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# Self-assembled drug delivery systems 1. Properties and in vitro/in vivo behavior of acyclovir self-assembled nanoparticles (SAN)

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**Abstract**

Self-assembled drug delivery systems (SADDS) were designed in the paper. They can be prepared from the amphiphilic conjugates of hydrophilic drugs and lipids through self-assembling into small-scale aggregates in aqueous media. The outstanding characteristic of SADDS is that they are nearly wholly composed of amphiphilic prodrugs. The self-assembled nanoparticles (SAN) as one of SADDS had been prepared from the lipid derivative of acyclovir (SGSA) in the previous paper. They were further studied on the properties and the in vitro/in vivo behavior in this paper. The SAN kept the physical state stable upon centrifugation or some additives including some inorganic salts, alkaline solutions, surfactants and liposomes except for HCl solution, $CaCl_2$ solution and animal plasma. Autoclave and bath heat for sterilization hardly influenced the SAN. However, gamma-irradiation strongly destroyed the structure of SAN and SGSA was degraded. SGSA in SAN showed good stability in weak acidic or neutral buffers although it was very sensitive to alkaline solutions and carboxylester enzymes, the half-lives ($t_{1/2}$) of which in the buffer at pH 7.4, the alkaline solution at pH 12.0, pig liver carboxylester enzyme solution, rabbit plasma, and rabbit liver tissue homogenate were 495, 21, 4.7, 25 and 8.7 h, respectively. Compared with SGSA in a disordered state, the specific bilayer structures of SAN could protect SGSA from hydrolysis through hiding the sensitive ester bonds. The SAN showed hemolytic action because the amphiphilic SGSA could insert into rabbit erythrocyte membranes. Both the high concentration of SGSA in samples and the long incubation time improved hemolysis. No hemolysis was observed if the additional volume of the SAN was less than 10% of rabbit whole blood in spite of the high concentration of SGSA. Plasma proteins could interfere the interaction between the SAN and erythrocytes by binding the SAN. The in vitro antiviral activity of acyclovir SAN was limited possibly because of the weak hydrolysis of SGSA in Vero cells, and the SAN showed a little cell toxicity possible due to the amphiphilicity of SGSA. A macrophage cell line of QXMSC1 cells showed uptake of the SAN but not significantly. The SAN were rapidly removed from blood circulation after bolus iv administration to rabbits with the very short distribution $t_{1/2}$ (1.5 min) and the elimination $t_{1/2}$ (47 min). The SAN were mainly distributed in liver, spleen and lung after iv administration, and SGSA was eliminated slowly in these tissues ($t_{1/2}$, about 7 h). It would appear that the nanosized SAN were trapped by the mononuclear phagocyte system. SADDS including SAN combine prodrugs, molecular self-assembly with nanotechnology, and hopefully become novel drug delivery approaches.


*Keywords:* Prodrugs; Molecular self-assembly; Nanotechnology; Amphiphiles; Acyclovir; Mononuclear phagocyte system

## 1. Introduction

The efficacy and safety of drugs is always the key of pharmacotherapy. To achieve the purpose, a variety of methods are applied to drug delivery. An ideal drug delivery system should possess two elements: the ability to target and drug controlled release. Drug targeting will ensure high therapeutic efficacy. But maybe even more important it will reduce side effects. The reduction or even prevention of side effects can also be achieved by controlled release. Drug carriers such as particulates (liposomes, nanoparticles, microemulsions) and externally triggered (pH-, temperature-, or magnetic-sensitive) carriers have widely been explored (Torchilin, 2000; Allen and Cullis, 2004). However, almost all of current delivery systems load drugs passively

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so that the problems of low drug-loaded efficiency and drug leakage in preparation, preservation and transport in vivo always appear (Barenholz, 2001; Glavas-Dodov et al., 2005). Carriers could have been destroyed in vivo before reaching target sites. In addition, lipophilic biomembranes including cell membranes usually prevent hydrophilic drugs from entering into target sites (Lipinski et al., 1997). Generally, carriers also cannot override cell membranes unless endocytosis/phagocytosis by cells/macrophages, so that the loaded drugs are probably released on target surfaces. A majority of drugs could not reach target sites at all on account of the poor properties of carriers and drugs.

It is well known that the surfactant-like amphiphiles would like to self-assemble into ordered aggregates such as micelles, vesicles, hexagosomes and cubosomes. Some of the aggregates have been applied to deliver drugs (Drummond and Fong, 2000; Kim et al., 2000; Shah et al., 2001; Whitesides and Grzybowski, 2002; Zarif, 2002). Unfortunately, surface-active drugs especially self-assembling drugs are few (Schreier et al., 2000). However, some amphiphiles with potential pharmacological action (drugs or prodrugs) can be prepared, and they likely self-assemble into ordered aggregates. Optimally, the aggregates can deliver themselves in vivo. We name the aggregates as self-assembled drug delivery systems (SADDS). Based on the aim of drug delivery, SADDS had better be small-scale systems and they would show drug targeting and sustained release. But the outstanding characteristic of SADDS over common nanoparticles or liposomes is that they are nearly wholly composed of amphiphilic prodrugs, so that high drug-loaded amount and very low drug leakage are archived easily. In addition, the amphiphilic monomers of SADDS would like to permeate biomembranes of targets provided that SADDS were decomposed on target surfaces.

Vaizoglu and Speiser used the word ‘pharmacosomes’ to describe the colloidal dispersions prepared from drug-lipid conjugates with or without additional surfactants (Vaizoglu and Speiser, 1986). Pindolol pharmacosomes (vesicle-like) were prepared from pindolol diglyceride by the authors. Pharmacosomes have been not deeply studied, possibly because no appropriated theory supports the new dosage form and no appropriated drugs and lipids are selected. Obviously, pharmacosomes can be considered as one of SADDS based on the theory of the paper.

The structural modification of drugs is necessary to obtain amphiphilicity. The lipid derivation of hydrophilic drugs should be preferentially considered because lipid derivatives can be well degraded in vivo and permeate biomembranes (Lambert, 2000). Nucleoside antivirals have to be activated to phosphates in cell plasma to resist virus although many of them permeate into cells not well due to high molecular polarity (Pastor-Anglada et al., 1998). Some lipophilic derivatives of nucleosides self-assemble in organic solvents based on hydrogen bonding (Gottarelli et al., 2000; Giorgi et al., 2002; Davis, 2004), and some phospholipid-nucleoside conjugates form ordered aggregates in aqueous solutions (Itojima et al., 1992; Choi et al., 2005; Moreau et al., 2005). Therefore, nucleoside antivirals are the optimal precursors to prepare self-assembling amphiphilic prodrugs.

