

# Review

## Lipid Carrier Systems for Targeted Drug and Gene Delivery

Mitsuru HASHIDA,\* Shigeru KAWAKAMI, and Fumiyoshi YAMASHITA

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University;  
Sakyo-ku, Kyoto 606–8501, Japan.

Received April 4, 2005

For effective chemotherapy, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and side effects. The use of lipid dispersion carrier systems, such as lipid emulsions and liposomes, as carriers of lipophilic drugs has attracted particular interest. A drug delivery system can be defined as a methodology for manipulating drug distribution in the body. Since drug distribution depends on the carrier, administration route, particle size of the carrier, lipid composition of the carrier, electric charge of the carrier and ligand density of the targeting carrier, these factors must be optimized. Recently, the lipid carrier system has also been applied to gene delivery systems for gene therapy. However, in both drug and gene medicine cases, a lack of cell-selectivity limits the wide application of this kind of drug and/or gene therapy. Therefore, lipid carrier systems for targeted drug and gene delivery must be developed for the rational therapy. In this review, we shall focus on the progress of research into lipid carrier systems for drug and gene delivery following systemic or local injection.

**Key words** drug delivery system; liposomes; lipid emulsion; gene delivery; targeting

### 1. Introduction

For effective chemotherapy, it is necessary to deliver therapeutic agents selectively to their target sites, since most drugs are associated with both beneficial effects and side effects. In general, the lack of selectivity of most conventional drugs is closely related to their pharmacokinetic properties. The *in vivo* fate of a drug given by a particular administration route is determined by both the physicochemical properties of drug and the anatomical and physiological characteristics of the body. Most conventional drugs diffuse freely throughout the body and show relatively even tissue distribution due to their low molecular weight.<sup>1)</sup>

The use of lipid dispersion carrier systems, such as lipid emulsions and liposomes, as carriers of lipophilic drugs has attracted particular interest. A drug delivery system can be defined as a methodology for manipulating drug distribution in the body. Since the drug distribution of loaded lipid carriers varies depending on; i) administration route (*i.e.*, local or systemic injection) of the carrier, ii) drug release from the carrier, iii) lipid composition and electric charge of the carrier, and iv) particle size of the carrier, these factors must be considered.

Recently, lipid carrier systems have also been applied to gene delivery systems for gene therapy.<sup>2)</sup> The most important factor for successful gene therapy is the development of novel gene vectors; therefore, various viral vectors and non-viral vectors have been developed. Although the gene transfer efficacy of the current non-viral vector systems is lower than that of viral vectors, the approach seems useful for many applications that require gene expression from the viewpoint of safety. Among the various types of non-viral

vectors, cationic liposome mediated gene transfection seems to be one of the most promising approaches because of its relatively high transfection efficiency.

In both drug and gene medicine cases, however, lack of site (or cell)-selectivity limits the wide application of this kind of drug and/or gene therapy. In this review, we shall focus on the progress of research into lipid carrier systems for drug and gene delivery following systemic or local injection.

### 2. Lipid Emulsions for Drug Delivery Following Local Injection

Lipid emulsions are considered to be superior to liposomes due to the fact that they can be produced on an industrial scale, are stable during storage, are highly biocompatible, and have a high solubilizing capacity as far as lipophilic drugs are concerned because lipid emulsions possess an oil phase in particulate form, so they can dissolve large amounts of highly lipophilic drugs.<sup>3)</sup> An important prerequisite for success in the application of pharmacologically active drugs is site-specificity. Local injection into the diseased tissues is one promising approach. This is particularly applicable to cancer chemotherapy, in which the supply of antitumor drugs to non-diseased tissue leads to serious side effects.

The local retention of anticancer agents injected intratumorally is very low because of the large diffusion capability due to their small molecular size. In our series of studies, we have demonstrated an increased transport and prolonged supply of antitumor drugs to lymphatics with water-in-oil (W/O) emulsions.<sup>4,5)</sup> In addition, the intratumoral injection of antitumor drugs is one of the most promising approaches for solid

\* To whom correspondence should be addressed. e-mail: hashidam@pharm.kyoto-u.ac.jp

local tumors, to minimize the side effects and maximize cytotoxicity at the tumor site.<sup>6-8)</sup> To enhance the retention and/or distribution in the lymph or tumor, lipid emulsions have been used because of their favorable characteristic as a biodegradable drug reservoir. In this section, we shall focus on lipid emulsions for local injection.

**2.1. Distribution Characteristics of Lipid Emulsions or Liposomes Following Intramuscular or Intra-gastric Injection** Figure 1 represents a model of drug transfer to the lymph after topical injection of lipid carrier formulation. As shown in this model, the drug injected into interstitial spaces of tissues is transported away from the injection site by the circulating blood, but reaches the regional lymph nodes to varying degrees, depending on the site of injection.

The lymphatic transport of bleomycin in different formulations; oil-in-water (O/W) and W/O emulsions was investigated.<sup>4)</sup> When O/W and W/O emulsions were utilized as the delivery system, the W/O emulsion was effective in increasing the lymph level, in both cases of intraperitoneal and intramuscular injection. In the emulsion system, the hydrophilic anticancer drug is predominantly located not in the oily phase, but in the aqueous phase; consequently, bleomycin is distributed in the outer phase in the case of the O/W emulsion, and it is encapsulated in the inner phase in the case of the W/O emulsion. Although the utilization of an emulsion seems promising for the facilitation of drug transportation into the lymph, the instability of the emulsion is one of the problems from the viewpoints of pharmaceutical technology. In order to enhance the stability, gelatin sphere-in-oil (S/O) emulsions were developed as a new formulation

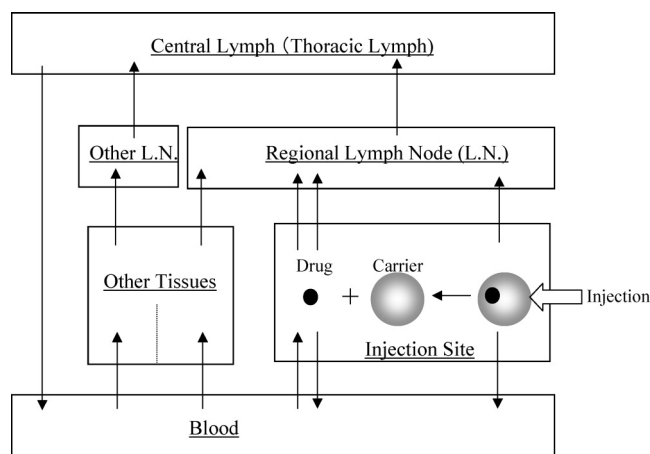


Fig. 1. Schematic Diagram of a Model of Drug Transfer Following the Injection of Lipid Carrier Formulations

for anticancer drugs. The greatest enhancement of the drug delivery and successful prevention of lymphatic metastasis was obtained with S/O emulsions following intramuscular and intragastric injection.<sup>5,9,10)</sup> The lymphatic transport of the S/O emulsion was accelerated by the increase of injection volume and massage of the injection site, suggesting that hydrostatic tissue pressure plays a role in lymphatic delivery.<sup>11)</sup> Furthermore, sphere-in-oil-in-water (S/O/W) multiple emulsions were developed to reduce the viscosity and improve the storage stability of multiple emulsions.<sup>12)</sup> Both S/O and S/O/W emulsions exhibited enhanced lymphatic transfer of bleomycin following injection into the appendices of rabbits (Fig. 2).

In clinical trials, 27 of 33 patients received a bleomycin S/O emulsion injected directly into the tumors with satisfactory results. Comparative studies of treatments between the bleomycin S/O emulsion and surgery indicated that injection therapy of the bleomycin S/O emulsion would be more beneficial than surgical excision.<sup>13)</sup>

As for the O/W emulsion, large logPCoct values or a high lipophilicity are required for drugs in order to keep them in the O/W emulsion. One of the most interesting potential approaches to prolong the retention time in emulsions after local injection is to increase the lipophilicity of the drug by chemical modification, leading to a prodrug.<sup>14,15)</sup> That is, the combined application of lipophilic prodrug to the lipid carrier should achieve controlled drug release.<sup>16)</sup> In fact, the lipophilic prodrug mitomycin C<sup>17)</sup> and 5-fluorouracil<sup>18)</sup> were more stably incorporated into the O/W emulsion and liposomes after intramuscular injection. This approach, the combined application of lipophilic prodrug to lipid carrier, could be applied to the liposomes. After intramuscular injection, liposomes appeared to accumulate at the lymph nodes to a greater degree than O/W emulsions<sup>19)</sup>; accordingly, liposome formulation is an effective approach for the lymph-selective drug delivery carrier. However, a distribution study of liposomes with incorporated drugs demonstrated that hydrophilic drugs were rapidly released from the liposomal formulations after intramuscular injection. In contrast, nonylloxycarbonyl mitomycin C was completely incorporated in the liposomes. In addition, we confirmed that nonylloxycarbonyl mitomycin C incorporated liposomes enhanced drug delivery to the regional lymph nodes after intramuscular injection. Drug incorporation efficacy into the liposomes depends not only on the lipophilicity of drugs but also on the type used in the lipid of liposomes<sup>20)</sup>; therefore, both the physicochemical properties of the drug and the lipid formulation should be considered with liposomal drug delivery systems.

