 is the rate constant of leakage from the surface (/min). Integration of Equations (1) and (2) gives

$$J = Ae^{-\alpha t} + Be^{-\beta t} \quad (3)$$

$$A = k_2 X_0 \frac{k_1 + (1 - R)(k_3 - k_2)}{k_1 + k_3 - k_2} \quad (4)$$

$$B = -\frac{k_1 k_2 R X_0}{k_1 + k_3 - k_2} \quad (5)$$

$$\alpha = k_2 \quad (6)$$

$$\beta = k_1 + k_3 \quad (7)$$

where J is the appearance rate in the venous outflow, which is equal to k_2X_1 (% dose/min); X_0 is the injected dose (100%); and R is the dosing ratio to the poorly-perfused region. The venous outflow pattern for each tumor preparation was then fitted to Equation (3) using the nonlinear regression program MULTI (25) to estimate the pharmacokinetic parameters.

RESULTS

Appearance of Various Lipid Carrier Formulations after Intratumoral Injection

Fig. 2 shows the mean venous appearance curves of various lipid carrier systems after intratumoral injection. The small emulsion and neutral liposome formulations appeared immediately in the venous outflow and about 30–50% of the injected dose was recovered 10 min after intratumoral injection. On the other hand, the appearance of the large emulsion formulation was highly restricted and its venous appearance rate was very low during the period of the perfusion experiment (2 hr). Furthermore, the venous appearance of the cationic liposome formulation was more restricted than that of the large emulsion formulation.

Recovery of Various Lipid Carrier Formulations in Outflow, Extruded Fluid, and Tumor Tissue after Intratumoral Injection

Recoveries of various lipid carrier formulations 120 min after intratumoral injection are shown in Fig. 3. In the case of injection of the small emulsion and neutral liposome formulations, a large amount of the injected dose appeared in the venous outflow (35–50% of dose recovered in first 1 min after injection) and tumor surface, and only about 10–40% of the injected dose remained in the tumor tissue. On the other hand, a large amount of the large emulsion and cationic liposome formulations remained in the tumor, and about 70% and 90% of injected dose remained in the tumor 2 hr after intratumoral injection, respectively.

Pharmacokinetic Analysis

Table II summarizes the pharmacokinetic parameters obtained in this experiment. Rate constants of transfer from the poorly-perfused region to the well-perfused region (k_1) for the large emulsion and cationic liposomes were significantly smaller than those for the small emulsion and neutral liposomes, on the other hand, the venous appearance rate constants (k_2) of these four formulations exhibited no significant differences.

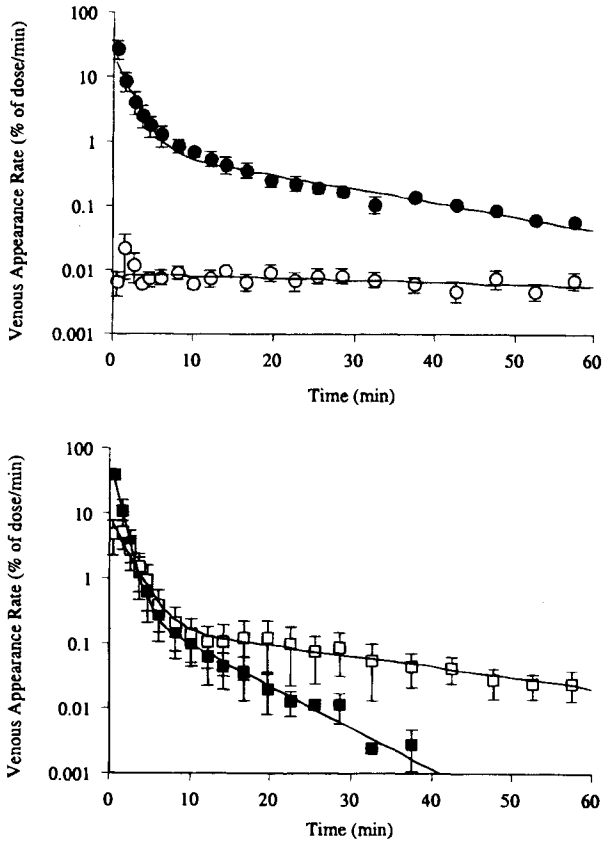

Fig. 2. Venous appearance curves of lipid carriers after intratumoral injection into tissue-isolated perfused tumors. ●, neutral liposomes; ○, cationic liposomes; ■, small emulsion; □, large emulsion. Each solid line represents the fitting line based on the pharmacokinetic model shown in Figure 1. Results are expressed as the mean ± SE. of three experiments.