Acyclovir is a typical nucleoside antiviral agent against herpes simplex virus (HSV) with the low oral bioavailability (20%) and a short circulation half-life ($t_{1/2}$, 2.5 h) (de Miranda and Blum, 1983). Some lipophilic derivatives of acyclovir showed higher bioavailability (Hostetler et al., 1993, 1997). We selected acyclovir as a model drug to study SADDS in this paper. A series of long-chained lipid derivatives of acyclovir had been synthesized and the self-assembly of them were characterized in our previous paper (Jin et al., 2005). It was found that stearyl-glycero-succinyl-acyclovir (SGSA), the single-chained lipid derivative self-assembled into cuboid-like self-assembled nanoparticles (SAN) in water based on the hydrophobic interaction of lipid chains and the layer-by-layer hydrogen bonding of nucleoside moieties. Acyclovir SAN had an average size of 83.2 nm, a negative surface charge of −31.3 mV. It should be rationally regarded as one of SADDS. The properties and the in vitro/in vivo behavior of acyclovir SAN were investigated in the paper, mainly including stability, the interaction with cells, pharmacokinetics and tissue distribution after iv administration.

## 2. Materials and methods

### 2.1. Materials

The lipid derivative of acyclovir, SGSA was prepared according to the previous research (Jin et al., 2005). Analytical reagents were used, and chromatographic reagents were used in HPLC determination. Distilled water was always used. Surfactants used in the stability investigation of SAN were from Amresco (sodium dodecyl sulphate, SDS), Sigma (Brij 35), Beijing Chemical Reagents Company (Tween 80 and cetyltrimethylammonium bromide, CTAB), Shenyang Yaoda Jiqi Pharmaceutical Factory (poloxamer 188) and Lucas Meyer GmbH (soybean phosphatidylcholine, SPC). Liposomes were prepared from SPC by the REV method (Szoka and Papahadjopoulos, 1978), which contained about 7 mg/ml SPC.

Pig liver carboxylester enzyme (PLCE, Sigma) was dissolved in Tris–HCl buffer (20 mM, pH 7.4) before use. A macrophage cell line of QXMSC1 cells was from Department of Biotechnology, Beijing Institute of Radiation Medicine (BIRM). African green monkey kidney (Vero) cells were from Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences.

Male albino rabbits (1.9–2.6 kg) from Laboratory Animal Center of BIRM were used. Principles in good laboratory animal care were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in BIRM. The rabbits were sacrificed by euthanasia to remove tissues. The homogenates used in the experiments of chemical stability and tissue distribution were prepared in tissue/water (1:1, w/w).

### 2.2. Preparation of SAN

Acyclovir SAN were prepared with a controlled process according to the previous research (Jin et al., 2005). The preparation method is described as follows. SGSA solutions of 5 mg/ml

in tetrahydrofuran (THF) were slowly and continually injected into vortexed water under surface via a 100-μl micro-syringe until a homogeneous and slightly opalescent suspension was obtained. The suspensions were incubated in a 37 °C water bath under vacuum for removing solvents and being concentrated. The final suspensions could contain high-concentration SGSA of more than 15 mg/ml, and appeared homogeneously.

### 2.3. HPLC determination of acyclovir and its derivatives

High-performance liquid chromatographic (HPLC) experiments were performed at room temperature on a Shimadzu 10A HPLC system (Japan) that consisted of LC-10Avp pump, SPD-10Avp UV detector, SCL-10Avp controller, and Shimadzu CLASS-VP 6.02 chromatographic workstation software. The Diamonsil™ C18-ODS HPLC columns (5 μm, 250 mm × 4.6 mm) and the EasyGuard™ C18-ODS HPLC guard columns (5 μm, 8 mm × 4 mm) were purchased from Dikma (China). A manual injection valve and a 20-μl loop (7725i, Rheodyne, USA) were used. UV detector was fixed at 254 nm.

Since the polarity of acyclovir, succinyl-acyclovir (SACV, the synthesis intermediate or the possible hydrolysis product) and SGSA was significantly different, the various mobile phases were used for their HPLC determination. Acyclovir and SACV in all samples except for tissue homogenates were determined in the mobile phase of methanol/water (19:81, v/v) containing 40 mM ammonium acetate (pH 7.0) at 0.8 ml/min. The retention times ($t_R$) of acyclovir and SACV were about 6.8 and 7.6 min, respectively. The mobile phase used to determine acyclovir in tissue homogenates was methanol/water (12:88, v/v) containing 40 mM ammonium acetate (pH 7.0), and the flow rate was 1.0 ml/min. The $t_R$ of acyclovir was about 8.7 min. SGSA in all samples was determined with the mobile phase of methanol/water (80:20, v/v) containing 40 mM ammonium acetate (pH 7.0) at 0.8 ml/min. The $t_R$ of SGSA was about 8.1 min.

### 2.4. Stability investigation of SAN

#### 2.4.1. Physical stability

The turbidity method was often used to investigate the physical changes of small-scale particles (Defrise-Quertain et al., 1984; Zhai et al., 2005). The turbidity of acyclovir SAN was equal to the absorbance at 550 nm with water as references in the paper. Particle size increasing or particle aggregation can be expressed by turbidity increasing. The effects of centrifugation and additives on the physical stability of the SAN were investigated. The SAN were centrifuged at various rotate speeds for 5 min and then resuspended thoroughly. The turbidity was measured by a Shimadzu UV-2501PC spectrophotometer. The water diluted SAN (containing about 120 μg/ml SGSA) of 2.5 ml were added into cuvettes, followed by, respectively, adding with a variety of additives, agitated thoroughly, and then the turbidity was measured. The additives included 150 mM NaCl, 0.1 M NaOH, 100 mM SDS, 10 mM CTAB, 10% Tween 80, 10% Brij 35, 10% poloxamer 188, SPC liposomes and rabbit plasma. The additional volume of additives was, respectively, 20, 50, 100 and 500 μl.