Mitsuru Hashida, Ph.D. is the Dean and Professor of the Graduate School of Pharmaceutical Sciences at Kyoto University. He received his B.S., M.S., and Ph.D. degrees from the Faculty of Pharmaceutical Sciences, Kyoto University in 1974, 1976, and 1979. Dr. Hashida is currently serving as Vice-President of the FIP and Academy of Pharmaceutical Science and Technology, Japan (APSTJ), and President of the Japan Society of Drug Delivery Systems (JSDDS). His research interests include the development and evaluation of novel drug delivery systems and the analysis of oral and transdermal drug absorption and is the author or co-author of about 300 articles.



Mitsuru Hashida

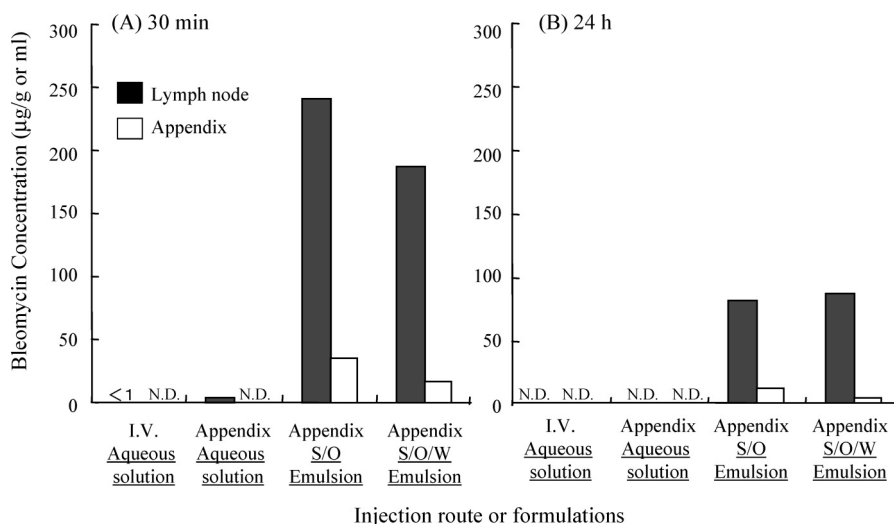


Fig. 2. Bleomycin Concentration in the Lymph Node and Appendix after Injection in Rabbits Using Various Formulations

Each result is the mean value of three experiments. N.D.; not detected. I.V.; intravenous.

**2.2. Distribution Characteristics of Lipid Emulsions Following Intratumoral Injection** Efficient cancer chemotherapy requires a high degree of selective localization of antitumor drug in the tumor. In this context, various drug delivery systems have been proposed and extensively investigated for their potential therapeutic application. Among the various routes of administration, the intratumoral route is a promising approach for solid local tumors to minimize the side effects and maximize cytotoxicity at the tumor site.<sup>6–8</sup> However, in most cases, the retention of antitumor drugs injected intratumorally is considered to be very low because of their low molecular size. In order to improve the retention of these drugs, lipid carriers could be used as a drug reservoir.

In order to clarify the distribution characteristics of injected drugs or carrier formulations after direct intratumoral injection, a Walker 256 tissue-isolated tumor perfusion system was employed.<sup>21–23</sup> This is a unique system, composed of a solid tumor with a pair of supplying arteries and a draining vein, and enabled us to study the intratumoral pharmacokinetics of a variety of materials, independent of the systemic circulation. The pharmacokinetics of anticancer drugs, macromolecular prodrugs and drug carriers following intra-arterial infusion or direct injection into the tumor have been studied with this system.

Using tumor-perfusion systems, the effect of the size of emulsions was studied.<sup>24</sup> A large emulsion (250 nm in diameter) and a small emulsion (85 nm in diameter) were prepared. Each formulation was labeled with [<sup>3</sup>H]cholesteryl hexadecyl ether. In the case of the small emulsion formulations, a large fraction of the injected dose appeared in the venous outflow, 35–50% of the dose was recovered in the first minute after injection, on the tumor surface, and only about 10–40% of the injected dose remained in local tumor tissue. On the other hand, the large emulsion formulations remained in the tumor for a considerably longer time, and about 70% of the injected dose remained in the tumor 2 h after intratumoral injection. These results indicate that particle size is an important determinant of the retention in the tumor after intratumoral injection.

### 3. Lipid Emulsions for Drug Delivery Following Systemic Injection

O/W lipid emulsions and liposomes, as carriers of lipophilic drugs, have attracted particular interest following systemic injection. In particular, lipid emulsion formulations are considered to be superior to others due to the fact that they can be produced on an industrial scale, are stable during storage, and are highly biocompatible. In fact, emulsion formulations of lipophilic drugs, such as prostaglandin E<sub>1</sub>, diazepam and non-steroidal anti-inflammatory drugs, have already been developed and marketed.<sup>25–27</sup> With recent pharmaceutical and therapeutic developments, lipid emulsions have been introduced as parental drug carriers offering sustained release and organ targeting. In this section, we shall focus on the progress of research into passive and active targeting systems of emulsions after systemic injection.

**3.1. Distribution Characteristics of Lipid Emulsion Following Systemic Injection** In order to clarify the distribution characteristics of emulsions after systemic injection, a pharmacokinetic study was performed using a [<sup>14</sup>C]-labeled cholesteryl oleate labeled emulsion. After intravenous injection, the large emulsion (about 280 nm in diameter) rapidly disappeared from the blood and about 60% of the dose was recovered in the liver within 10 min of its intravenous injection in mice.<sup>28</sup> On the other hand, small emulsions (about 100 nm in diameter) showed a reduced hepatic uptake and a prolonged blood circulation time. A pharmacokinetic analysis revealed that the small emulsion has an 8- to 100-times smaller organ distribution clearance by the liver, spleen and lungs and about a 4-times greater area under the plasma concentration–time curve (*AUC*) than the large emulsion. Single-pass rat liver perfusion experiments have shown that more than 70% of the large emulsion was extracted by the liver, indicating extensive uptake of the large emulsion during a single passage.<sup>29</sup> In addition, the large emulsion was predominantly recovered from liver non-parenchymal cells (NPC), including Kupffer cells, and showed a higher accumulation in the NPC fraction.

Sphingomyelin (SM) is known to stabilize the membrane structure of liposomes and the addition of SM to liposomes

has been reported to be effective in reducing their clearance by the RES.<sup>30–32</sup> In order to develop a stable emulsion in blood, we developed novel emulsions composed of soybean oil and egg yolk SM (SM emulsion).<sup>28</sup> After intravenous injection, the SM emulsion showed a prolonged retention in the blood circulation. The uptake clearance of the SM emulsions in the liver was about 4-times less than that of conventional emulsions, suggesting reduced clearance by the RES.

**3.2. Galactosylated Emulsions for Asialoglycoprotein Receptor-Mediated Drug Delivery to Hepatocytes** Cell-specific drug targeting is sometimes urgently required for a variety of clinical purposes; however, there are few reports of cell-specific drug targeting using lipid emulsions. Recently, Rensen *et al.* developed novel apo E associated emulsions for hepatocyte targeting.<sup>33</sup> These apo E associated emulsions are reported to be selectively taken up by liver parenchymal cells (PC) and are useful for the delivery of antiviral drugs, such as iododeoxyuridine, to hepatocytes. However, the introduction of apo E to the carrier is rather complicated, and so there can be problems as far as the reproducibility and stability of apoE emulsions are concerned.

Receptors for carbohydrates, such as the asialoglycoprotein receptor on hepatocytes and the mannose receptor on several macrophages and liver endothelial cells, recognize the corresponding sugars on the non-reducing terminal of sugar chains. The lipid emulsion (oil-in-water) surface exhibits aqueous properties; thus a galactose moiety could cover the emulsion surface. It was reported that a lipophilicity exceeding  $\log PC_{oct} = 8$ <sup>34</sup> or 18<sup>35</sup> was required for the stable entrapment of drugs in O/W emulsions after intravenous injection; accordingly, ligand modified lipids should possess a high lipophilicity for efficient delivery *in vivo*.

