Journal of Pharmaceutical Investigation
Vol. 40, No. 6, 357-362 (2010)

Complexation of Adiponectin-encoding Plasmid DNA with Rosiglitazone-loaded Cationic Liposomes

Enkhzaya Davaa[1], Ui-Hyeon Jeong[1], Baek-Ki Shin[1], Soon Gil Choi[1], Chang-Seon Myung[2,3] and Jeong-Sook Park[1,3†]

[1]*Department of Physical Pharmacy, College of Pharmacy, Chungnam National University*
[2]*Department of Pharmacology, College of Pharmacy, Chungnam National University*
[3]*Institute of Drug Research and Development, Chungnam National University*

(Received October 27, 2010 · Revised November 26, 2010 · Accepted November 27, 2010)

ABSTRACT − To enhance therapeutic effects of insulin-sensitizing adipokine, ADN gene and potent agonists, rosiglitazone for the PPARγ, cationic liposomes as non-viral vectors were formulated. The particle size and zeta potential of drug loaded and unloaded cationic liposomes were investigated. The complex formation between cationic liposomes and negatively charged plasmid DNA was confirmed and the protection from DNase was observed. *In vitro* transfection was investigated in HepG2, HeLa, and HEK293 cells by mRNA expression of ADN. Encapsulation efficacy of rosiglitazone-loaded liposomes was determined by UV detection. Particle sizes of cationic liposomes were in the range of 110-170 nm and those of rosiglitazone-loaded cationic liposomes were in the range of 130-180 nm, respectively. Gel retardation of complexes indicated that the complex was formed at weight ratios of cationic lipid to plasmid DNA higher than 20:1. Both complexes protected plasmid DNA from DNase either drug free or drug loading. Encapsulation efficiency of rosiglitazone-loaded emulsion was increased by drug dose. The mRNA expression levels of ADN were dose-dependently increased in cells transfected with plasmid DNA. Therefore, cationic liposomes could be potential co-delivery system for drug and gene.

Key words − Cationic liposome, Gene delivery, Type 2 diabetes mellitus, Adiponectin, Rosiglitazone

Type 2 diabetes mellitus (T2DM) is characterized by a progressive decline in pancreatic β-cell function and increased insulin resistance. Oral antihyperglycaemic agents are extensively used in the treatment of T2DM. Thiazolidinediones are insulin sensitizers developed specifically for T2DM, which act via activation of peroxisome proliferator-activated receptors (PPARs). Rosiglitazone is a thiazolidinedione that displays high affinity for PPARγ, which is predominately expressed in adipose tissue. Rosiglitazone can cause adipocyte differentiation, leading to simultaneously increase in both glucose uptake and lipid accumulation. Thus, this action may leads to weight gain, an unwanted side effect in an era that is rapidly associating obesity with T2DM (Lebovitz, 2002). Recently, the use of rosiglitazone to patients with T2DM has been restricted by FDA, due to data that suggest an elevated risk of cardiovascular events, such as heart attack and stroke, in patients treated with rosiglitazone.

In patients with T2DM, the plasma levels of adiponectin (ADN), adipokine hormone, are significantly lowered in which ADN stimulates glucose uptake and is abundant in plasma at normal state (Hotta et al., 2000). ADN decreases insulin resistance by lowering triglyceride content in muscle and liver in obese mice via the increased expression of molecules involved in both fatty-acid combustion and energy dissipation in muscle (Yamauchi et al., 2001). It has been reported that ADN is correlated with the homeostasis model assessment (HOMA), which is an indirect index of insulin resistance (Inoue et al., 2005). ADN also acts in the brain to decrease body weight mainly by stimulating energy expenditure (Qi et al., 2004). Previously we constructed ADN-encoding plasmid DNA and administered into T2DM mouse model, since successful delivery of ADN protein to targets by direct administration is difficult due to its peptide characteristics (Nan et al., 2010). Moreover, it is reported that co-delivery of drug and gene by cationic micelles could achieve a synergistic therapeutic effect as well as improve patients' compliance. Thus, if rosiglitazone is available in combination with ADN, it is supposed that ADN alleviates the side effect of rosiglitazone by dose reduction (Wiradharma et al., 2009).