#### 2.4.2. Chemical stability

Acyclovir SAN were diluted with different buffers, including 20 mM phosphate buffers (pH 5.0 and 7.4) and 20 mM Tris–HCl buffers (pH 9.0 and 12.0), and the dilutions were incubated in a 37 °C bath. At predetermined time intervals, 20-μl aliquots were removed, dissolved with methanol, and assayed by HPLC. SGSA solutions in dimethylsulphoxide (DMSO) were also mixed with buffers, and the stability was measured to compare the degradation kinetics with SGSA in SAN.

The effects of PLCE solution (10 U/ml), rabbit plasma and tissue homogenates on the chemical stability of acyclovir SAN at 37 °C were investigated as above. The samples were deproteinized with methanol, followed by vortex for 1 min, and centrifuged at 10,000 rpm for 10 min. SGSA in supernatants was determined by HPLC. Acyclovir in samples was also determined as the above procedure except for deproteinization with methanol/5% $ZnSO_4$ (9:1, v/v).

#### 2.4.3. Sterilization of SAN

Gamma-irradiation of $1.5 \times 10^4$ Gy from a $^{60}Co$ source (BIRM, China) was used to sterilize acyclovir SAN in glass bottles at room temperature. The heat sterilization of the SAN in glass bottles was performed through autoclave for 30 min, or the 100 °C bath for 30 min. Whether SGSA in the sterilized SAN was hydrolyzed was evaluated by HPLC determination.

### 2.5. Interaction between SAN and erythrocytes

Rabbit erythrocyte suspension (2%, v/v) was prepared as follows. Whole blood was obtained from rabbits via marginal ear vein puncture and collected in a clean beaker, and agitated with a glass stick to remove fibrinogen. Erythrocytes were separated by centrifugation at 3000 rpm for 3 min, and washed three times with 0.9% NaCl solution. The sediment cells were diluted with 0.9% NaCl solution to obtain the erythrocyte suspension that was stored at 4 °C and used within 24 h.

Hemolytic action was investigated briefly as the followings. The 2-ml samples containing 1% erythrocytes, a series of SAN suspensions and supplementary 0.9% NaCl solutions were prepared. After 12 h of incubation at 37 °C, hemolytic phenomena were observed by naked eyes against the completely hemolytic sample prepared by adding water into erythrocytes, and the cells were counted with light microscopy. The cells and the supernatants were separated, and SGSA in them was determined by HPLC. Acyclovir solutions instead of SAN were as control. The interaction between acyclovir SAN and rabbit whole blood (fresh heparinized) was also investigated.

### 2.6. Toxicity and antiviral activity on cell model

Vero cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$. The cultural medium was the MEM (Sigma) solution supplemented with 10% calf serum and $1 \times 10^5$ unit penicillin and streptomycin. Vero cells were cultured in 96-well plates

at a concentration of $2 \times 10^4$ cells/well. After 24 h of culture, the autoclaved SAN suspensions and acyclovir solutions diluted with the cultural medium not containing serum were, respectively, added to the wells. Eight samples of the SAN with SGSA concentration increasing from 31.2 to 4000 μg/ml were added to the wells in triplicates. So did eight samples of acyclovir solutions from 7.8 to 1000 μg/ml. The wells not containing the drugs were as control. After 48 h of incubation at 37 °C, the cytopathic effect (CPE) assay was performed with light microscope, and the 50% toxic concentrations ($TC_{50}$) of SGSA in SAN and acyclovir were calculated (de La Iglesia et al., 1998).

Anti-HSV therapeutic effect was also investigated like the above procedure. After Vero cells were cultured in 96-well plates for 24 h, they were infected by herpes simplex virus type-1 (HSV-1) strain (VR733, ATCC, USA) of $10^4$-fold dilution. The cultural media were withdrawn after 2 h of virus adsorption. Eight samples of the SAN with SGSA concentration increasing from 1.9 to 250 μg/ml were added to the wells in triplicates. So did eight samples of acyclovir solutions from 0.8 to 100 μg/ml. The wells without HSV-1 infection were as control. After 48 h of incubation at 37 °C, the CPE was examined, and the 50% inhibitory concentrations ($IC_{50}$) of SGSA in SAN and acyclovir were calculated (de La Iglesia et al., 1998).

### 2.7. Macrophage culture and uptake of SAN

A macrophage cell line of QXMSC1 cells in 6-well plates was cultured at 37 °C in a humidified atmosphere of 5% $CO_2$. The cultural medium was the RPMI-1640 (Sigma) solution supplemented with 10% calf serum, 2.0 mM glutamine, 0.05 mM 2-mercaptoethanol, 4.5 g/l glucose, 1.5 g/l $NaHCO_3$, 1.0 mM sodium pyruvate, 10 mM HEPES, and $1 \times 10^5$ unit penicillin and streptomycin (Qin and Xie, 1998). At a concentration of $1 \times 10^5$ cells/well, the autoclaved SAN diluted with the cultural medium not containing serum, were added to each well. After the plates had been incubated at 37 °C for a predetermined period, the supernatants in the wells were drawn off, and the cells were washed with the cold Tris–HCl buffer (20 mM, pH 7.4). The washing was collected and added to the supernatants. The remaining cells were mixed with Tris–HCl buffer (0.5 ml), scraped off, collected in a centrifuge tube, and experienced probe sonication in a 0 °C bath for 20 s to prepare cell lysates. SGSA in supernatants and cell lysates was determined. Acyclovir solutions were as control.

### 2.8. SAN iv administration to rabbits

Pharmacokinetics and tissue distribution were studied after acyclovir SAN bolus iv administration to rabbits. SGSA in SAN should be concentrated to 15 mg/ml or more to reduce injection volume. The SAN were autoclaved and determined before use, and then administered to rabbits at SGSA dose of 30 mg/kg through ear vein. Half of one milliliter of blood sample was collected into heparinized centrifuge tubes at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 8, 10, 15, 20, 30, 40, 50, 60, 90, 120, 180, 240 min after medication. Plasma was separated by centrifugation at 3000 rpm for 10 min. The rabbits were sacrificed at 0.5, 2, 4 and 12 h, and the tissues were removed, weighted and disrupted to homogenates. The plasma samples and the homogenate samples were stored at −20 °C until HPLC analysis (see Section 2.4.2).

## 3. Results and discussion

### 3.1. Physical stability of SAN

#### 3.1.1. Effect of centrifugation

Like other colloidal dispersions, SAN may aggregate into large particles. After centrifugation with rotate speed from 2000 to 8000 rpm, the particle size of acyclovir SAN increased slightly with speed dependency according to the turbidity measurement (data not shown). However, the particles size increased significantly after 10,000-rpm centrifugation. The surface charge repulsion among SAN improves to keep stable (Langer et al., 2003). In fact, nearly no significant precipitants or flocculation appeared after the SAN containing SGSA of less than 5 mg/ml kept at room temperature for one year.