Our strategy for the efficient targeting of lipid carriers by glycosylation is to achieve stable fixation of the sugar moiety on the surface of the liposomes under *in vivo* conditions. Therefore, cholesterol was chosen as a hydrophobic anchor, which is stably associated with the liposomal membrane<sup>36,37</sup> and only mono-galactoside was introduced to the lipid as a ligand because the introduction of many hydrophilic galactose moieties to a lipid anchor would result in their removal from liposomes by interacting with lipoproteins and/or other lipid compartments under *in vivo* conditions.<sup>38</sup> We synthesized a novel galactosylated cholesterol derivative, *i.e.*, cholesten-5-yloxy-*N*-(4-((1-imino-2-D-thiogalactosylethyl)amino)butyl) formamide (Gal-C4-Chol), to modify lipid carriers with galactose moieties for hepatocyte targeting.<sup>39</sup> When Gal-C4-Chol was added to O/W emulsions, a hydrophilic galactose moiety was fixed to the particle surface. Figures 3A and B show the scheme of ligand modified lipid carriers for cell-selective drug delivery.

After intravenous injection, galactosylated emulsions (Gal-emulsions) were rapidly eliminated from the blood and accumulated in the liver, in contrast to the bare-emulsions.<sup>40</sup> The liver uptake clearance of the Gal-emulsions was 3.2-times greater than that of the bare-emulsions. The uptake ratio in liver PC and NPC of the Gal-emulsions was higher than that of the bare-emulsions, suggesting that Gal-emulsions are effective PC-selective carriers. The hepatic uptake of Gal-emulsions, but not that of bare-emulsions, was significantly inhibited by predosing not only with lactoferrin but also Gal-liposomes, suggesting an asialoglycoprotein recep-

tor-mediated endocytosis. Thus, Gal-emulsions have been proven to be an alternative carrier for hepatocyte-selective drug targeting after intravenous injection.

#### 4. Liposomes for Drug Delivery Following Local Injection

Similar to emulsions, the intratumoral injection of antitumor drugs or genes with liposomes is one of the most promising approaches for solid local tumors to minimize side effects and maximize cytotoxicity at the tumor site. Liposomes have also been used because of their favorable characteristics as a biodegradable drug or gene medicine reservoir. In this section we shall focus on the local distribution characteristics of liposome formulations after intratumoral injection have become an important issue in drug or gene delivery.

**4.1. Distribution Characteristics of Liposomes Following Intratumoral Injection** Since drugs incorporated in liposomes are distributed with liposomes, the distribution characteristics of liposomes after intratumoral administration are important. To investigate the effect of size or charge on distribution, neutral liposomes (120-nm in diameter), and cationic liposomes (125-nm in diameter) were prepared.<sup>24</sup> Each formulation was labeled with [<sup>3</sup>H]cholesteryl hexadecyl ether. The zeta potentials of neutral liposomes and cationic liposomes were  $-5.4$  and  $47.6$  mV. The pharmacokinetic properties of the gene were studied after direct intratumoral injection using a Walker 256 tissue-isolated tumor perfusion system. After intratumoral injection, approximately 90% of the administered cationic liposomes remained in the tumor while the corresponding figure for neutral liposomes was 18%. Since the size of each liposome was almost the same, cationic liposomes may remain in the tumor due to the electrostatic interaction after intratumoral injection.

**4.2. Distribution and Gene Expression Characteristic of pDNA and Its Complex with Cationic Liposomes after Intratumoral Injection** Wolff *et al.* reported that naked pDNA in the skeletal muscle after intramuscular injection is specifically located in T tubules and/or caveolae specific to striated muscle, and these structures may play an important role in the uptake rather than physical disruption of the membrane of myotubes with direct injection.<sup>41</sup> We applied this method to the pharmacokinetic evaluation of naked pDNA and its cationic liposome complexes.<sup>42</sup> The pharmacokinetic properties of the gene were studied after direct intratumoral injection using a Walker 256 tissue-isolated tumor perfusion system.<sup>21–23</sup> Approximately 50% of the naked pDNA was eliminated from the tumor 2 h after injection and intact pDNA was found in the venous outflow, while more than 90% of the pDNA was retained in the tumor when complexed with cationic liposomes (Lipofectin<sup>®</sup>), suggesting that the cationic liposomes increase the retention of pDNA in the tumor tissue due to electrostatic interaction which results in less appearing in the venous outflow.

Gene expression was assessed in three types of solid mouse tumors after the direct injection of naked pDNA encoding the luciferase gene (pCMV-Luc) and its DC-Chol liposome complexes.<sup>43</sup> The intratumoral injection of naked pCMV-Luc into subcutaneously inoculated mouse colon tumor (CT-26), fibrosarcoma (MCA-15) and bladder carcinoma (MBT-2) resulted in significant gene expression regardless of the rapid clearance from the injection site. Sur-

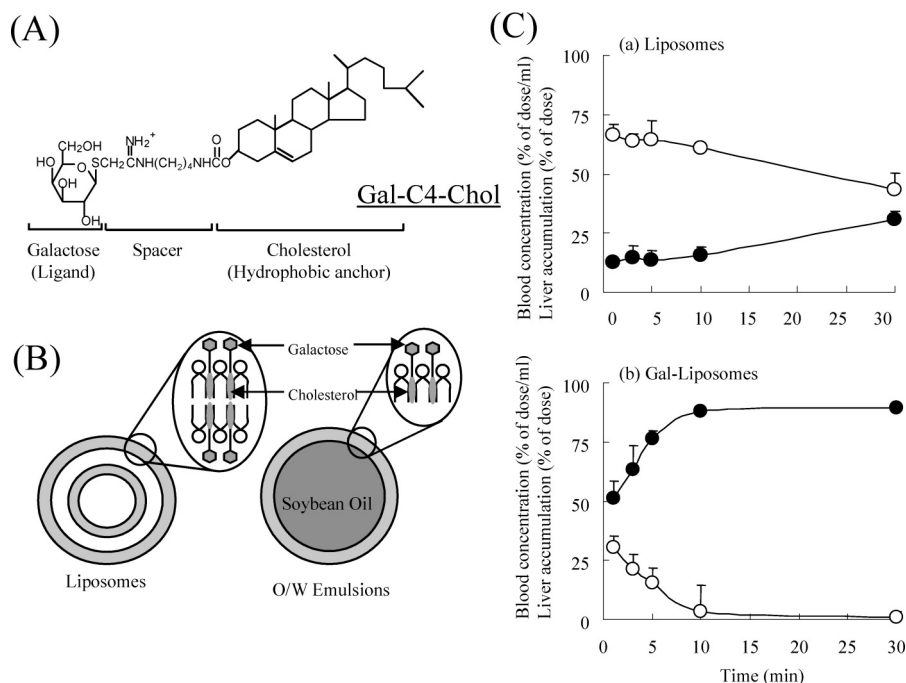


Fig. 3. (A) Structure of Gal-C4-Chol, (B) Scheme of Galactosylated Liposomes and Emulsions Using Gal-C4-Chol, and (C) Blood Concentration (○) and Liver Accumulation (●) of [ $^3$ H]cholesteryl Hexadecyl Ether Labeled Liposomes (a) and Gal-Liposomes (b) Following the Intravenous Injection into Mice

Liposomes and Gal-liposomes were composed of DSPC, Chol, Gal-C4-Chol at a molar ratio of 12:8 and 12:7:1, and the lipid concentration was adjusted to 5 mg/ml. Each value represents the mean  $\pm$  S.D. of three experiments.

prisingly, a cationic liposome formulation, which was expected to have a longer retention in the tumor, showed a lower level of gene expression in these tumor models. Moreover, increasing the cationic charge of the lipoplex decreased the gene expression in the tumors. These results suggested that free pDNA might affect the gene expression following intratumoral injection.

**4.3. Gene Expression Characteristics of pDNA and Its Complex with Cationic Liposomes after Intravitreal Injection** To optimize the *in vivo* ocular transfection efficiency of pDNA/cationic liposome complexes, DOTMA/DOPE liposomes and DOTMA/cholesterol liposomes were prepared with varying amounts of pDNA.<sup>44</sup> pDNA/cationic liposome complexes were intravitreally injected in rabbits, and the luciferase activity in the cornea, aqueous humor, iris-ciliary body, lens, vitreous body and retina were measured. In the case of intravitreal injection, the gene expression in ocular tissues of the lipoplex was markedly higher than those of naked pDNA. Taking the intratumoral injection results into consideration, the gene expression characteristics after local injection (*i.e.*, naked pDNA *vs.* lipoplex) differed from the injection site.