Here, we prepared cationic liposomes for co-delivery therapeutic gene adiponectin and drug rosiglitazone. Then, we investigated the physicochemical properties of rosiglitazone-loaded cationic liposomes and the effect of drug loading on the features of complexed DNA.

†Corresponding Author :
Tel : +82-42-821-5932, E-mail : eicosa@cnu.ac.kr
DOI : 10.4333/KPS.2010.40.6.357

Materials and Methods

Materials

Rosiglitazone was purchased from Masung & Co., Ltd. (Seoul, Korea). 1,2-Dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP), 1,2-distearyltrimethyl ammonium propane (DSTAP) or 1,2-dimyristoyltrimethyl ammonium propane (DMTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Gibco® BRL (NY, USA). Lipofectamine™ 2000 and Trizol® were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals used were of reagent grade.

Cell culture

Human hepatocellular carcinoma cells (HepG2), human cervical adenocarcinoma cells (HeLa) and human embryonic kidney cells (HEK293) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 unit/mL penicillin and 100 μg/mL streptomycin at 37°C in humidified incubator supplied with 5% CO_2.

Purification of plasmid DNA

The plasmid DNA encoding adiponectin (pVAX/ADN) (Nan et al., 2010) was amplified in the *Escherichia coli* DH5α and purified with the Qiagen Plasmid Mega kit (Qiagen, CA, USA) according to the manufacturer's instruction. The integrity of DNA preparation was confirmed on a 1% agarose gel.

Preparation of cationic liposomes and drug-loaded liposomes

Cationic liposomes were prepared by lipid film method. Stock solutions of each lipid were mixed at the molar ratio of cationic lipid/DOPE/Chol=5:4:1 in chloroform (Kang et al., 2010), in which cationic lipids were varied such as DOTAP, DMTAP, or DSTAP. After choosing cationic lipid, the mixing ratio of lipid was optimized to obtain better physicochemical properties. The organic phase was removed at 37°C on a rotary evaporator (KU-NLW, Sunil Instrument Co., Korea). The dried lipid film was flushed with nitrogen gas to remove traces of organic solvents. After 2 mL of phosphate-buffered saline (PBS, pH 7.4) was added to hydrate liposomes, the lipid solution was vortexed and subsequently sonicated in a bath type sonicator (Bransonic, Branson Ultrasonic Co., Danbury, CT, USA). Following sonication at about 37°C for 90 min, the solution was extruded through a Nucleopore polycarbonate membrane of 0.1 μm pore size with nitrogen gas. Then, the prepared cationic liposomes were stored at 4°C. Drug-loaded cationic liposomes were prepared by hydration buffer (monobasic potassium phosphate buffer, pH 2.3) containing rosiglitazone diluted.

Preparation of cationic liposomes/DNA complexes

The cationic liposomes/DNA complexes were prepared by mixing plasmid DNA and cationic lipid formulation at the various ratios. The mixtures were incubated for 15 min at 37°C to facilitate complex formation.

Measurement of particle size and zeta potential

Particle size distribution of cationic liposomes and complex was measured by light scattering spectrophotometer (ELS-8000, Otsuka Electronics Co., Japan). The samples were diluted with deionized water, and then transferred into a quartz cuvette in an ELS-8000 dynamic light scattering instrument. Zeta potential of the cationic liposomes and complex was measured using an electrophoretic light scattering spectrophotometer at room temperature. Data were analyzed using a software package (ELS-8000 software) supplied by the manufacturer.