#### 3.1.2. Effects of additives

The low-concentration SAN would like to keep stable when being mixed with 150 mM NaCl, 0.1 M NaOH, 100 mM SDS, 10 mM CTAB, 10% Tween 80, 10% Brij 35, 10% poloxamer 188 and SPC liposomes because the turbidity of the mixtures had no significant changes, even though the large volume (500 μl) of additives was used to mix with the SAN of 2.5 ml. However, 0.1 M HCl solution, 150 mM $CaCl_2$ solution and plasma significantly increased the turbidity even when a few additives (50 μl) were added. $H^+$ and $Ca^{2+}$ could promote the negatively charged SAN aggregating or fusion by possibly improving surface hydrophobicity (Ohki and Arnold, 2000). The effect of plasma on SAN could mainly be resulted from plasma protein binding to the SAN, and the protein could improve the aggregation of SAN like liposomes (Lauraeus et al., 1998). In addition, the sensitivity of SAN to NaCl-like electrolytes was depended on the particle number per unit volume of suspensions. The low-concentration SAN (less than 5 mg/ml) were not sensitive to 150 mM NaCl. However, when SGSA in SAN was over 7 mg/ml, i.e. high particle number per unit volume, 150 mM NaCl solution led the SAN aggregation easily.

Although the SAN were relatively insensitive to various surfactants and liposomes, these surfactants including phospholipids did not benefit the preparation of SAN. They were apt to interfere the formation of SAN. A lot of large particles appeared if the surfactants were co-dissolved with SGSA in THF and injected into water. The surfactants could insert the bilayers of SAN on preparing so that the SAN could not be ready to form.

#### 3.1.3. Effects of sterilization methods

As a sterilization method, $^{60}Co$ gamma-irradiation strongly facilitated the SAN aggregation and damage. A lot of flocculation appeared after irradiation, and the content of SGSA decreased. Like liposomes, gamma-irradiation may destroy the bilayer structures of SAN and promote monomer degradation (Zuidam et al., 1995), whereas short-time heat treatment has fewer influences than radiation on bilayers (Zuidam et al., 1993).

The autoclave or the 100 °C bath for sterilization neither influenced the appearance of the SAN nor improved SGSA hydrolysis. Therefore, autoclave or 100 °C bath is the good sterilization method for SAN.

### 3.2. Chemical stability of SAN

#### 3.2.1. Effect of pH

SGSA in SAN was very sensitive to alkaline solutions (pH 9.0 and 12.0), and kept stable in weak acidic buffers (pH 5.0) and neutral buffers (pH 7.4) (Fig. 1). SGSA possesses the carboxylester structure and would like to be hydrolyzed with $OH^{-1}$ as catalyzer (Shameem et al., 1993). The main product of hydrolysis was acyclovir while the possible intermediate SACV hardly appeared, so that the ester bond between succinyl and acyclovir moieties was more sensitive than the bond between succinyl and glycerol moieties. The pseudo-first order kinetics was used to describe SGSA degradation. The $t_{1/2}$ of SGSA in SAN at pH 5.0, 7.4, 9.0 and 12.0 were 1733, 495, 94 and 21 h, respectively.

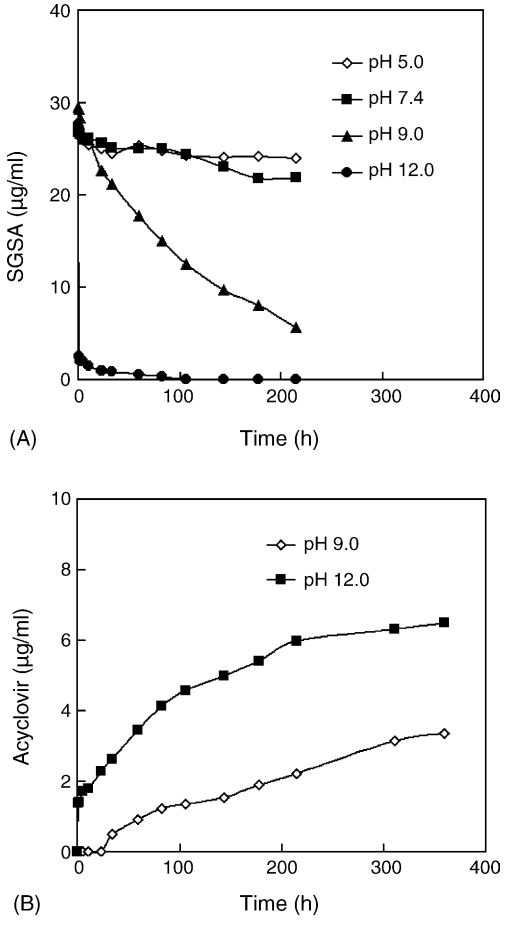


Fig. 1. The pH dependency of the chemical stability of acyclovir SAN. The SAN were diluted with different buffers and incubated at 37 °C. The buffers were 20 mM phosphate buffers (pH 5.0 and 7.4) and 20 mM Tris–HCl buffers (pH 9.0 and 12.0), respectively. (A) The degradation profiles of SGSA; (B) the production profiles of acyclovir. The half-lives of SGSA at pH 5.0, 7.4, 9.0 and 12.0 were 1733, 495, 94 and 21 h, respectively, based on the pseudo-first order kinetics.

Interestingly, after SGSA solutions in DMSO were diluted with buffers, SGSA degraded more rapidly than SGSA in SAN. The $t_{1/2}$ of SGSA in the DMSO/buffers mixtures were 495 h at pH 5.0 (about 1/4 of SGSA in SAN) and 210 h at pH 7.4 (less than 1/2 of SGSA in SAN), and disappeared completely within 20 min at pH 12.0. The degradation of SGSA is mainly determined by the exposure probability of sensitive ester bonds to water according to the above analysis. The sensitive bonds of SGSA in SAN are hidden in bilayers. However, when SGSA solutions in DMSO were diluted with buffers, SGSA molecules fell into an irregular state where a lot of sensitive ester bonds maybe directly exposed to water and catalyzers. Therefore, it is probably the specific ordered structures of SAN that protect SGSA from hydrolysis.