## 5. Liposomes for Drug Delivery Following Systemic Injection

Liposomes are an established example of a lipid carrier system that has been researched extensively. After intravenous injection, they are commonly retained in the blood circulation and removed by the RES. Thus, their application is mainly limited to persistent retention in the blood circulation and passive targeting to the RES or solid tumors with a highly permeable capillary endothelium. Since an ideal drug therapy has high therapeutic efficacy with few side effects, cell-specific targeting of liposomes is sometimes urgently re-

quired for a variety of clinical purposes.

**5.1. Galactosylated Liposomes for Asialoglycoprotein Receptor-Mediated Drug Delivery to Hepatocytes** Receptor-mediated drug delivery is a promising approach to site-selective drug delivery. Receptors for carbohydrates, such as the asialoglycoprotein receptor on hepatocytes (liver PC) and the mannose receptor on several macrophages and liver endothelial cells, recognize the corresponding sugars on the non-reducing terminal of sugar chains.<sup>2</sup> This mechanism would be an effective way to achieve hepatocyte targeting.

For the application of drug targeting systems to liposomes, we developed Gal-C4-Chol to modify liposomes with galactose moieties for hepatocyte-selective drug delivery (Figs. 3A, B).<sup>45</sup> As mentioned for Gal-emulsions, our strategy for the efficient targeting of lipid carriers by glycosylation is achieved by stable fixation of the sugar moiety on the surface of the liposomes under *in vivo* conditions. Since cationic charge enhances the non-specific interaction, galactosylated liposomes for drug delivery were prepared with Gal-C4-Chol, neutral lipid (distearoylphosphatidylcholine) and cholesterol. Each formulation was labeled with [ $^3$ H]cholesteryl hexadecyl ether. After intravenous injection, galactosylated liposomes (Gal-liposome) rapidly disappeared from the blood and 85% of the dose had accumulated in the liver within 10 min, while the hepatic accumulation of bare liposomes was 12% (Fig. 3C). The liver was perfused with collagenase, and liver PC and NPC were separated by centrifugal differentiation to determine the cellular distribution. The PC/NPC ratios for Gal-liposomes and bare-liposomes were 15.1 and 1.1, respectively, indicating the PC-selectivity in Gal-liposomes. Furthermore, the hepatic uptake of Gal-liposome liposomes was significantly inhibited by the predosing of galactosylated bovine serum albumin, but not by that of bare-liposomes. These results indicated that Gal-liposomes

Table 1. Various Factors on *in Vivo* Gene Expression by Lipoplex

Factors	Effect on gene expression	Ref.
1. Lipoplex		
Charge	High cationic charge enhances gene expression	60, 61, 62, 63, 66
2. Cationic liposomes		
Helper lipid	Cholesterol containing liposomes enhance gene expression	58, 62, 64, 67
Size	Large sized liposomes enhance gene expression	62
3. pDNA		
Dose	High dose enhances gene expression	59, 60, 61, 63, 64, 66
CpG motif	CpG motif in pDNA induces the inflammatory cytokines; as a consequence, the terms of gene expression are decreased	81,82

are efficiently taken up by the asialoglycoprotein receptor on PC after intravenous injection.

These Gal-liposomes were able to effectively deliver prostaglandin E<sub>1</sub><sup>46)</sup> and probucol<sup>47)</sup> to hepatocytes *in vivo*, indicating that Gal-liposomes function as hepatocyte-selective drug carriers of lipophilic drugs. Moreover, the recognition by asialoglycoprotein receptors of Gal-liposomes *in vivo* may be affected by the cholesterol contents<sup>48)</sup> and lipid compositions<sup>47,49)</sup> in Gal-liposomes.

**5.2. Mannose and Fucose Liposomes for Mannose and Fucose Receptor-Mediated Drug Delivery to Liver NPC** After intravenous injection, we have demonstrated that mannosylated<sup>50)</sup> and fucosylated<sup>51)</sup> proteins are efficiently taken up by NPC, mainly composed of sinusoidal endothelial cells and Kupffer cells, and this uptake was mediated by mannose and fucose receptor mediated endocytosis.<sup>52)</sup> Based on these observations, we synthesized two glycolipids, cholesten-5-yloxy-*N*-(4-((1-imino-2-*D*-thiomannosylethyl)amino)butyl) formamide (Man-C4-Chol) and cholesten-5-yloxy-*N*-(4-((1-imino-2-*D*-thiofucosylethyl)amino)butyl)formamide (Fuc-C4-Chol), to prepare the mannosylated (Man-) and fucosylated (Fuc-) liposomes for NPC-selective drug delivery *via* mannose and fucose receptor mediated uptake.<sup>53,54)</sup> Furthermore, we have demonstrated that mannose-specific lectin in the serum, MBP, which binds to pathogens having mannose units on their surface, can bind to Man-liposomes, and these MBP-bound Man-liposomes are more efficiently recognized by macrophages.

## 6. Cationic Liposomes for Gene Delivery Following the Systemic Injection

In 1987, Felgner *et al.* reported that the use of cationic liposomes was more effective than either the calcium phosphate or the DEAE-dextran transfection technique in various cultured cells.<sup>56)</sup> This technique is simple, highly reproducible and effective for both the transient and stable expression of transfected DNA. In 1993, Zhu *et al.* reported *in vivo* gene expression could be observed by the intravenous injection of pDNA complexed with DOTMA (*N*-[1-(2,3-dioleilyloxy)propyl]-*n,n,n*-trimethylammonium chloride)/DOPE (dioleoylphosphatidylethanolamine) liposomes; however, its transfection efficacy seemed to relatively low.<sup>57)</sup> In the late 1990s, several studies showed that various factors enhanced the gene expression *in vivo*.<sup>58–67)</sup> These factors for *in vivo* gene expression due to lipoplex are listed in Table 1. After the intravenous administration of lipoplex, the lung shows the highest amount of gene expression among the various organs and the lung endothelial cells are the main contributor

to transgene expression. We and others have confirmed that the gene expression level in the lung is 100–10000 times higher than that in the liver and spleen. Using these gene expression characteristics of the lung, lipoplex was applied to the prevention of lung cancer metastasis in mice.<sup>68,69)</sup> Since the physicochemical properties of pDNA are almost the same, irrespective of the encoding cDNA for the therapy, targeting technologies are especially required for their broad clinical application.

For successful *in vivo* gene delivery using these carrier systems, there are many barriers to overcome.<sup>70)</sup> Such factors include; i) the extent of DNA condensation, ii) particle size of the lipoplex, iii) interaction with endogenous components and tissues, iv) the route of administration, v) stability against nucleases, vi) controlled *in vivo* distribution, vii) binding to cell surface receptors and internalization, and viii) how intracellular trafficking affects *in vivo* gene delivery and expression.<sup>71)</sup> Since many barriers from the injection site to the target cells exist for cell-selectivity with *in vivo* gene transfection, there are few *in vivo* reports compared with *in vitro* observation. In order to develop an effective cell-selective *in vivo* gene carrier, therefore, the distribution characteristics of gene medicine must be clarified.<sup>72,73)</sup> Based on the pharmacokinetic information, we have developed novel cell-selective gene transfection carriers. In this section, we shall focus on the progress of research into targeted delivery systems of lipoplexes after systemic administration.