Measurement of drug encapsulated in cationic liposomes

The encapsulation efficiency was calculated according to a method as reported previously by Ishii and Nagasaka (2001).

$$\text{Encapsulation efficiency (\%)} = \frac{C_{total} - C_{out}}{C_{total}} \times 100$$

where C_{out} is the liposomes suspension diluted with water and ultrafiltered through a 0.45 μm PVDF syringe filter (Leur Lock type; NSW Norm-Ject®, Whatman International Ltd) to remove the liposomes and C_{total} is the liposomes suspension diluted with heated ethanol (70°C) in order to disrupt the liposomes completely and release the encapsulated drugs to the solvent. The ethanol solution was cooled and ultrafiltered through a 0.45 μm PVDF syringe filter.

Agarose gel retardation

Complex formation between the plasmid DNA and the cationic liposomes was assessed using an agarose gel electrophoresis. Various ratios (4-40, w/w) of lipid/DNA mixtures with fixed amount of DNA (1 μg of pVAX/ADN) were incubated for 15 min to facilitate complex formation and loaded onto a 1% agarose gel. As a control, Lipofectamine®/DNA complex containing 0.5 μg of pVAX/ADN at the optimal ratio

of 6 (Lipofectamine® to DNA, w/w) was also loaded on the gel. Gel electrophoresis was carried out in a TBE buffer (40 mM Tris, boric acid, 1 mM EDTA) at 50 mV. Ethidium bromide stained DNA was visualized using an UV illuminator.

Complex stability against DNase I digestion

The cationic liposomes were mixed with 0.2 μg of pVAX/ADN to the ratio of 40 (DOTAP to DNA, w/w) and allowed to form complexes for 15 min at room temperature and 37ºC. For DNase I digestion, the cationic liposomes were prepared in water instead of PBS to avoid the interfering effect of high salt in DNase I activity. Naked DNA (0.2 μg) or the cationic liposomes/DNA complex containing the same amount of DNA was exposed to DNase I for 30 min at 37ºC to investigate the stability of the complex against nuclease digestion. DNA was retrieved by phenol/chloroform extraction followed by ethanol precipitation and visualized on an agarose gel containing ethidium bromide.

***In Vitro* transfection**

Cells were seeded at a density of 5×10^5 cells/well in 6-well plates (Nunc, Denmark) and grown to 70-80% confluence. The cells were washed with PBS and the transfection complex was added to each well. After transfection for 3 h, the transfection complex was removed and the cells were fed with 2 mL fresh DMEM containing 10% FBS and incubated for 24 h at 37ºC. Commercially available Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used as a control. All transfection experiments were performed in triplicate. Twenty-four hours post-transtection, the mRNA expression levels of ADN in cells were measured by RT-PCR.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The transfected cells were lysed with Trizol® (Invitrogen) and protein were extracted with chloroform. Total RNAs were precipitated with isopropanol and washed twice with 70% ethanol diluted by DEPC-treated water. The purity and concentration of RNAs were analyzed using a spectrophotometer at 260 nm and 280 nm. The cDNA was synthesized from total RNA in total reaction mixture containing 5× reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 100 μg/mL Oligo-dT, 10 mM dNTP, 100 U M-MLV reverse transcriptase (Promega, Madison, WI). The RT reaction product was amplified by PCR with specific primers (forward: 5'-GGA ATT CAT GCT ACT GTT GCA AGC TCT-3', reverse: 5'-GCT CTA GAT CAG TTG GTA TCA TGG TAG AGA AG-3') for ADN and (forward: 5'-CTG TCT GGC GGC ACC ACC AT-3', reverse: 5'-GCA ACT AAG TCA TAG TCC GC-3') for human b-actin as the house-keeping gene. Amplification of mouse ADN was the performed by 30 cycles at 94ºC for 30 s, 59ºC for 40 s, 72ºC for 1 min. PCR products were separated on a 1% agarose gel and visualized by UV. The amount of cDNA was measured using ChemiDoc™ XRS System (Quantity One®, Bio-Rad) to quantitate ADN mRNA or b-actin mRNA in separate reactions.