#### 3.2.2. Effects of hydrolase, plasma and tissue homogenates

Hydrolases including carboxylester enzymes exist widely in organism. PLCE, rabbit plasma and rabbit tissue homogenates were used to study the enzymatic hydrolysis of SGSA. SGSA in SAN was nearly completely hydrolyzed in PLCE solutions (10 U/ml) after 15 h of incubation at 37 °C with the $t_{1/2}$ of 4.7 h, and the hydrolysis speed was more than 100 folds as rapid as in pH 7.4 buffers (Fig. 2A). Therefore, SGSA is very sensitive to carboxylester enzymes. Also, rabbit plasma hydrolyzed SGSA much more rapidly than pH 7.4 buffers with the $t_{1/2}$

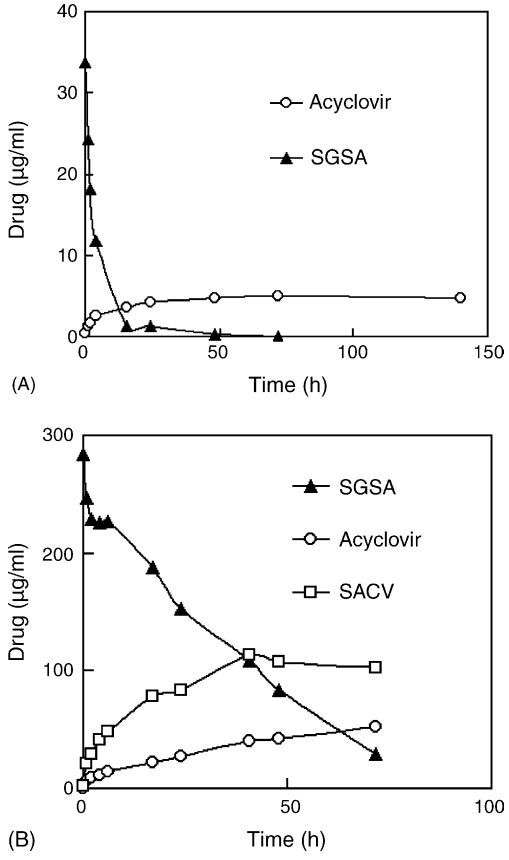


Fig. 2. The effects of: (A) pig liver carboxylester enzyme (PLCE); and (B) rabbit plasma on the chemical stability of acyclovir SAN at 37 °C. PLCE solution (10 U/ml) was prepared with Tris–HCl buffer (20 mM, pH 7.4) before use. The half-lives of SGSA were 4.7 h in PLCE solution and 25 h in rabbit plasma.

of 25 h (Fig. 2B). Some intermediate SACV appeared in the plasma samples, which indicated that some enzymes in plasma could hydrolyze the succinyl-glycerol ester bonds of SGSA. The degradation of SGSA in rabbit tissue homogenates depended on the types of homogenates. The degradation $t_{1/2}$ of SGSA in heart, liver, lung, spleen, kidney and brain were 5.0, 8.7, 11.8, 13.7, 30.7 and 73.7 h, respectively, which must result from diverse enzyme activity between tissue homogenates.

### 3.3. Hemolytic action of SAN

Hemolytic phenomena partly appeared with varied extent in the SAN/erythrocyte mixtures after 12 h of incubation at 37 °C. Both the high-concentration SGSA and the long incubation time improved hemolysis. SGSA of 18 μg/ml did not show significantly hemolytic action, while marked hemolysis happened when SGSA in samples was over 36 μg/ml. At the same time, the cell number in the sample containing 18 μg/ml SGSA was $18 \times 10^7$, close to the primitive cell number ($24 \times 10^7$), while the sample containing 36 μg/ml SGSA had $12 \times 10^7$ cells. When SGSA of 90 μg/ml was in the sample, the cell number was only $3.0 \times 10^7$. The concentration ratio of SGSA in cells and solutions ($C_{cell}/C_{solution}$) had positive linear relationship with the cell number in samples, i.e. the more cell number was, the more fraction SGSA distributed in cells. When enough SGSA molecules distributed in cells, cell membranes would like to be disrupted, and then SGSA together with hemoglobin was released to solutions, which resulted in cell number and $C_{cell}/C_{solution}$ reducing. The high-concentration SGSA in samples would accelerate and strengthen the process. In the case of 90 μg/ml SGSA, $C_{cell}/C_{solution}$ were 1.2, compared with 3.3 of 18 μg/ml SGSA. Therefore, it is likely that the amphiphilic SGSA molecules may insert into erythrocyte membranes until membrane breakdown like some surface-active chemicals (Ross et al., 2004). Hemolysis did not immediately happen on mixing probably because the transfer of SGSA from SAN to erythrocyte membranes needed time. Acyclovir solution did not show any hemolytic action. Cell-membrane insertion would give SGSA a chance to go into cells.

Surprisingly, SAN seemed to have no hemolytic effect on whole blood. When the additional SAN was less 10% (v/v) of rabbit whole blood, no hemolysis happened even though SGSA in the sample was near to 1000 μg/ml. However, as soon as the additional SAN or water was over 10% (v/v) of whole blood, hemolysis preferred to happen. Obviously, the hemolysis of whole blood was just relevant to the descent of osmotic pressure of samples. The binding plasma proteins would markedly reduce the interaction between SAN and erythrocytes, and the binding action also named opsonization would lead the uptake of SAN by the mononuclear phagocyte system (MPS) in vivo like liposomes and nanoparticles (Ishida et al., 2002; Moghimia and Szebeni, 2003).

### 3.4. In vitro toxicity and antiviral activity of SAN

Acyclovir SAN showed weak toxicity to Vero cells. $TC_{50}$ of SGSA was 750 μg/ml, i.e. 1126 μM. Acyclovir solutions did not show any toxicity even when acyclovir was over 1000 μg/ml, i.e. 4444 μM. Referred to the interaction between SAN and erythrocytes, the strong cell-membrane insertion of amphiphilic SGSA could be the primary reason of cell toxicity (Prisbe et al., 1986).

The anti-HSV $IC_{50}$ of SGSA in SAN on Vero cell model was 46.8 μM, higher than that of acyclovir (7.1 μM). Antiviral selection index (SI) was equal to $TC_{50}/IC_{50}$. The SI of SGSA and acyclovir were 24 and more than 626, respectively. The above data just demonstrate that the antiviral activity of SGSA in SAN is much weaker than acyclovir, but whether the in vivo results is the same needs further proofs.

