

Note

Modifying Cationic Liposomes with Cholesteryl-PEG Prevents Their Aggregation in Human Urine and Enhances Cellular Uptake by Bladder Cancer Cells

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Intravesical drug delivery by cationic liposomes (Cat-LPs) represents a potent nanotechnology for enhancing therapeutic effects against bladder disorders. However, preventing the aggregation of Cat-LPs in urine poses a significant barrier. We report on an examination of the effect of modifying liposomes with polyethylene glycol (PEG) lipids to prevent Cat-LPs from aggregating in human urine. Although Cat-LPs underwent significant aggregation in human urine, introducing 5 mol% of PEG2k lipid or 2 mol% of PEG5k lipid completely inhibited the aggregation of the Cat-LPs. When 2 mol% of PEG2k lipids were introduced, the lipid structures of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and 1,2-distearoyl-*sn*-glycerol (DSG) greatly prevented aggregation compared with cholesterol. By contrast, when Cat-LPs, after incubation in urine, were exposed to bladder cancer cells, only introducing cholesteryl-PEG into the Cat-LPs showed a significant enhancement in cellular uptake. These results offer the potential for incorporating cholesteryl-PEG into Cat-LPs for achieving both stability in urine and effective cellular uptake.

Key words cationic lipid; drug delivery system; liposome; pegylation; urine stability; nanoparticle

Major disorders affecting the urinary bladder are bladder cancer and interstitial cystitis, which occur at the urothelium. Thus, the intravesical injection of drugs can be effective compared with systemic injection. Although, in systemic injection, only a small fraction of the drug reaches the affected area, intravesically injected drugs directly attack the affected area. However, the unique features of the bladder, such as the presence of urine, a mucus layer and low urothelial permeability, are obstacles for effective intravesical treatment. Thus, nano carriers for intravesical drug delivery have been developed to overcome these obstacles.¹⁾

Of the numerous drug delivery platforms, liposomal drugs have been approved in several countries and liposomes are now recognized as a safe and effective nano carrier. In recent years, studies regarding the intravesical delivery of several drugs (chemo drugs, toxins, nucleic acids, genes) by liposomes have been reported.^{2–8)} We also reported that Bacille Calmette–Guerin cell wall skeleton (BCG-CWS) loaded liposomes have potential for use as an immunotherapeutic drug against bladder cancer.⁹⁾ However, the effect of the nature of liposomes on the features of bladder or intravesical delivery have not been extensively examined.¹⁰⁾

In this study, we focused on the effect of urine on liposome stability. The characteristics of liposomes can affect their stability in urine, because urine contains urea, a variety of ions (Cl, Na, Mg, H₃PO₄, etc.), creatinine, uric acid, ammonia and hormones. The aggregation of liposomes results in a decrease in the efficiency of delivery to the affected area. Cationic liposomes (Cat-LPs) have certain advantages in mucoadhesivity and cellular affinity, whereas their cationic properties are assumed to make them susceptible to the components in urine. Modification of the liposomal surface with polyethylene glycol (PEG) can be a potent strategy for decreasing the susceptibility of Cat-LPs. Thus, we investigated the effect of introducing PEG lipids on preventing the aggregation of Cat-LPs in human urine.

MATERIALS AND METHODS

Preparation of Liposomes Cat-LPs were prepared by the hydration method. Cat-LPs composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids, Alabaster, AL, U.S.A.), cholesterol (Chol) (Avanti Polar Lipids) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (POPE) (NOF Corporation, Tokyo, Japan) (mol ratio for DOTAP: Chol: POPE=30:30:40). *N*-(Carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG2k), DSPE-PEG5k, 1,2-distearoyl-*sn*-glycerol, methoxypolyethylene glycol 2000 (DMG-PEG2k), DMG-PEG5k, cholesteryl-PEG2k (Chol-PEG2k) and Chol-PEG5k (NOF Corporation) were used at 2 or 5 mol% of the total lipid. Additional details can be found in the supplementary materials.

Evaluation of Stability of Liposomes in Human Urine Human urine was obtained from a healthy volunteer. The research protocols involving human subjects were approved by the institutional review board of the Faculty of Pharmaceutical Sciences in Hokkaido University (2015-003-2). Fifty microliters of liposomes were added to 450 μ L of human urine or 450 μ L of 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4). The mixtures were incubated for 30 min at 37°C, and the absorbance at 660 nm was measured. Additional details can be found in the supplementary materials.