Statistics

All data were expressed as the mean ± the standard error of the mean (S.E.M) from three independent experiments.

Results and Discussion

Physicochemical characterization of cationic liposomes

The cationic liposomes with DOTAP lipid formulation were smaller in particle size than other cationic lipid (Table I). From particle size data, DOTAP was chosen as cationic lipid and the various ratios of lipid were applied to preparation of liposomes. The cationic liposomes at lipid ratio of DOTAP:DOPE:Chol=1:1:1 showed smaller particle size of 170.0±31.2 nm and cationic liposomes at all ratios showed positive charges of 68.0±5.1 mV (Table II).

Figure 1a shows the effect of sonication time on the particle size of cationic liposomes. Although the particle size of the cationic liposomes decreased dependency on sonication time until 90 min, the zeta potential of the cationic liposomes had no significant correlation on sonication time. Figure 1b shows the effect of extrusion cycles on the particle size and zeta

Table I. *Effect of Cationic Lipid on Particle Size of Cationic Liposomes*

Cationic lipid	Particle size (nm)
DOTAP	249.7 ± 35.7
DMTAP	257.5 ± 41.2
DSTAP	539.8 ± 241.9

Cationic liposomes were prepared at the ratio of cationic lipid : DOPE : Chol = 5:4:1 (n=3). The lipid ratio was adopted from Kang et al. (2010).

Table II. *Particle Size and Zeta Potential of Cationic Liposomes at Various Lipid Ratios (n=3)*

DOTAP:DOPE:Chol	Particle size (nm)	Zeta potential (mV)
1:0.5:0.5	313.2 ± 35.3	45.7 ± 18.5
1:1:1	170.0 ± 31.2	68.0 ± 5.1
1:1:0	313.7 ± 57.0	41.4 ± 12.3
1:0:1	374.5 ± 58.8	50.7 ± 13.8

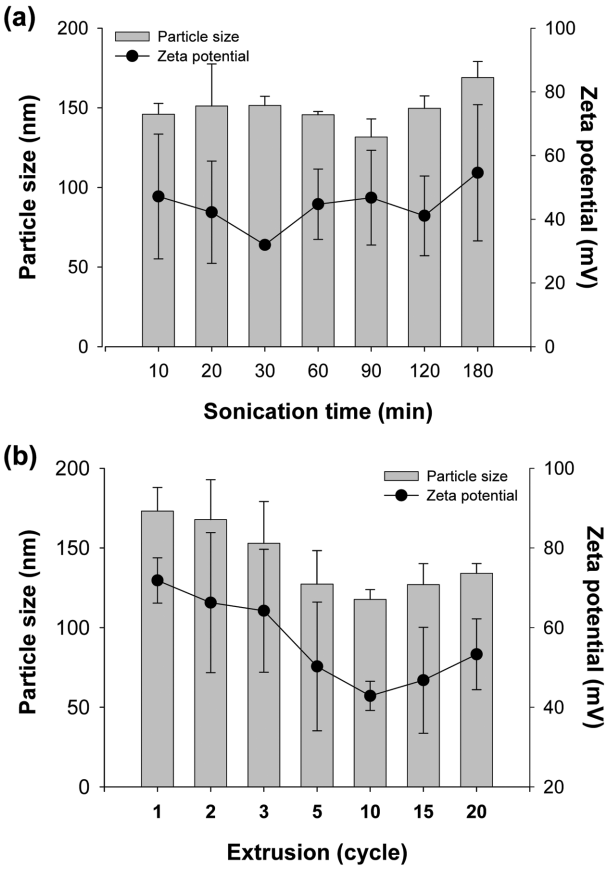

Figure 1. Preparation parameters of cationic liposomes. (a) Effect of sonication time on the particle size and zeta potential of cationic liposomes (n=3). (b) Effect of extrusion cycles on the particle size and zeta potential of cationic liposomes (n=3).