Table I. Characteristics of Lipid Carriers Tested in this Perfusion System

Formulations and Composition	Total Lipid Concentration (%)	Mean[c] Diameter (nm)	Zeta[d] Potential (mV)
Large Emulsion[a] (eggPC/soybean oil 0.12:1)	5	254.0 ± 5.1	−26.2
Small Emulsion[b] (eggPC/soybean oil 1.1)	5	85.5 ± 18.4	−1.9
Neutral Liposomes (eggPC/DOPE 1:1)	5	120.0 ± 15.2	−5.4
Cationic Liposomes (eggPC/DOPE/DDAB 2:1:1)	5	125.0 ± 29.4	+47.6

[a] O/W emulsion used clinically for parenteral nutrition (Intralipid®).
[b] O/W emulsion prepared by sonication method.
[c] Measured by Dynamic Light Scattering Spectrophotometer.
[d] Measured by Electrophoretic Light Scattering Spectrophotometer.

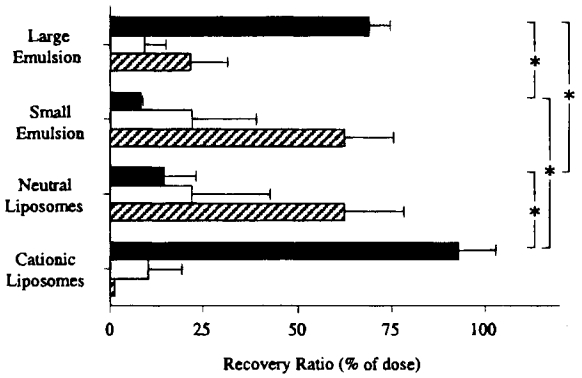

Fig. 3. Recovery ratios of lipid carriers 2 h after intratumoral injection into tissue-isolated perfused tumors. ▨, recovery in venous outflow; □, leakage from surface of tumor; ■, remaining in tumor tissue. Results are expressed as the mean ± SE. of three experiments. There is a statistically significant difference by ANOVA: (*) $P < 0.001$.

The value of k_1 for the small emulsion is about 12 times that for the large emulsion, and the cationic charged liposomes significantly reduced (1/180) rate of transfer compared with the neutral liposomes in spite of having the same particle size. On the other hand, the value of k_3 varied greatly among the tumors tested in this study.

DISCUSSION

Direct injection of tumors with various anticancer agents has several advantages over systemic administration. For example, lower doses can be used when injecting the tumor site, thereby reducing side-effects. However, most anticancer agents are small molecules and their intratumoral clearance is relatively rapid. In a previous study, we evaluated the intratumoral behavior of mitomycin C (MMC) and its dextran conjugates (MMC-D) after intratumoral injection using tissue-isolated tumor perfusion systems (12). In contrast to MMC in the free form, which immediately appeared in the venous outflow perfusate following intratumoral injection, the intratumoral clearance of MMC can be greatly retarded by conjugation with cationic and anionic dextran.

Another approach to promote intratumoral retention of low molecular weight drugs after intratumoral injection is to use lipid carrier systems, such as liposomes and emulsions. Many investigators have reported the accumulation of lipid carrier systems in tumors after i.v. injection, and evaluated the effect of particle size and surface charge (17–22). However, in the case of intratumoral injection, there is little information on the actual disposition characteristics of lipid carrier systems in the tumor tissue. We have assessed the effect of the physicochemical properties of the lipid carriers, such as particle size and surface charge, on their disposition in the tumor after direct injection using tissue-isolated tumor perfusion systems.

The size of the small emulsion and neutral liposomes used in this study (mean diameter, 85 nm and 120 nm, respectively) is comparable with or smaller than the size of the vascular fenestrae in tumor tissue (21–23). In the perfusion experiments, the intratumoral clearance of the small emulsion and neutral liposomes was relatively large, however in the case of the large emulsion (mean diameter, 250 nm), it was very restricted. These results indicate that particle size is an important determinant of the retention time of these lipid carriers in the isolated tumors after intratumoral injection, and it is considered that there is a critical size, between 120 and 250 nm, which markedly alters the intratumoral clearance of lipid carriers.

Although the cationic and neutral liposomes have a similar particle size distribution, 90% of the administered cationic liposomes remained in tumor tissue 2 hr after intratumoral injection. Our previous studies demonstrated that cationic macromolecules (mitomycin C-cationic dextran conjugates *Mr;* 70,000) largely remained in tumor after intratumoral injection compared with anionic ones due to electrostatic interaction (12). The same mechanism can account for the interaction of positively charged liposomes with the negative charges of the glycocalyx on the tumor cell surface and components in the interstitium such as proteoglycans. In contrast, neutral lipid carriers are only distributed in the interstitial fluid, without any tissue interaction, and quickly appear in the venous outflow with convective flow.