**6.1. Distribution Characteristic of pDNA Following the Systemic Injection** To develop a strategy for establishing liposomal carrier systems of pDNA, it is necessary to understand their *in vivo* distribution characteristics. Thus, the distribution characteristics of pDNA were analyzed by [<sup>32</sup>P] labeled pDNA. After intravenous injection, [<sup>32</sup>P] pDNA was rapidly eliminated from the plasma, involving extensive uptake by the liver.<sup>74)</sup> Pharmacokinetic analysis demonstrated that the hepatic uptake clearance of pDNA is almost identical to the plasma flow rate in the liver, suggesting highly effective elimination by the liver. As for the uptake mechanism by the liver, a competitive inhibition study demonstrated that [<sup>32</sup>P] pDNA is taken up preferentially by the liver NPC *via* a scavenger receptor-mediated process, in a manner specific for polyanions. The involvement of scavenger receptors in the hepatic uptake of pDNA has also been supported by a single-pass rat perfusion study<sup>75)</sup> and an uptake study using primary cultured mouse peritoneal macrophages.<sup>76,77)</sup>

**6.2. Distribution and Gene Expression Characteristics of Lipoplexes Following Systemic Injection** The distribution characteristics of lipoplexes at the early period is

Table 2. Cell-Selective Gene Delivery Carriers Using Ligands According to Their *in Vivo* Applications

Receptor	System	Results	Ref.
<b>Asialoglycoprotein</b>			
Wu, 1988	Asialoorosomucoid-polylysine	Expression in liver after i.v. injection	84
Perales, 1994	Galactose-polylysine	Expression in liver after i.v. injection	85
Hara, 1995	Asialofetuin-liposome	Expression in liver after intraportal injection	86
Kawakami, 2000	Galactose-liposome	Expression in hepatocytes after intraportal injection	87
Nishikawa, 2000	Galactose-polyornithine-HA2	Expression in hepatocytes after i.v. injection	88
Morimoto, 2003	Galactose-PEI	Expression in hepatocytes after intraportal injection	89
Fumoto, 2004	Galactose-liposome	Expression in hepatocytes after intraportal injection	90
<b>Mannose</b>			
Kawakami, 2000	Mannose-liposome	Expression in liver NPC after i.v. injection	91
Kawakami, 2004	Mannose-liposome	Expression in liver NPC after i.v. injection	92
Hattori, 2004	Mannose-liposome	Enhancement of immune responses by DNA vaccination	93
<b>Transferrin</b>			
Ogris, 1999	Transferrin-PEG-PEI (800 kDa)	Expression in cancer cells (s.c.) after i.v. injection	94
Kirchheis, 2001	Transferrin-PEI (22 kDa)	Expression in cancer cells (s.c.) after i.v. injection	95
Shi, 2001	Transferrin-liposome	Expression in brain after i.v. injection	96
Kursa, 2003	Transferrin-PEG-PEI (22 kDa)	Expression in cancer cells (s.c.) after i.v. injection	97
Zhang, 2003	Transferrin-liposome	Expression in brain after i.v. injection	98
<b>Folate</b>			
Hofland, 2002	Folate-liposome	Expression in cancer cells (s.c.) after i.v. injection	99
Reddy, 2002	Folate-liposome	Efficient expression in intraperitoneal cancer cells after intraperitoneal injection	100

important for gene expression. In fact, Barron *et al.* recently demonstrated that lipoplex-mediated gene expression to the lung occurs within 60 min after intravenous injection.<sup>78)</sup> We have emphasized the importance of distribution for the development of gene carriers; therefore, we evaluated the distribution characteristics of [<sup>32</sup>P] lipoplex.<sup>79,80)</sup> After the intravenous injection of a [<sup>32</sup>P] lipoplex, a rapid clearance of pDNA from the circulation was observed with extensive accumulation in the lung and liver. As far as the type of liver cells involved was concerned, the [<sup>32</sup>P] lipoplexes were predominantly taken up by liver NPC. As for the uptake mechanism by liver NPC, a competitive inhibition study demonstrated that the hepatic uptake of lipoplexes was significantly inhibited by the preceding administration of dextran sulfate, but not by poly [C] and poly [I], suggesting the involvement of a phagocytic process.

Recent studies have demonstrated that the intravenous administration of a lipoplex induced significant proinflammatory cytokine production in the blood and inhibited transgene expression in the pulmonary endothelial cells.<sup>81,82)</sup> Even if gene expression is exhibited at a favorable level, a high toxicity would lead to failure in clinical application. We have demonstrated that tissue macrophages involving liver Kupffer cells and spleen macrophages are closely involved in TNF- $\alpha$  production.<sup>83)</sup> This result corresponded with our previous distribution results that the [<sup>32</sup>P] lipoplex was mainly distributed in the liver NPC.<sup>84,85)</sup> Thus, it was suggested that avoiding lipoplex uptake and subsequent cytokine production by Kupffer cells and spleen macrophages would be a useful method of maintaining a high level of gene expression in the lung after repeated injections.

**6.3. Galactosylated Cationic Liposomes for Asialoglycoprotein Receptor-Mediated Gene Delivery to Hepatocytes** For cell-specific delivery, receptor-mediated endocytosis (RME) systems possessed by various cell types would be useful and a number of gene delivery systems have been developed to introduce foreign DNA into specific cells with RME. Table 2 summarizes the cell-selective *in vivo* gene de-

livery systems using ligand modified cationic liposomes or polymer.<sup>84–100)</sup> Since there are many barriers under *in vivo* conditions, there are few reports about the cell-selective gene delivery using ligand-modified lipoplexes. In order to overcome these barriers, gene carrier systems must be developed by consideration of the effect of physicochemical properties on the *in vivo* distribution. In this section, we shall focus on the research into our targeted gene systems of lipoplexes using asialoglycoprotein receptors.

Hepatocytes exclusively express large numbers of high affinity cell-surface receptors that can bind asialoglycoproteins and subsequently internalize them to the cell interior. Remy *et al.* reported the feasibility of using galactose-presenting lipopolyamine vectors for targeted gene transfer into hepatoma cells under *in vitro* conditions.<sup>101)</sup> The inclusion of galactose residues in the electrically neutral complex increased the transgene expression approaching the level obtained with a large excess of cationic liposomes alone. For *in vivo* hepatocyte-selective gene transfection, we designed Gal-C4-Chol for the preparation of the galactosylated cationic liposomes.<sup>39)</sup>

A distribution study demonstrated that the radioactivity in the liver from the [<sup>32</sup>P] pDNA/Gal-C4-Chol incorporated complex (Gal-lipoplex) was about 75% of the dose, even 1 min after intraportal injection.<sup>87)</sup> The hepatic gene expression of the Gal-lipoplex was more than 10-times greater than that of the pDNA complexed with conventional cationic liposomes. When the gene expression was examined by determining the intrahepatic cellular levels, the gene expression of liver PC of Gal-lipoplex was significantly higher than that of liver NPC. In contrast, there was little difference in the gene expression of PC and NPC of conventional cationic liposomes. In addition, an excess amount of galactosylated bovine serum albumin was intravenously injected prior to the injection of the Gal-lipoplex; the gene expression in the liver was significantly reduced, suggesting uptake via asialoglycoprotein receptor-mediated endocytosis.

However, the level of *in vivo* gene expression due to the

Gal-lipoplex was not as high as that expected from the *in vitro* results. There must be several barriers associated intrinsically with *in vivo* situations, such as convective blood flow in the liver, passage through the sinusoids and tissue interactions. To investigate these barrier processes, we studied the hepatic distribution profiles of Gal-lipoplexes using rat liver perfusion techniques<sup>102</sup> that allowed us to determine the uptake characteristics of various substances under different experimental conditions with the structure of the liver remaining intact.<sup>103–105</sup> In that study, we demonstrated that the penetration of the Gal-lipoplex through the hepatic fenestrated endothelium to the PC was greatly restricted in perfused rat liver in spite of the small size of the Gal-lipoplex (about 120 nm), as far as crossing the fenestrae was concerned.

In the next step, therefore, we tried to enhance the gene expression in the liver by preparing the novel stabilizing Gal-lipoplex.<sup>96</sup> Lipoplexes are often prepared in a nonionic solution due to their well-known tendency to aggregate out of solution as the salt concentration is increased.<sup>106,107</sup> Aggregation during lipoplex formation in ionic solution may be due to neutralization of the surface positive charge of the lipoplex intermediate by the associated counter-ion. Taking into account neutralization by the counter-ion, we hypothesized that the presence of an essential amount of sodium chloride (NaCl) during lipoplex formation might regulate the repulsion between cationic liposomes and thereby, the fusion of cationic liposomes in the lipoplex would be accelerated by the partial neutralization of the positive charge. Consequently, pDNA in the lipoplex could be largely covered by cationic lipids while retaining enough positive charge to prevent aggregate formation. Such types of lipoplex are expected to be more stable than the conventional lipoplex, which is prepared using a nonionic solution. After intraportal administration, the hepatic transfection activity of the Gal-SCR-lipoplex was approximately 10- to 20-times higher than that of the conventional galactosylated lipoplex in mice. The transfection activity in hepatocytes of the Gal-SCR-lipoplex was significantly higher than that of the conventional lipoplex, and pre-injection of asialoglycoprotein-receptor blocker markedly reduced the hepatic gene expression, suggesting that hepatocytes are responsible for high hepatic transgene expression of the Gal-SCR-lipoplex.