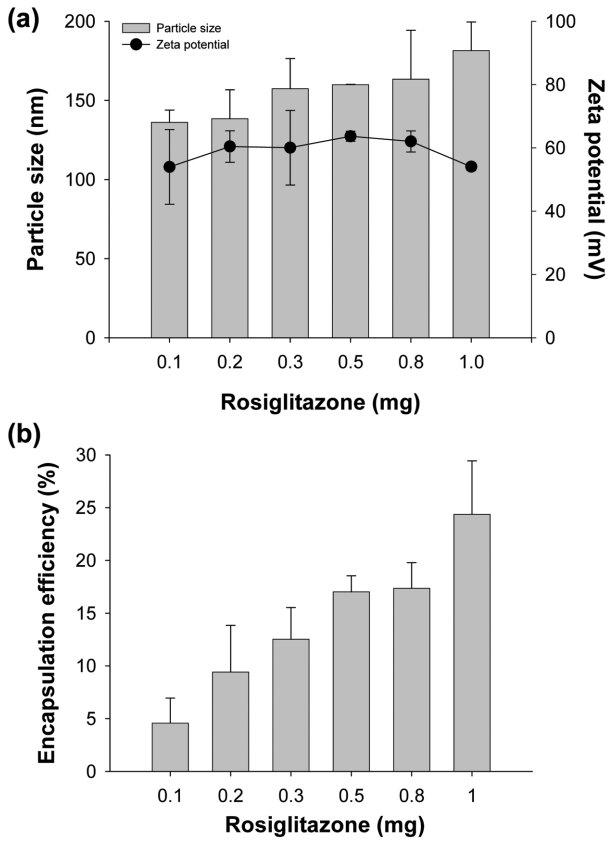

Figure 2. Physicochemical properties of rosiglitazone-loaded cationic liposomes. Total lipids were fixed as 1 mg on each preparation. (a) Particle size and zeta potential of drug-loaded cationic liposomes (n=3). (b) Encapsulation efficiency of rosiglitazone in cationic liposomes with varying the loading amount (n=3).

potential of cationic liposomes. The particle size and zeta potential of the cationic liposomes were decreased depending on extrusion cycles, and cycle number of 10 times indicated smallest particle sizes.

In order to investigate the effect of drug loading on particle size and zeta potential, we determined of rosiglitazone-loaded cationic liposomes at the various amount of drug. Whereas the particle sizes of drug-loaded liposomes were increased from 130 nm to 180 nm, and zeta potentials of drug-loaded liposomes were not changed by increasing the drug loading into cationic liposomes (Figure 2a). It is supposed that the increasing incorporation of rosiglitazone did not affect the zeta potential (from 54 to 64 mV) of cationic liposomes because the drug might be incorporated into lipid bilayer not on the surface of liposomes.

High encapsulation efficiency of rosiglitazone was obtained by increasing loading amount in cationic liposomes (Figure 2b). The encapsulation efficiency of rosiglitazone in the cationic liposomes was enhanced according to the loading amount, suggesting that complex formation occurs between rosiglitazone and cationic lipid composition. However, the loading efficiency of lipophilic drug would be reduced at certain ratio of drug to lipid due to limit in encapsulation capacity of lipid bilayer (Gulati et al., 1998).