After intratumoral injection of lipid carriers, there are two processes of transfer in tumors resulting in their appearance in the venous outflow. One is the transfer in the interstitial space from the poorly-perfused injected site (center of the tumor) to the well-vascularized peripheral tissue. The other is transfer from the well-perfused region to the vascular side. There are many reports about the vascular permeability of blood-oriented lipid carriers in tumors and the maximum diameter of vessel pores in tumors is less than 200 nm (21,23) or larger (22). However, there is little information about their permeability from the interstitial space to the vascular side after direct intratumoral injection. In order to estimate the rate of transfer of the lipid carriers in these processes in tumor tissue, their venous appearance curves were analyzed and their pharmacokinetic parameters were derived using a two-compartment model (Table II). The values of k_2 for four types of lipid carriers were not

Table II. Pharmacokinetic Parameters of Lipid Carriers After Intratumoral Injection

	Pharmacokinetic Parameters			
	$k_1{}^a$ ($\times 10^{-3}$ min^{-1})	k_2 ($\times 10^{-1}$ min^{-1})	$k_3{}^a$ ($\times 10^{-2}$ min^{-1})	R^a
Large Emulsion	2.32 ± 1.82*	6.65 ± 1.21	3.02 ± 0.55***	0.862 ± 0.067***
Small Emulsion	24.42 ± 6.58	24.96 ± 13.07	19.96 ± 2.44	0.226 ± 0.044
Neutral Liposomes	14.81 ± 8.28	7.74 ± 2.23	3.66 ± 0.25***	0.702 ± 0.125**
Cationic Liposome	0.081 ± 0.017*	4.44 ± 1.62	0.96 ± 0.69***	0.999 ± 0.000059***

Results are expressed as the mean ± S.E. of three experiments.
[a] Statitically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by ANOVA test as compared with that of the group injected with small emulsion.

so different, however they slightly increased as the particle size decreased. In contrast, the values of k_1 were significantly smaller than k_2 and showed great variation with the particle size and charge of the lipid carriers. Increase of the mean particle size from 85.5 nm (small emulsion) to 254.0 nm (large emulsion) and the cationic charge (cationic liposomes) greatly altered the rate of transfer from the poorly-perfused region to the well-perfused region represented by the value of k_1. These results indicate that the value of k_1 is the rate-limiting step that determines the retention time of carriers in the tumor after direct injection. The value of R increased especially in the case of cationic liposomes and large emulsion compared to small emulsion (Table II). The reason, we believe, is that the values of R are determined not only by the anatomical architectures of tumor tissues but also by the physicochemical properties of the carriers. Immediately after the carriers were injected into the tissues, they would spread into the tissues according to the injection pressure. In this case, the degree of distribution of the carriers may be influenced by the interaction with the tissues and the size-dependent repulsion by the interstitial network. This may be the reason why the values of R for cationic liposome and large emulsion were larger.

The amounts in tumor, leakage, and venous outflow at the end of the experiment were calculated using disposition parameters listed in Table II. The amount in tumor was underestimated as compared to the observed one, whereas the amount in leakage was overestimated (data not shown). This suggests that k_3 may not necessarily reflect the rate of leakage out of tumor. It may include irreversible binding to the tumor tissues and transfer to the other nontumorous capillaries than indicated by the amount recovered.

We have previously tested other substances which have different physicochemical characteristics, such as low-molecular weight drugs (mitomycin C; MMC) (11,12), macromolecules (mitomycin C-dextran conjugates; MMC-D) (12) and a gene (plasmid DNA) (13) in the same experimental system. Interestingly, the small emulsion and neutral liposomes showed similar pharmacokinetic properties to low-molecular weight drugs such as MMC. An increase in particle size restricted the rate of transfer represented by k_1 and the large emulsion exhibited a higher retention than macromolecules such as plasmid DNA and a macromolecular prodrug (MMC-D). The addition of a cationic charge to the neutral liposomes greatly retarded this rate and cationic liposomes showed the highest retention in tumor tissue. Overall, alteration of the physicochemical properties of drugs and carriers changed the value of k_1 markedly and the variation in the value of k_2 was small, indicating that the rate of transfer from the poorly-perfused region to the well-perfused region is the determining factor which alters the intratumoral behavior of drugs and carriers after direct injection.

In summary, the present study demonstrates that the particle size and surface charge of lipid carriers are important factors affecting their pharmacokinetic properties after intratumoral injection. These findings will be useful for the development of novel lipid carriers for local cancer chemotherapy and gene therapy.

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