Evaluation of the Cellular Uptake of Liposomes by Bladder Cancer Cells Fifty microliters of DiD-labeled liposomes were added to 250 μ L of human urine, and the mixtures were incubated for 30 min at 37°C. After washing the MB49 cells with phosphate buffered saline (PBS), 100 μ L of the mixtures and 600 μ L of Dulbecco's modified Eagle's medium (DMEM) without fetal bovine serum (FBS) were added to the cells. After a 1 h incubation, the cells were analyzed by flow cytometry (Gallios). The relative geometric mean (GeoMean) of the fluorescent intensity (FI) was calculated by setting the value

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for the GeoMean of the non-treatment (NT) to 1. Additional details can be found in the Supplementary materials.

Statistical Analysis Statistical analysis of multiple comparisons were performed by one-way ANOVA, followed by the Tukey–Kramer test. A *p* value of <0.05 was considered to be a significant difference.

RESULTS AND DISCUSSION

Stability of PEGylated Cat-LPs in Human Urine The physical characteristics of the Cat-LP are shown in the accompanying Table 1. After incubating the Cat-LP in human urine for 30 min at 37°C, the turbidity of solution was measured. As expected, an increase in the turbidity of the urine was observed, indicating that the Cat-LP had undergone aggregation in the urine (Fig. 1). To prevent this aggregation, we introduced PEGylated lipids into Cat-LP and investigated the effect of the lipid structure, the length of the PEG chain and the PEG density on aggregation. The physical characteristics of the PEGylated Cat-LPs are shown in the accompanying Table 1. Introducing PEGylated lipids clearly drastically decreased the turbidity in urine (Fig. 1). That is, the aggregation of Cat-LP was inhibited by modifying the liposomal surface with PEG. This suggests that the PEG layer attenuated the interaction between the Cat-LP and urine components. Interestingly,

the efficiency of inhibiting aggregation was dependent on the lipid structure of the PEGylated lipid. When Chol-PEG2k was introduced at 2 mol%, the turbidity was significantly higher than the values for the other PEGylated Cat-LPs (Fig. 1). The turbidity in the case of 2 mol% DSG-PEG2k also appeared to be high, but not significant, compared with other PEGylated Cat-LPs (Fig. 1). Complete inhibition was observed in the cases of 5 mol% of PEG2k and 2 mol% of PEG5k, regardless of lipid structures (Fig. 1). Under these experimental conditions, the pH of urine, collection time and foods had no effect on liposomal stability in human urine. This suggests that the order of stabilizing effect was DSPE-PEG>DSG-PEG>Chol-PEG. The clear difference between 2 mol% DSPE-PEG2k, 2 mol% DSG-PEG2k and 2 mol% Chol-PEG2k appears to be due to the efficiency of masking the cationic charge and the conformational flexibility of the PEG chains. DSPE-PEG can compensate for the positive charge of Cat-LP in addition to the shielding effect by the PEG layer, compared with DSG-PEG, because DSPE-PEG has a negative charge derived from the phosphorylethanolamine group. Meanwhile, the conformational flexibility of PEG chains in Chol-PEG may be minor compared with DSPE-PEG and DSG-PEG, because the Chol anchor is likely located deeper in the liposomal membrane, resulting in the PEG layer having a minor shielding effect.¹¹⁾

Cellular Uptake of PEGylated Cat-LPs after the Exposure of Human Urine To kill bladder cancer cells, drug-loaded liposomes must be taken up by bladder cancer cells in the bladder. However, the PEGylation of liposomes decrease causes a decreased cellular affinity, resulting in the inhibition of cellular uptake.¹²⁾ Thus, we next investigated the cellular uptake of PEGylated Cat-LPs by MB49 cells, mouse bladder cancer cells. After incubating fluorescence (DiD)-labeled liposomes in human urine, the liposome suspension was added to the MB49 cells. After a 1 h incubation, the cells were analyzed by flow cytometry. For all liposomes, a peak shift was observed, indicating that all types of liposomes were taken up by MB49 cells (Fig. 2a). Meanwhile, the peaks for cells treated with PEGylated Cat-LPs were sharp compared with that of Cat-LPs, because the Cat-LPs aggregated in human urine. The homogeneity of cellular uptake was so high that the coefficient of variation (CV) value was small, because the CV value indicates the degree of variation. That is, the homogeneity was high, thus making the CV value similar to the CV value for the NT group. Figure 2b clearly shows that the PEG modification increased the homogeneity of cellular uptake. Figure 2c shows the average data for the relative FI. Interestingly, the