Gel retardation assay

The DNA binding ability of cationic liposomes was studied through gel retardation assay (Jeong et al., 2009). To verify the cationic liposomes/DNA complex formation, agarose gel electrophoresis was performed after the complexes were formed with 1 μg of pVAX/ADN. Free DNA that failed to form complex with cationic liposomes was clearly visible at complex ratios of 4-20 (μg:μg). However, free DNA disappeared when the ratio of DOTAP to DNA was above 24 (μg:μg) (Figure 3). Lipofectamine® formed complexes with DNA without a trace of free DNA at 6:1 (Lipofectamine® :DNA, w/w). The movement of plasmid DNA on a 1% agarose gel was retarded as the amount of liposomes increased. At the ratios over 24, cationic

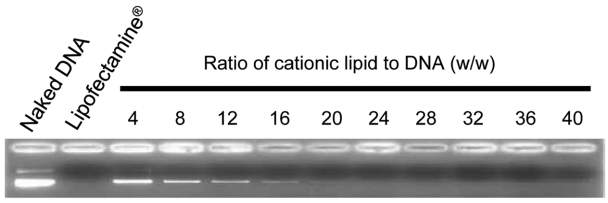

Figure 3. Agarose gel retardation of plasmid DNA by drug free cationic liposomes.

liposomes were able to bind DNA efficiently, and the complete retardation of DNA mobility was achieved at ratio 24. Complexes formed at under ratios 20 shows incomplete complexation (Figure 3).

Stability of the liposomes/DNA complex

We also examined whether DNA in the complex could be protected from DNase I digestion (Jeong et al., 2009), since complexes are not able to work *in vivo* owing to the interrupting effect of nuclease. Naked DNA was completely digested by 0.25 units of DNase I (Figure 4). However, the complexed DNA was protected from DNase I at 37°C independent of drug loading. It means that the drug loading did not alter the complexation between DNA and cationic liposomes.

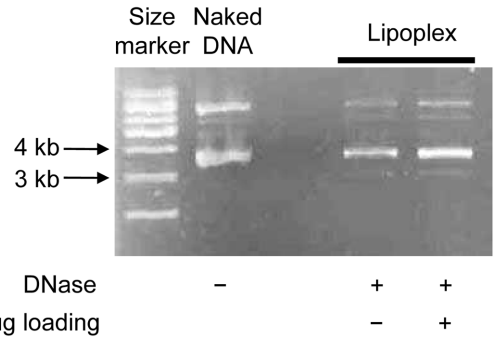

Figure 4. Stability of plasmid DNA complexed with drug free and drug-loaded cationic liposomes. The cationic liposomes were prepared at a mixing ratio of DOTAP:DOPE:Chol=1:1:1.

***In Vitro* transfection of liposomes/DNA complexes**

To examine effective ADN gene delivery into cells, various cell lines were transfected with plasmid DNA by cationic liposomes. The amount of cationic lipid was fixed as 16 μg and the dose of DNA was increased, and vice versa. As shown in Figure 5a, the mRNA expression levels of ADN were increased dose-dependently increased in most cell lines transfected with plasmid. Meanwhile, the mRNA expression of ADN at equal amount of plasmid DNA was increased with cationic lipid content from 0 to 16 μg (Figure 5b). Moreover, the mRNA expression patterns were different depending on cell types. Thus, a new approach to modify a plasmid DNA or vehicle is necessary to improve the expression of ADN in target organ, liver.

Conclusion

The encapsulation efficiency of rosiglitazone in cationic liposomes was high and did not affect the physicochemical characteristics of cationic liposomes except particle sizes. The complex formation of cationic liposomes and plasmid DNA was not influenced by drug loading. Moreover, drug loading did not affect the stability of complexed DNA against DNase I. From the results, it was confirmed that co-delivery of rosiglitazone, a therapeutic for T2DM, and ADN gene would be possible without significant physicochemical alteration of cationic liposomes and complexes. Further studies are needed to improve the transfection efficiency of rosiglitazone and ADN