Acyclovir has to be transformed to its triphosphate in cell plasma to resist HSV. The in vitro anti-HSV action of the SAN demonstrated that SGSA could enter into cell plasma where it was hydrolyzed to acyclovir. The degradation of SGSA in organism was carboxylester enzyme-dependency (see Section 3.2). Unfortunately, the carboxylester enzyme activity of Vero cells is low according to the literature (McGuigan et al., 2000). In fact, some prodrugs of antivirals show strong antiviral action in vivo but weak activity on cell model because the enzyme activity in vivo and in vitro is significantly different (Neyts and de Clercq, 1998). For example, a good antiviral famciclovir (a prodrug of acyclovir) do not show any anti-HSV action on Vero cell model (Li et al., 2003).

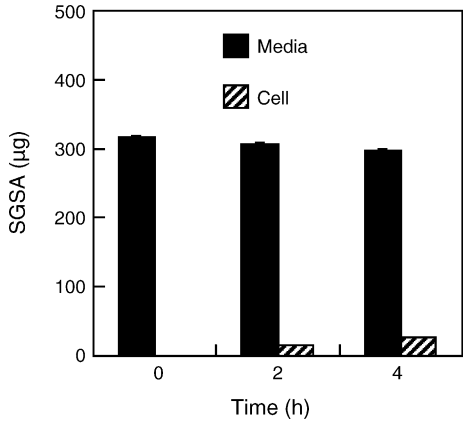


Fig. 3. The uptake of acyclovir SAN by a macrophage cell line of QXMSC1 cells after co-culturing them at 37 °C. Results were expressed as the mean ± S.D. ($n = 3$). No significant uptake was found.

### 3.5. Uptake of SAN by macrophages

QXMSC1 cells are established from murine marrow and show phagocytic ability (Qin and Xie, 1998). The uptake of acyclovir SAN by QXMSC1 cells was not significant (Fig. 3). The in vitro uptake of no-ligand modified liposomes by macrophages is not significant too (Opanasopit et al., 2001). However, after being modified by ligands such as mannose residues and serum poteins (opsonins), the liposomes or the nanoparticles can be markedly trapped by macrophages (Opanasopit et al., 2001; Ahsan et al., 2002; Ishida et al., 2002). No uptake was found for acyclovir solutions.

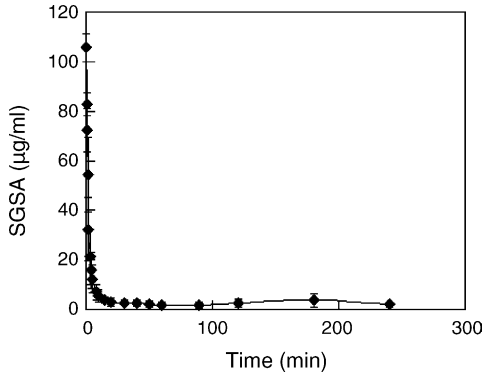


Fig. 4. The time profile of SGSA concentration in plasma after acyclovir SAN bolus iv administration to rabbits with 30 mg SGSA/kg through ear vein. Results were expressed as the mean ± S.D. ($n = 5$). The field of 0–5 min was considered as the distribution phase with the half-life ($t_{1/2\alpha}$) of 1.5 min, and 5–90 min as the elimination phase with the half-life ($t_{1/2\beta}$) of 47 min based on the first order kinetics. A little higher SGSA concentration at 180 min than the minimum level was maybe due to some lung-blocked SAN-aggregating large particles releasing SAN or SGSA to circulation.

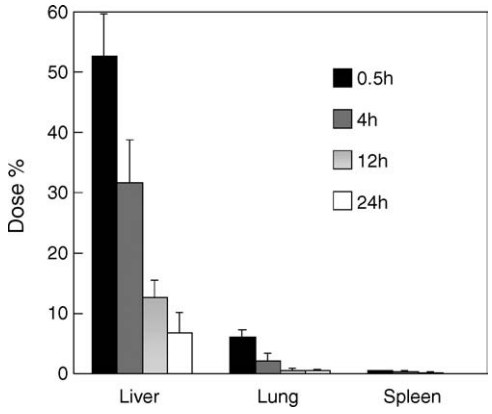


Fig. 5. The tissue distribution of SGSA after acyclovir SAN bolus iv administration to rabbits. Results were expressed as the mean ± S.D. ($n = 3$). SGSA was mainly distributed in liver, spleen and lung although only a little SGSA appeared in kidney and heart, and nothing was found in brain. The elimination $t_{1/2}$ of SGSA in liver, spleen and lung were 7.6, 6.8 and 6.6 h, respectively, near to the hydrolysis $t_{1/2}$ of SGSA in tissue homogenates. The main tissue elimination way of SGSA could be resulted from the metabolism.

### 3.6. *In vivo fate of SAN*

#### 3.6.1. *Pharmacokinetics*

The SAN were cleared from blood circulation very quickly after rabbits received acyclovir SAN by bolus iv administration (Fig. 4). The plasma concentration of SGSA had descended for about 90% at 5 min, and approached the minimum level at 30 min. The field of 0–5 min was considered as the distribution phase with the half-life ($t_{1/2\alpha}$) of 1.5 min, and 5–90 min as the elimination phase with the half-life ($t_{1/2\beta}$) of 47 min based on the first-order kinetics. The pharmacokinetic characteristics of SAN were similar to the particulate preparations such as liposomes and nanoparticles (Harashima and Kiwada, 1996; Groll et al., 2000; Bibby et al., 2005). Integrating the in vitro result (Section 3.5), it was likely that the SAN were opsonized in vivo by plasma proteins, and then it would appear that the nanosized SAN were trapped by the MPS. Some physicochemical properties such as size, surface charge would also influence the in vivo clearance rate of the SAN. In addition, the clearance of the SAN seemed more rapid than liposomes or nanoparticles (Harashima and Kiwada, 1996).