**6.4. Mannosylated Cationic Liposomes for Mannose Receptor-Mediated Gene Delivery to Macrophages**  
Macrophages are important targets for the gene therapy of a number of diseases, such as Gaucher's disease<sup>108</sup> and human immunodeficiency virus (HIV) infection,<sup>109</sup> but the process of gene transfection in such cases is not easy. The use of non-viral vectors is attractive for *in vivo* gene delivery because it is simpler than using viral systems and is free from some of

the risks inherent in the latter. Erbacher *et al.* investigated the suitability of various glycosylated poly(L-lysine) derivatives for introducing pDNA into human monocyte-derived macrophages and found that mannosylated poly(L-lysine) exhibited high transfection activity.<sup>110</sup> However, they also reported that the transfection activity was markedly enhanced in the presence of chloroquine due to the prevention of endosomal and/or lysosomal degradation of pDNA after mannose receptor-mediated endocytosis; for this reason, their *in vivo* use remains limited.

Hence, a cationic liposome-based targeted gene delivery system is a better method under *in vivo* conditions. Recently, we synthesized Man-C4-Chol for mannose receptor-mediated gene transfection to macrophages,<sup>91</sup> which are known to express large numbers of mannose receptors on their surface. In primary cultured mouse peritoneal macrophages, a Man-C4-Chol containing lipoplex (Man-lipoplex) showed higher transfection activity than that of the conventional lipoplex. The presence of 20 mM mannose significantly inhibited the transfection efficiency of Man-lipoplex, suggesting that the mannosylated lipoplex is recognized and taken up by the mannose receptors on macrophages. To further enhance gene transfection, polyethylenimine (PEI) was incorporated into this liposome complex (DNA/Man-PEI-complexes), taking note of the pH-buffering capacity in endosomes and DNA-condensing activity of PEI.<sup>111</sup> It was demonstrated that multifunctional DNA/Man-PEI-complexes exhibit highly improved gene transfection in primary cultured macrophages *via* mannose receptor-mediated endocytosis.

After intravenous injection, the highest gene expression was observed in the liver after the intravenous injection of the Man-lipoplex in mice.<sup>91</sup> In addition, gene expression with Man-lipoplex in the liver was observed preferentially in the liver NPC and was significantly reduced by predosing with mannosylated bovine serum albumin. These results suggest that Man-lipoplex exhibits high transfection activity in NPC due to recognition by mannose receptors. Unlike the case of the Gal-lipoplex, cell-selective gene transfection can be achieved by the intravenous administration of the Man-lipoplex.<sup>92</sup> This phenomenon could be explained by the fact that in the liver and spleen, macrophages are present around endothelial cells; therefore, they are in contact with the lipoplex without passing through the sinusoids (100–200 nm). Hence, the Man-lipoplex is effective in an NPC-selective gene transfection system, even when administered intravenously. The same phenomenon may be achieved with the intraportally administered Man-lipoplex.<sup>112</sup> In order to obtain a theoretical strategy to develop an efficiently targetable gene carrier to the liver by mannosylation, we studied the tissue, intrahepatic distribution and subcellular localiza-

Table 3. Various Factors on *in Vivo* Cell-Selective Gene Expression by the Glycosylated Lipoplex

Factors	Effect on gene expression	Ref.
1. Glycosylated lipoplex		
Charge	Moderate cationic charge ratio (– : +), 1.0 : 2.3—1.0 : 3.1 is suitable	87, 92, 102
Size	Small and/or stabilized lipoplexes enhance gene expression	90
2. Glycosylated cationic liposomes		
Helper lipid	Depending on the administration routes	87, 112
3. pDNA		
Dose	High dose enhances gene expression	87, 90



tion of a [<sup>32</sup>P]- or [<sup>111</sup>In]-labeled Man-lipoplex after intravenous injection.<sup>113</sup> The radioactivity in the cytosolic fraction of liver homogenate of [<sup>111</sup>In] Man-lipoplex was 2-times higher than that of the [<sup>111</sup>In] lipoplex, indicating that Man liposomes facilitate the release of pDNA into the cytosolic space. However, a rapid sorting of the radioactivity from endosomes to lysosomes was observed with the [<sup>111</sup>In] Man-lipoplex. Also, the amplification of pDNA by PCR suggested that the Man-lipoplex is more rapidly degraded within the intracellular vesicles than the lipoplex. These results suggested that modulation of the intracellular sorting may improve the transfection efficiency of the Man-lipoplex. Table 3 summarizes the various factors for *in vivo* cell-selective liposomal gene delivery obtained in our studies.

**6.5. Application of Man-Lipoplex to Gene Therapy**  
DNA vaccination, the administration of DNA-encoding antigen genes into the body, is of great interest in gene therapy for the immunotherapy of cancer and infectious diseases. Animal studies have shown that DNA immunization induces not only an antibody response but also a potent cell-mediated immune response against the encoding antigen.<sup>114–116</sup> This cell-mediated immune response plays a crucial role in the immune response against cancer and infectious diseases.<sup>117</sup> Recently, we showed that the targeted delivery of DNA vaccine by Man-C4-Chol liposomes is a potent method of DNA vaccine therapy.<sup>93</sup> Although further improvements in transfection efficacy are required, the targeted delivery of DNA vaccine to DCs may improve future *in vivo* DNA vaccine therapies.

## 7. Conclusions

Successful drug and gene therapy requires the development of a rational delivery technology that satisfies various requirements for each target disease. We developed various lipid carrier systems for targeted drug and/or gene delivery following local or systemic injection. This information will be of value for the future use, design, and development of drug and/or gene delivery systems based on lipid carriers.

**Acknowledgements** This research was supported by the following researchers and their helpful contribution was greatly appreciated: Professor Hitoshi Sezaki (Kyoto University), Professor Yoshinobu Takakura (Kyoto University), Dr. Makiya Nishikawa (Kyoto University), and laboratory members in Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University.