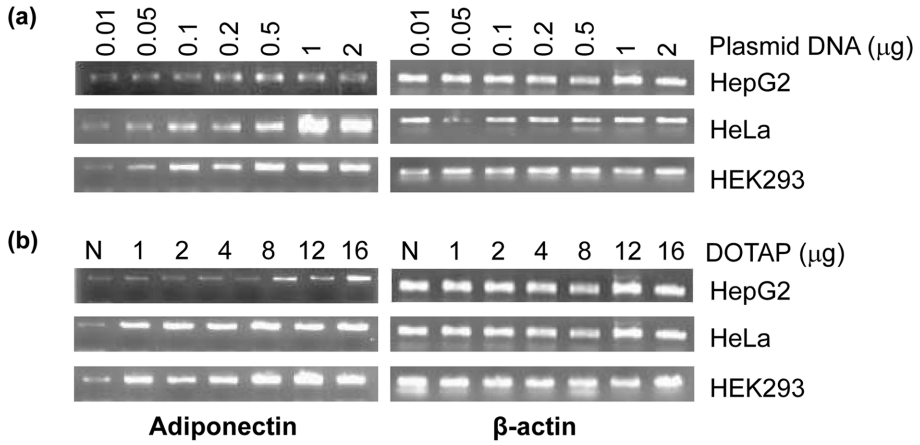

Figure 5. Representative mRNA expressions of ADN and β-actin in HepG2, HeLa and HEK293 cells after transfection of drug loaded complexes. (a) Dose-dependent mRNA expression of ADN transfected at the fixed amount of DOTAP as 16 μg, (b) Effect of DOTAP content on mRNA expression at the fixed amount of plasmid DNA as 1 μg.

gene by modifying vehicles or by subcloning promoter for both *in vitro* and *in vivo*.

Acknowledgements

This work was supported by Mid-career Researcher Program (No. 2007-0054811) and Priority Research Centers Program (No. 2009-0093815) through the National Research Foundation of Korea (NRF) funded by the Korea government (Ministry of Education, Science and Technology).

References

Gulati, M., Grover, M., Singh, S., Singh, M., 1998. Lipophilic drug derivatives in liposomes. Int. J. Pharm. 165, 129-168.

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T., Matsuzawa, Y., 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler. Thromb. Vasc. Biol. 20, 1595-1599.

Inoue, M., Maehata, E., Yano, M., Taniyama, M., Suzuki, S., 2005. Correlation between the adiponectin-leptin ratio and parameters of insulin resistance in patients with type 2 diabetes. Metabolism 54, 281-286.

Ishii, F., Nagasaka, Y., 2001. Simple and convenient method for estimation of marker entrapped in liposomes. J. Dispersion Sci. Technol. 22, 97-101.

Jeong, U.H., Jung, J.H., Davaa, E., Park, S.J., Myung, C.S., Park, J.S., 2009. Effect of drug loading on the physicochemical properties and stability of cationic lipid-based plasmid DNA complexes. J. Kor. Pharm. Sci. 39, 339-343.

Kang, J.W., Davaa, E., Kim, Y.T., Park, J.S., 2010. A new vaginal delivery system of amphotericin B: a dispersion of cationic liposomes in a thermosensitive gel. J. Drug Target. 18, 637-644.

Lebovitz, H.E., 2002. Differentiating members of the thiazolidinedione class: a focus on safety. Diabetes Metab. Res. Rev. 18, S23-29.

Nan, M.H., Park, J.S., Myung, C.S., 2010. Construction of adiponectin-encoding plasmid DNA and gene therapy of non-obese type 2 diabetes mellitus. J. Drug Target. 18, 67-77.

Qi, Y., Takahashi, N., Hileman, S.M., Patel, H.R., Berg, A.H., Pajvani, U.B., Scherer, P.E., Ahima, R.S., 2004. Adiponectin acts in the brain to decrease body weight. Nat. Med. 10, 524-529.

Wiradharma, N., Tong, Y.W., Yang, Y.Y., 2009. Self-assembled oligopeptide nanostructures for co-delivery of drug and gene with synergistic therapeutic effect. Biomaterials 30, 3100-3109.

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M.L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., Kadowaki, T., 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat. Med. 7, 941-946.