#### 3.6.2. *Tissue distribution*

To further evaluate the in vivo fate of acyclovir SAN, the tissue distribution of SGSA was investigated. SGSA was mainly distributed in liver, spleen and lung after acyclovir SAN bolus iv administration to rabbits (Fig. 5). SGSA content in liver was very high to over 50% of the whole injected dose at 0.5 h, and more than 30% at 4 h. SGSA concentration in liver, spleen and lung was very high to more than 200 μg/g at 0.5 h. Only a little SGSA appeared in kidney (about 30 μg/g at 0.5 h) and heart (about 4 μg/g at 0.5 h). Nothing was found in brain. Therefore, the nanosized SAN were mainly trapped by the MPS including liver, spleen and lung. Also, lung capillary vessels could block some large particles formed due to SAN aggregation. SGSA in tissues was cleared in a first order kinetic mode and disappeared completely at 48 h (Fig. 5). The elimination $t_{1/2}$ of SGSA in liver, spleen and lung were 7.6, 6.8 and 6.6 h, respectively, near to the hydrolysis $t_{1/2}$ of SGSA in tissue homogenates, so that the metabolism of SAGS in cell plasma could be the main elimination way. From the plasma concentration-time curve (Fig. 4), SGSA concentration in plasma at 180 min was a little higher than the minimum level, which was thought that some lung-blocked SAN-aggregating large particles released SAN or SGSA to circulation again. Acyclovir as the in vivo metabolism product was also determined, and no acyclovir was found in plasma, heart, kidney and brain. Acyclovir with less than 10 μg/g appeared in liver, spleen and lung within 12 h after administration because acyclovir could be eliminated rapidly from plasma and tissues (Biron et al., 1982).

The site-specific distribution of SAN indicates that, acyclovir SAN or other SAN prepared from antivirals can be expected to benefit the therapy of virosis in liver, spleen and lung. The SAN would be considered as the novel preparations of acyclovir with targeting and sustained-release functions although acyclovir was a model drug in the paper. SADDS including SAN would like to become targeted drug delivery systems although only the physical targeting to the MPS is achieved now. In the future, SAN will be modified to got more functions such as long circulating, pH sensitivity.

## 4. Conclusions

SADDS are novel drug delivery approaches, and enlarge the fields of pharmaceutical researches. SADDS combine prodrugs, molecular self-assembly with nanotechnology. Acyclovir SAN in the paper become the successful examples of SADDS, and show the well site-specific distribution in vivo. Much more SADDS including SAN will be prepared from more hydrophilic drugs such as nucleosides and lipids such glycerides on the basis of this paper. It can be predicted that SADDS will be useful to anti-viral, anti-cancer and gene therapy.

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## References

Ahsan, F., Rivas, I.P., Khan, M.A., Suarez, A.I.T., 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. J. Control. Release 79, 29–40.

Allen, T.M., Cullis, P.R., 2004. Drug delivery systems: entering the mainstream. Science 303, 1818–1822.

Barenholz, Y., 2001. Liposome application: problems and prospects. Curr. Opin. Colloid Interface Sci. 6, 66–77.

Bibby, D.C., Talmadge, J.E., Dalal, M.K., Kurz, S.G., Chytil, K.M., Barry, S.E., Shand, D.G., Steiert, M., 2005. Pharmacokinetics and biodistribution of RGD-targeted doxorubicin-loaded nanoparticles in tumor-bearing mice. Int. J. Pharm. 293, 281–290.

Biron, K.K., Noblin, J.E., Miranda, P.D., Elion, G.B., 1982. Uptake, distribution, and anabolism of acyclovir in herpes simplex virus-infected mice. Antimicrob. Agents Chemother. 21, 44–50.

Choi, S.K., Vu, T.K., Jung, J.K., Kim, S.J., Jung, H.R., Chang, T., Kim, B.H., 2005. Nucleoside-based phospholipids and their liposomes formed in water. Chembiochem 6, 432–439.

Davis, J.T., 2004. G-quartets 40 years later: from 5′-GMP to molecular biology and supramolecular chemistry. Angew. Chem. Int. Ed. 43, 668–698.

Defrise-Quertain, F., Chatelain, P., Delmelle, M., Ruysschaert, J., 1984. Model studies for drug entrapment and liposome stability. In: Gregoriadis, G. (Ed.), Liposome Technology, vol. 2. CRC Press, Florida, Boca Raton, pp. 1–17.

de La Iglesia, P., Melo, S., Lopez, B., Rodriguez, M., Blanco, M.I., Mellado, P., de Ona, M., 1998. Rapid screening tests for determining in vitro susceptibility of herpes simplex virus clinical isolates. J. Clin. Microbiol. 36, 2389–2391.

de Miranda, P., Blum, M.R., 1983. Pharmacokinetics of acyclovir after intravenous and oral administration. J. Antimicrob. Chemother. 12 (Suppl. B), 29–37.

Drummond, C.J., Fong, C., 2000. Surfactant self-assembly objects as novel drug delivery vehicles. Curr. Opin. Colloid Interface Sci. 4, 449–456.

Giorgi, T., Grepioni, F., Manet, I., Mariani, P., Masiero, S., Mezzina, E., Pieraccini, S., Saturni, L., Spada, G.P., Gottarelli, G., 2002. Gel-like lyomesophases formed in organic solvents by self-assembled guanine ribbons. Chem. Eur. J. 8, 2143–2152.

Glavas-Dodov, M., Fredro-Kumbaradzi, E., Goracinova, K., Simonoska, M., Calis, S., Trajkovic-Jolevska, S., Hincal, A.A., 2005. The effects of lyophilization on the stability of liposomes containing 5-FU. Int. J. Pharm. 291, 79–86.

Gottarelli, G., Masiero, S., Mezzina, E., Pieraccini, S., Rabe, J.P., Samori, P., Spada, G.P., 2000. The self-assembly of lipophilic guanosine derivatives in solution and on solid surfaces. Chem. Eur. J. 6, 3242–3248.

Groll, A.H., Mickiene, D., Werner, K., Petraitiene, R., Petraitis, V., Calendario, M., Field-Ridley, A., Crisp, J., Piscitelli, S.C., Walsh, T.J., 2000. Compartmental pharmacokinetics and tissue distribution of multilamellar liposomal nystatin in rabbits. Antimicrob. Agents Chemother. 44, 950–957.

Harashima, H., Kiwada, H., 1996. Liposomal targeting and drug delivery: kinetic consideration. Adv. Drug Deliv. Rev. 19, 425–444.

Hostetler, K.Y., Parker, S., Sridhar, C.N., Martin, M.J., Li, J.L., Stuhmiller, L.M., Wijk, G.M.T., Bosch, H., Gardner, M.F., Aldern, K.A., Richman, D.D., 1993. Acyclovir diphosphate dimyristoylglycerol: a phospholipid prodrug with activity against acyclovir-resistant herpes simplex virus. Proc. Natl. Acad. Sci. U.S.A. 90, 11835–11839.