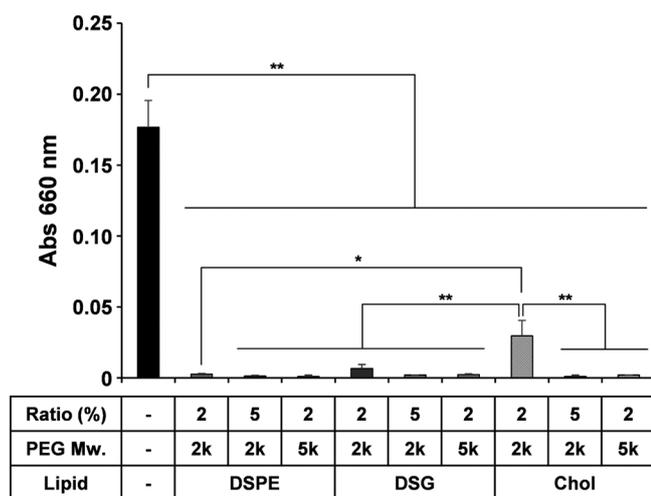


Fig. 1. Stability of PEGylated Cat-LPs in Human Urine

Cat-LPs (black bar) or PEGylated Cat-LPs were incubated in human urine for 30 min at 37°C, and the turbidity of the solution was then measured. Data are the mean±S.D. (*n*=3, ***p*<0.01, **p*<0.05).

Table 1. Physical Characteristics of the Liposomes Used in This Study

Lipid composition	Diameter (nm)	PDI	ζ-Potential (mV)
DOTAP/Chol/POPC (30/30/40)	99±10	0.22±0.01	48±1
DOTAP/Chol/POPC/DSPE-PEG2k (30/30/40/2)	92±7	0.21±0.01	41±5
DOTAP/Chol/POPC/DSPE-PEG2k (30/30/40/5)	95±2	0.21±0.01	41±9
DOTAP/Chol/POPC/DSPE-PEG5k (30/30/40/2)	94±6	0.21±0.06	35±7
DOTAP/Chol/POPC/DSG-PEG2k (30/30/40/2)	100±11	0.33±0.08	50±4
DOTAP/Chol/POPC/DSG-PEG2k (30/30/40/5)	112±36	0.29±0.09	39±4
DOTAP/Chol/POPC/DSG-PEG5k (30/30/40/2)	114±32	0.28±0.09	38±9
DOTAP/Chol/POPC/Chol-PEG2k (30/30/40/2)	104±10	0.25±0.01	56±2
DOTAP/Chol/POPC/Chol-PEG2k (30/30/40/5)	98±4	0.26±0.01	42±4
DOTAP/Chol/POPC/Chol-PEG5k (30/30/40/2)	132±40	0.37±0.06	44±7

PDI: polydispersity index. Data are the mean±S.D. (*n*=3).