## References

- Sezaki H., Hashida M., *Crit. Rev. Ther. Drug Carrier Syst.*, **1**, 1–38 (1984).
- Hashida M., Nishikawa M., Yamashita F., Takakura Y., *Adv. Drug Deliv. Rev.*, **52**, 187–196 (2001).
- Kawakami S., Yamashita F., Hashida M., *Adv. Drug Deliv. Rev.*, **45**, 77–88 (2000).
- Nakamoto Y., Hashida M., Muranishi S., Sezaki H., *Chem. Pharm. Bull.*, **23**, 3125–3131 (1975).
- Hashida M., Egawa M., Muranishi S., Sezaki H., *J. Pharmacokin. Biopharm.*, **5**, 225–239 (1977).
- Takakura Y., Hashida M., *Crit. Rev. Oncol. Hematol.*, **18**, 207–231 (1995).
- Knepp W. A., Jayakrishnan A., Quigg J. M., Sitren H. S., Bagnall J. J., Goldberg E. P., *J. Pharm. Pharmacol.*, **45**, 887–891 (1993).
- Landrito J. E., Yoshiga K., Sakurai K., Takada K., *Anticancer Res.*, **14**, 113–118 (1994).
- Hashida M., Takahashi Y., Muranishi S., Sezaki H., *J. Pharmacokin. Biopharm.*, **5**, 241–255 (1977).
- Hashida M., Muranishi S., Sezaki H., *Chem. Pharm. Bull.*, **25**, 2410–2418 (1977).
- Hashida M., Liao M. H., Muranishi S., Sezaki H., *Chem. Pharm. Bull.*, **28**, 1659–1666 (1980).
- Yoshioka T., Ikeuchi K., Hashida M., Muranishi S., Sezaki H., *Chem. Pharm. Bull.*, **30**, 1408–1415 (1982).
- Tanigawa N., Shimomatsuya T., Takahashi K., Inomata Y., Tanaka K., Satomura K., Hikasa Y., Hashida M., Muranishi S., Sezaki H., *Cancer*, **60**, 741–749 (1987).
- Sasaki H., Mukai E., Hashida M., Kimura T., Sezaki H., *Int. J. Pharm.*, **15**, 49–59 (1983).
- Sasaki H., Fukumoto M., Hashida M., Kimura T., Sezaki H., *Chem. Pharm. Bull.*, **31**, 4083–4090 (1983).
- Sasaki H., Kakutani T., Hashida M., Sezaki H., *J. Pharm. Sci.*, **75**, 1166–1170 (1986).
- Sasaki H., Kakutani T., Hashida M., Kimura T., Sezaki H., *Chem. Pharm. Bull.*, **33**, 2968–2973 (1985).
- Sasaki H., Kakutani T., Hashida M., Sezaki H., *J. Pharm. Pharmacol.*, **37**, 461–465 (1985).
- Hashida M., Sato K., Takakura Y., Sezaki H., *Chem. Pharm. Bull.*, **36**, 3186–3189 (1988).
- Teshima M., Kawakami S., Nishida K., Nakamura J., Sakaeda T., Terazono H., Kitahara T., Nakashima M., Sasaki H., *J. Control. Release*, **97**, 211–218 (2004).
- Ohkouchi K., Imoto H., Takakura Y., Hashida M., Sezaki H., *Cancer Res.*, **50**, 1640–1644 (1990).
- Imoto H., Sakamura Y., Ohkouchi K., Atsumi R., Takakura Y., Sezaki H., Hashida M., *Cancer Res.*, **52**, 4396–4401 (1992).
- Nakajima S., Koshino Y., Nomura T., Yamashita F., Agrawal S., Takakura Y., Hashida M., *Antisense Nucleic Acid Drug Dev.*, **10**, 105–110 (2000).
- Nomura T., Koreeda N., Yamashita F., Takakura Y., Hashida M., *Pharm. Res.*, **15**, 128–132 (1998).
- Singh M., Ravin L. J., *J. Parenter. Sci. Technol.*, **40**, 34–41 (1986).
- Yamaguchi T., Mizushima Y., *Crit. Rev. Ther. Drug Carrier Syst.*, **11**, 215–229 (1994).
- Constantinides P. P., *Pharm. Res.*, **12**, 1561–1572 (1995).
- Takino T., Konishi K., Takakura Y., Hashida M., *Biol. Pharm. Bull.*, **17**, 121–125 (1994).
- Takino T., Nagahama E., Sakaeda T., Yamashita F., Takakura Y., Hashida M., *Int. J. Pharm.*, **114**, 43–54 (1995).
- Finkelstein M. C., Weissmann G., *Biochim. Biophys. Acta*, **587**, 202–216 (1979).
- Allen T. M., *Biochim. Biophys. Acta*, **640**, 385–397 (1981).
- Senior J., Gregoriadis G., *Life Sci.*, **30**, 2123–2136 (1982).
- Rensen P. C., Herijgers N., Netscher M. H., Meskers S. C., van Eck M., van Berkel T. J., *J. Lipid Res.*, **38**, 1070–1084 (1997).
- Takino T., Nakajima C., Takakura Y., Sezaki H., Hashida M., *J. Drug Target.*, **1**, 117–124 (1993).
- Sakaeda T., Hirano K., *J. Drug Target.*, **6**, 119–127 (1998).
- Tokunaga Y., Iwasa T., Fujisaki J., Sawai S., Kagayama A., *Chem. Pharm. Bull.*, **36**, 3060–3069 (1988).
- Tokunaga Y., Iwasa T., Fujisaki J., Sawai S., Kagayama A., *Chem. Pharm. Bull.*, **36**, 3557–3564 (1988).
- Slidregt L. A., Rensen P. C., Rump E. T., van Santbrink P. J., Bijsterbosch M. K., Valentijn A. R., van der Marel G. A., van Boom J. H., van Berkel T. J., Biessen E. A., *J. Med. Chem.*, **42**, 609–618 (1999).
- Kawakami S., Yamashita F., Nishikawa M., Takakura Y., Hashida M., *Biochem. Biophys. Res. Commun.*, **252**, 78–83 (1998).
- Ishida E., Managit C., Kawakami S., Nishikawa M., Yamashita F., Hashida M., *Pharm. Res.*, **21**, 932–939 (2004).
- Wolff J. A., Malone R. W., Williams P., Chong W., Acsadi G., Jani A., Felgner P. L., *Science*, **247**, 1465–1468 (1990).
- Nomura T., Nakajima S., Kawabata K., Yamashita F., Takakura Y., Hashida M., *Cancer Res.*, **57**, 2681–2686 (1997).
- Nomura T., Yasuda K., Yamada T., Okamoto S., Mahato R. I., Watanabe Y., Takakura Y., Hashida M., *Gene Ther.*, **6**, 121–129 (1999).
- Kawakami S., Harada A., Sakanaka K., Nishida K., Nakamura J., Sakaeda T., Ichikawa N., Nakashima M., Sasaki H., *Int. J. Pharm.*, **278**, 255–262 (2004).
- Kawakami S., Munakata C., Fumoto S., Yamashita F., Hashida M., *J. Pharm. Sci.*, **90**, 105–113 (2001).
- Kawakami S., Munakata C., Fumoto S., Yamashita F., Hashida M., *J. Drug Target.*, **8**, 137–142 (2000).
- Hattori Y., Kawakami S., Yamashita F., Hashida M., *J. Control. Re-*