Hostetler, K.Y., Beadle, J.R., Kini, G.D., Gardner, M.F., Wright, K.N., Wu, T.H., Korba, B.A., 1997. Enhanced oral absorption and antiviral activity of 1-*O*-octadecyl-*sn*-glycero-3-phospho-acyclovir and related compounds in hepatitis B virus infection, in vitro. Biochem. Pharmacol. 53, 1815–1822.

Ishida, T., Harashima, H., Kiwada, H., 2002. Liposome clearance. Biosci. Rep. 22, 197–224.

Itojima, Y., Ogawa, Y., Tsuno, K., Hands, N., Yanagawa, H., 1992. Spontaneous formation of helical structures from phospholipid-nucleoside conjugates. Biochemistry 31, 4757–4765.

Jin, Y., Qiao, Y., Li, M., Ai, P., Hou, X., 2005. Langmuir monolayers of the long-chain alkyl derivatives of a nucleoside analogue and the formation of self-assembled nanoparticles. Colloid. Surf. B Biointerfaces 42, 45–51.

Kim, I.S., Jeong, Y.I., Cho, C.S., Kim, S.H., 2000. Thermo-responsive self-assembled polymeric micelles for drug delivery in vitro. Int. J. Pharm. 205, 165–172.

Lambert, D.M., 2000. Rationale and applications of lipids as prodrug carriers. Eur. J. Pharm. Sci. 11, S15–S27.

Langer, K., Balthasar, S., Vogel, V., Dinauer, N., von Briesen, H., Schubert, D., 2003. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. Int. J. Pharm. 257, 169–180.

Lauraeus, S., Holopainen, J.M., Taskinen, M., Kinnunen, P.K.J., 1998. Aggregation of dimyristoylphosphatidylglycerol liposomes by human plasma low density lipoprotein. Biochim. Biophys. Acta 1373, 147–162.

Li, J., Teng, L., Chen, H., Jiang, N., Jiang, J., 2003. Comparison of efficacies of famciclovir with acyclovir against herpes simple virus type 1 and 2 in vitro and in vivo. Chin. Pharm. J. 38, 423–426.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 23, 3–25.

McGuigan, C., Slate, M.J., Parry, N.R., Perry, A., Harris, S., 2000. Synthesis and antiviral activity of acyclovir-5′-(phenyl methoxy alaninyl) phosphate as a possible membrane-soluble nucleotide prodrug. Bioorg. Med. Chem. Lett. 10, 645–647.

Moghimia, S.M., Szebeni, J., 2003. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog. Lipid Res. 42, 463–478.

Moreau, L., Grinstaff, M.W., Barthélémy, P., 2005. Vesicle formation from a synthetic adenosine based lipid. Tetrahedron Lett. 46, 1593–1596.

Neyts, J., de Clercq, E., 1998. In vitro and in vivo inhibition of murine gamma herpesvirus 68 replication by selected antiviral agents. Antimicrob. Agents Chemother. 42, 170–172.

Ohki, S., Arnold, K., 2000. A mechanism for ion-induced lipid vesicle fusion. Colloid. Surf. B Biointerfaces 18, 83–97.

Opanasopit, P., Higuchi, Y., Kawakami, S., Yamashita, F., Nishikawa, M., Hashida, M., 2001. Involvement of serum mannan binding proteins and mannose receptors in uptake of mannosylated liposomes by macrophages. Biochim. Biophys. Acta 1511, 134–145.

Pastor-Anglada, M., Felipe, A., Casado, F.J., 1998. Transport and mode of action of nucleoside derivatives used in chemical and antiviral therapies. Trends Pharmacol. Sci. 19, 424–430.

Prisbe, E.J., Martin, J.C., McGee, D.P.C., Barker, M.F., Smee, D.F., Duke, A.E., Matthews, T.R., Verheyden, J.P.H., 1986. Synthesis and antiherpes virus activity of phosphate and phosphonate derivatives of 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine. J. Med. Chem. 29, 671–675.

Qin, F.H., Xie, S.S., 1998. Establishment and characterization of murine bone marrow stromal macrophage line. Chinese Sci. Bull. 43, 178–186.

Ross, B.P., Braddy, A.C., McGeary, R.P., Blanchfield, J.T., Prokai, L., Toth, I., 2004. Micellar aggregation and membrane partitioning of bile salts, fatty acids, sodium dodecyl sulfate, and sugar-conjugated fatty acids: correlation with hemolytic potency and implications for drug delivery. Mol. Pharm. 1, 233–245.

Schreier, S., Malheiros, S.V.P., de Paula, E., 2000. Surface active drugs: self-association and interaction with membranes and surfactants. Physicochemical and biological aspects. Biochim. Biophys. Acta 1508, 210–234.

Shah, J.C., Sadhale, Y., Chilukuri, D.M., 2001. Cubic phase gels as drug delivery systems. Adv. Drug Deliv. Rev. 47, 229–250.

Shameem, M., Imai, T., Otagiri, M., 1993. An in vitro and in vivo correlative approach to the evaluation of ester prodrugs to improve oral delivery of propranolol. J. Pharm. Pharmacol. 45, 246–252.

Szoka, F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 7, 4194–4198.

Torchilin, V.P., 2000. Drug targeting. Eur. J. Pharm. Sci. 11, S81–S91.

Vaizoglu, M.O., Speiser, P.P., 1986. Pharmacosomes—a novel drug delivery system. Acta Pharm. Suec. 23, 163–172.

Whitesides, G.M., Grzybowski, B., 2002. Self-assembly at all scales. Science 295, 2418–2421.

Zarif, L., 2002. Elongated supramolecular assemblies in drug delivery. J. Control. Release 81, 7–23.

Zhai, L., Zhang, J., Shi, Q., Chen, W., Zhao, M., 2005. Transition from micelle to vesicle in aqueous mixtures of anionic/zwitterionic surfactants studied by fluorescence, conductivity, and turbidity methods. J. Colloid Interface Sci. 284, 698–703.

Zuidam, N.J., Lee, S.S., Crommelin, D.J., 1993. Sterilization of liposomes by heat treatment. Pharm. Res. 10, 1591–1596.

Zuidam, N.J., Lee, S.S., Crommelin, D.J., 1995. Gamma-irradiation of non-frozen, frozen, and freeze-dried liposomes. Pharm. Res. 12, 1761–1768.