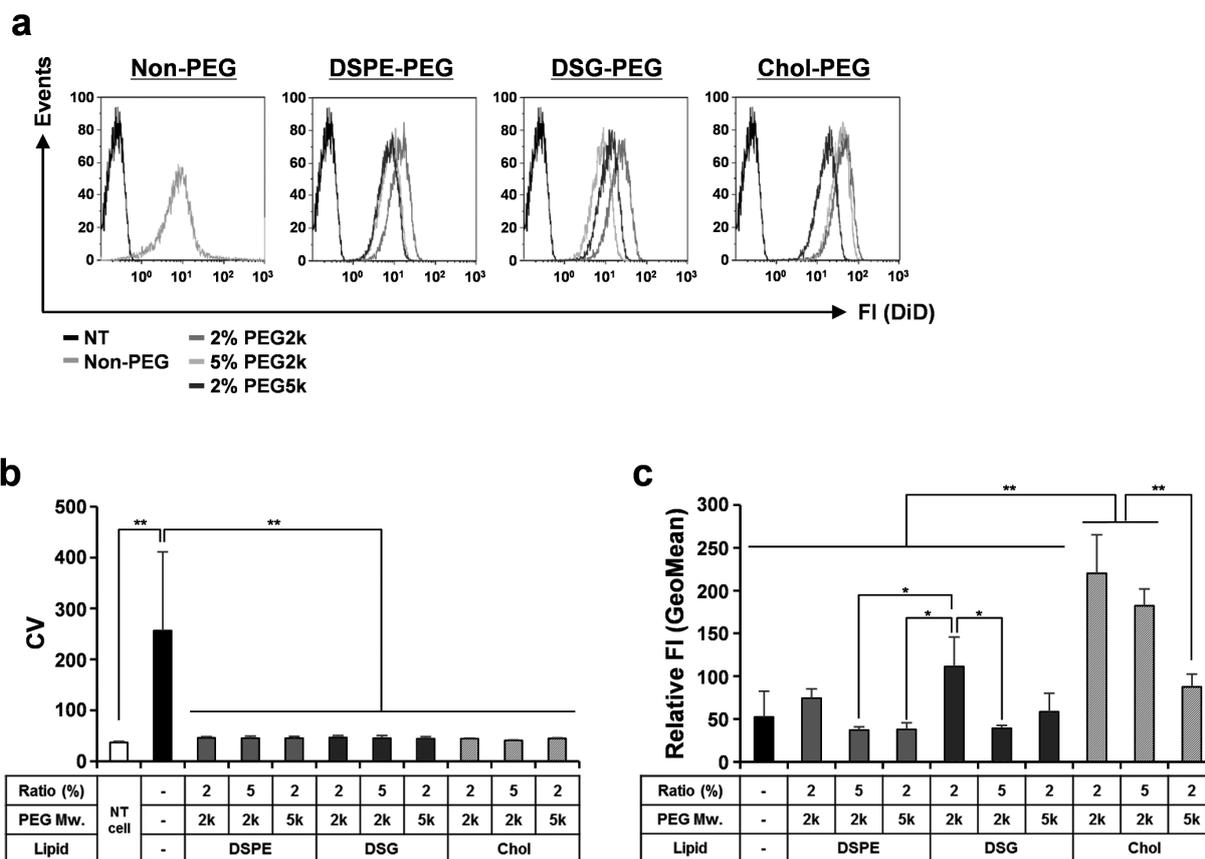


Fig. 2. Cellular Uptake of PEGylated Cat-LPs by MB49 Cells

After the DiD-labeled Cat-LPs (black bar) or PEGylated Cat-LPs were incubated with human urine, they were added to the MB49 cells. After a 1 h incubation, the cells were analyzed by flow cytometry. (a) Typical histograms. (b) The average data of CV. Data are the mean+S.D. ($n=3$, $**p<0.01$, $*p<0.05$). (c) The average data of relative FI (GeoMean) which was calculated by setting the value of GeoMean of NT to 1. Data are the mean+S.D. ($n=3$, $**p<0.01$, $*p<0.05$).

cellular uptakes of both the 2 or 5 mol% Chol-PEG2k modified Cat-LPs were significantly higher than those for the Cat-LP and other PEGylated Cat-LPs (Fig. 2b). This, therefore, indicates that modification of the Cat-LPs with Chol-PEG2k enhances the cellular uptake by bladder cancer cells after exposure to urine. These results were contrary to the stability of the particles in urine. As shown in Fig. 1, the 2 mol% Chol-PEG2k modified Cat-LPs aggregated in urine, while the 2 mol% Chol-PEG2k modified Cat-LPs were efficiently taken up by cells. It is likely that most of the Cat-LPs aggregated in urine, whereas only a portion of the PEGylated Cat-LPs aggregated in the case of 2 mol% Chol-PEG2k modified Cat-LPs. Thus, the non-aggregated PEGylated Cat-LPs can be taken up by cells. Moreover, the minor conformational flexibility of the PEG chains in Chol-PEG appeared to favor the extent of cellular uptake. The low shielding effect of Chol-PEG can be attributed to the increase in electrostatic interactions between cationic lipids and the cancer cells.

CONCLUSION

The incorporation of DSPE-PEG and DSG-PEG in to Cat-LPs effectively prevents them from undergoing agglomeration in human urine, while cellular uptake was abrogated. In contrast, when Chol-PEG2k was incorporated the Cat-LPs were stable in human urine and the cellular uptake of Cat-LPs was enhanced. Therefore, Chol-PEG represents a potent PEGylated lipid that can be used to control the stability of particles in

urine and permit them to be taken up by bladder cancer cells.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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