- lease, **69**, 369—377 (2000).
- 48) Murao A., Nishikawa M., Managit C., Wong J., Kawakami S., Yamashita F., Hashida M., *Pharm. Res.*, **19**, 1808—1814 (2002).
  - 49) Managit C., Kawakami S., Nishikawa M., Yamashita F., Hashida M., *Int. J. Pharm.*, **266**, 77—84 (2003).
  - 50) Ogawara K., Hasegawa S., Nishikawa M., Takakura Y., Hashida M., *J. Drug Target.*, **6**, 349—360 (1999).
  - 51) Opanasopit P., Nishikawa M., Yamashita F., Takakura Y., Hashida M., *J. Drug Target.*, **9**, 341—351 (2001).
  - 52) Higuchi Y., Nishikawa M., Kawakami S., Yamashita F., Hashida M., *Int. J. Pharm.*, **287**, 147—154 (2004).
  - 53) Kawakami S., Wong J., Sato A., Hattori Y., Yamashita F., Hashida M., *Biochim. Biophys. Acta*, **1524**, 258—265 (2000).
  - 54) Opanasopit P., Sakai M., Nishikawa M., Kawakami S., Yamashita F., Hashida M., *J. Control. Release*, **80**, 283—294 (2002).
  - 55) Opanasopit P., Higuchi Y., Kawakami S., Yamashita F., Nishikawa M., Hashida M., *Biochim. Biophys. Acta*, **1511**, 134—145 (2001).
  - 56) Felgner P. L., Gadek T. R., Holm M., Roman R., Chan H. W., Wenz M., Northrop J. P., Ringold G. M., Danielsen M., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413—7417 (1987).
  - 57) Zhu N., Liggitt D., Liu Y., Debs R., *Science*, **261**, 209—211 (1993).
  - 58) Solodin I., Brown C. S., Bruno M. S., Chow C. Y., Jang E. H., Debs R. J., Heath T. D., *Biochemistry*, **34**, 13537—13544 (1995).
  - 59) Thierry A. R., Lunardi-Iskandar Y., Bryant J. L., Rabinovich P., Gallo R. C., Mahan L. C., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 9742—9746 (1995).
  - 60) Li S., Huang L., *Gene Ther.*, **4**, 891—900 (1997).
  - 61) Liu F., Qi H., Huang L., Liu D., *Gene Ther.*, **4**, 517—523 (1997).
  - 62) Liu Y., Mounkes L. C., Liggitt H. D., Brown C. S., Solodin I., Heath T. D., Debs R. J., *Nat. Biotechnol.*, **15**, 167—173 (1997).
  - 63) Mahato R. I., Anwer K., Tagliaferri F., Meaney C., Leonard P., Wadhwa M. S., Logan M., French M., Rolland A., *Hum. Gene Ther.*, **9**, 2083—2099 (1998).
  - 64) Templeton N. S., Lasic D. D., Frederik P. M., Strey H. H., Roberts D. D., Pavlakis G. N., *Nat. Biotechnol.*, **15**, 647—652 (1997).
  - 65) Hong K., Zheng W., Baker A., Papahadjopoulos D., *FEBS Lett.*, **400**, 233—237 (1997).
  - 66) Hofland H. E., Nagy D., Liu J. J., Spratt K., Lee Y. L., Danos O., Sullivan S. M., *Pharm. Res.*, **14**, 742—749 (1997).
  - 67) Sakurai F., Nishioka T., Saito H., Baba T., Okuda A., Matsumoto O., Taga T., Yamashita F., Takakura Y., Hashida M., *Gene Ther.*, **8**, 677—686 (2001).
  - 68) Whitmore M., Li S., Huang L., *Gene Ther.*, **6**, 1867—1875 (1999).
  - 69) Sakurai F., Terada T., Maruyama M., Watanabe Y., Yamashita F., Takakura Y., Hashida M., *Cancer Gene Ther.*, **10**, 661—668 (2003).
  - 70) Mahato R. I., Takakura Y., Hashida M., *Crit. Rev. Ther. Drug Carrier Syst.*, **14**, 133—172 (1997).
  - 71) Kawakami S., Yamashita F., Nishida K., Nakamura J., Hashida M., *Crit. Rev. Ther. Drug Carrier Syst.*, **19**, 171—190 (2002).
  - 72) Takakura Y., Hashida M., *Pharm. Res.*, **13**, 820—831 (1996).
  - 73) Takakura Y., Nishikawa M., Yamashita F., Hashida M., *Eur. J. Pharm. Sci.*, **13**, 71—76 (2001).
  - 74) Kawabata K., Takakura Y., Hashida M., *Pharm. Res.*, **12**, 825—830 (1995).
  - 75) Yoshida M., Mahato R. I., Kawabata K., Takakura Y., Hashida M., *Pharm. Res.*, **13**, 599—603 (1996).
  - 76) Takagi T., Hashiguchi M., Mahato R. I., Tokuda H., Takakura Y., Hashida M., *Biochem. Biophys. Res. Commun.*, **245**, 729—733 (1998).
  - 77) Takakura Y., Takagi T., Hashiguchi M., Nishikawa M., Yamashita F., Doi T., Imanishi T., Suzuki H., Kodama T., Hashida M., *Pharm. Res.*, **16**, 503—508 (1999).
  - 78) Barron L. G., Gagne L., Szoka F. C., Jr., *Hum. Gene Ther.*, **10**, 1683—1694 (1999).
  - 79) Mahato R. I., Kawabata K., Nomura T., Takakura Y., Hashida M., *J. Pharm. Sci.*, **84**, 1267—1271 (1995).
  - 80) Mahato R. I., Kawabata K., Takakura Y., Hashida M., *J. Drug Target.*, **3**, 149—157 (1995).
  - 81) Li S., Wu S. P., Whitmore M., Loeffert E. J., Wang L., Watkins S. C., Pitt B. R., Huang L., *Am. J. Physiol.*, **276**, L796—L804 (1999).
  - 82) Yew N. S., Zhao H., Przybylska M., Wu I. H., Tousignant J. D., Scheule R. K., Cheng S. H., *Mol. Ther.*, **5**, 731—738 (2002).
  - 83) Sakurai F., Terada T., Yasuda K., Yamashita F., Takakura Y., Hashida M., *Gene Ther.*, **9**, 1120—1126 (2002).
  - 84) Wu G. Y., Wu C. H., *J. Biol. Chem.*, **263**, 14621—14624 (1988).
  - 85) Perales J. C., Ferkol T., Beegen H., Ratnoff O. D., Hanson R. W., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 4086—4090 (1994).
  - 86) Hara T., Aramaki Y., Takada S., Koike K., Tsuchiya S., *Gene Ther.*, **2**, 784—788 (1995).
  - 87) Kawakami S., Fumoto S., Nishikawa M., Yamashita F., Hashida M., *Pharm. Res.*, **17**, 306—313 (2000).
  - 88) Nishikawa M., Yamauchi M., Morimoto K., Ishida E., Takakura Y., Hashida M., *Gene Ther.*, **7**, 548—555 (2000).
  - 89) Morimoto K., Nishikawa M., Kawakami S., Nakano T., Hattori Y., Fumoto S., Yamashita F., Hashida M., *Mol. Ther.*, **7**, 254—261 (2003).
  - 90) Fumoto S., Kawakami S., Ito Y., Shigeta K., Yamashita F., Hashida M., *Mol. Ther.*, **10**, 719—729 (2004).
  - 91) Kawakami S., Sato A., Nishikawa M., Yamashita F., Hashida M., *Gene Ther.*, **7**, 292—299 (2000).
  - 92) Kawakami S., Hattori Y., Lu Y., Higuchi Y., Yamashita F., Hashida M., *Pharmazie*, **59**, 405—408 (2004).
  - 93) Hattori Y., Kawakami S., Suzuki S., Yamashita F., Hashida M., *Biochem. Biophys. Res. Commun.*, **317**, 992—999 (2004).
  - 94) Ogris M., Brunner S., Schuller S., Kircheis R., Wagner E., *Gene Ther.*, **6**, 595—605 (1999).
  - 95) Kircheis R., Wightman L., Schreiber A., Robitzka B., Rossler V., Kurska M., Wagner E., *Gene Ther.*, **8**, 28—40 (2001).
  - 96) Shi N., Zhang Y., Zhu C., Boado R. J., Pardridge W. M., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 12754—12759 (2001).
  - 97) Kurska M., Walker G. F., Roessler V., Ogris M., Roedel W., Kircheis R., Wagner E., *Bioconjug. Chem.*, **14**, 222—231 (2003).
  - 98) Zhang Y., Boado R. J., Pardridge W. M., *J. Gene Med.*, **5**, 1039—1045 (2003).
  - 99) Hofland H. E., Masson C., Iginla S., Osetinsky I., Reddy J. A., Leamon C. P., Scherman D., Bessodes M., Wils P., *Mol. Ther.*, **5**, 739—744 (2002).
  - 100) Reddy J. A., Abburi C., Hofland H., Howard S. J., Vlahov I., Wils P., Leamon C. P., *Gene Ther.*, **9**, 1542—1550 (2002).
  - 101) Remy J. S., Kichler A., Mordvinov V., Schuber F., Behr J. P., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 1744—1748 (1995).
  - 102) Fumoto S., Nakadori F., Kawakami S., Nishikawa M., Yamashita F., Hashida M., *Pharm. Res.*, **20**, 1452—1459 (2003).
  - 103) Nishida K., Tonegawa C., Kakutani T., Hashida M., Sezaki H., *Pharm. Res.*, **6**, 140—146 (1989).
  - 104) Nishida K., Mihara K., Takino T., Nakane S., Takakura Y., Hashida M., Sezaki H., *Pharm. Res.*, **8**, 437—444 (1991).
  - 105) Nishida K., Eguchi Y., Takino T., Takakura Y., Hashida M., Sezaki H., *Pharm. Res.*, **8**, 1253—1257 (1991).
  - 106) Ogris M., Steinlein P., Kurska M., Mechtler K., Kircheis R., Wagner E., *Gene Ther.*, **5**, 1425—1433 (1998).
  - 107) Wasan E. K., Harvie P., Edwards K., Karlsson G., Bally M. B., *Biochim. Biophys. Acta*, **1461**, 27—46 (1999).
  - 108) Ohashi T., Boggs S., Robbins P., Bahnson A., Patrene K., Wei F. S., Wei J. F., Li J., Lucht L., Fei Y., Clark S., Kimak M., He H., Mowery-Rushton P., Barranger J. A., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 11332—11336 (1992).
  - 109) Kohn D. B., Sarver N., *Adv. Exp. Med. Biol.*, **394**, 421—428 (1996).
  - 110) Erbacher P., Bousser M. T., Raimond J., Monsigny M., Midoux P., Roche A. C., *Hum. Gene Ther.*, **7**, 721—729 (1996).
  - 111) Sato A., Kawakami S., Yamada M., Yamashita F., Hashida M., *J. Drug Target.*, **9**, 201—207 (2001).
  - 112) Kawakami S., Sato A., Yamada M., Yamashita F., Hashida M., *S.T.P. Pharma Sci.*, **11**, 117—120 (2001).
  - 113) Yamada M., Nishikawa M., Kawakami S., Hattori Y., Nakano T., Yamashita F., Hashida M., *J. Control. Release*, **98**, 157—167 (2004).
  - 114) Ulmer J. B., Donnelly J. J., Parker S. E., Rhodes G. H., Felgner P. L., Dwarki V. J., Gromkowski S. H., Deck R. R., DeWitt C. M., Friedman A., Howe L. A., Leander K. R., Martinez D., Perry H. C., Shiver J. W., Montgomery D. L., Liu M. A., *Science*, **259**, 1745—1749 (1993).
  - 115) Schirmbeck R., Bohm W., Ando K., Chisari F. V., Reimann J., *J. Virol.*, **69**, 5929—5934 (1995).
  - 116) Doolan D. L., Sedegh M., Hedstrom R. C., Hobart P., Charoenvit Y., Hoffman S. L., *J. Exp. Med.*, **183**, 1739—1746 (1996).
  - 117) Nishimura T., Nakui M., Sato M., Iwakabe K., Kitamura H., Sekimoto M., Ohta A., Koda T., Nishimura S., *Cancer Chemother. Pharmacol.*, **46** S52—S61 (2000).