

## Research Paper

# Permeation Enhancer-Containing Water-In-Oil Nanoemulsions as Carriers for Intravesical Cisplatin Delivery

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**Purpose.** In the present work, we developed water-in-oil (w/o) nanoemulsions for the intravesical administration of cisplatin.

**Methods.** The nanoemulsions were made up of soybean oil as the oil phase and Span 80, Tween 80, or Brij 98 as the emulsifier system.  $\alpha$ -Terpineol and oleic acid were incorporated as permeation enhancers. The physicochemical characteristics of droplet size, zeta potential, and viscosity were determined. Nanoemulsions were administered intravesically for 1~4 h to rats *in vivo*. Animals were subsequently sacrificed, and the bladders were harvested to examine drug accumulation and histology.

**Results.** Ranges of the mean size and zeta potential were 30~90 nm and -3.4 to -9.3 mV, respectively. The addition of enhancers further reduced the size of the nanoemulsions. The viscosity of all systems exhibited Newtonian behavior. The cisplatin-loaded nanoemulsions were active against bladder cancer cells. The nanoemulsions with Brij 98 exhibited the complete inhibition of cell proliferation. The encapsulation of cisplatin and carboplatin, another derivative of cisplatin, in nanoemulsions resulted in slower and more-sustained release. The amount of drug which permeated into bladder tissues significantly increased when using carriers containing Brij 98, with the  $\alpha$ -terpineol-containing formulation showing the best result. The nanoemulsion with  $\alpha$ -terpineol prolonged the duration of higher drug accumulation to 3~4 h. At the later stage of administration (3~4 h), this system increased the bladder wall deposition of cisplatin and carboplatin by 2.4~3.3-fold compared to the control solution. Histological examination of the urothelium showed near-normal morphology in rats instilled with these nanoemulsions.  $\alpha$ -Terpineol possibly caused slight desquamation of umbrella cells.

**Conclusions.** The nanoemulsions are feasible to load cisplatin for intravesical drug delivery.

**KEY WORDS:** bladder; cisplatin; intravesical delivery; nanoemulsion; sustained release.

## INTRODUCTION

An estimated 261,000 new cases of bladder cancer are diagnosed worldwide each year (1). Bladder cancer is the fourth most common malignancy among men, but the high recurrence rates likely make it the most prevalent malignancy among all cancers, and certainly the most expensive per patient treated (2,3). More than 70% of bladder cancers present as non-muscle-invasive bladder cancer (NMIBC) (4). The current treatment consists of transurethral resection (TUR) of visible tumors, followed by intravesical chemotherapy to reduce disease recurrence and progression (5). The rationale for intravesical therapy is to maximize the exposure of tumors located in the bladder cavity to ther-

apeutic agents while limiting the systemic exposure and thereby limiting toxicity to the host. However, intravesical delivery has to overcome its own set of challenges, and most prominent among them is the low residence time of a drug in the bladder, which necessitates frequent instillation (6). Nanosystems with a well-defined particle size and shape have immense potential for intravesical delivery, as they can enhance the ability of drugs to cross the urothelium. A higher surface-to-volume ratio may also be responsible for increased absorption of encapsulated drugs (7).

Cisplatin is widely used for treating bladder cancer (8). Given the toxicity and mortality associated with cisplatin-based chemotherapy, intravesical administration would significantly reduce the systemic side effects, thus improving its therapeutic index (5-10). Cisplatin is a hydrophilic molecule with an n-octanol/water partitioning coefficient ( $\log P_{\text{octanol/water}}$ ) of -2.19. The efficacy of intravesical therapy for bladder cancer is in part limited by the poor permeation of hydrophilic drugs into the urothelium. Developing a formulation for intravesical cisplatin delivery not only for higher concentrations in the bladder wall but also for longer retention with reduced side effects would be very beneficial. Water-in-oil (w/o) nanoemulsions, a class of nanosystems with droplet sizes of 20~200 nm, are considered to be enhanced, prolonged-release systems for hydrophilic

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drugs with the oil and interfacial layers acting as release barriers (11,12). Nanosystems may be suitable for cisplatin to achieve these aims.

The purpose of this work was to develop w/o nanoemulsions as intravesical delivery systems to efficiently target cisplatin to the bladder. Permeation enhancers, such as  $\alpha$ -terpineol and oleic acid, were also loaded in the nanoemulsions, since the chemical enhancers can increase the permeability of drugs through the urothelium (13). Thus, the anti-cancer activity of cisplatin can be enhanced via the encapsulation into the nanoemulsions. The droplet size, zeta potential, and viscosity were determined as the physicochemical characteristics of the nanoemulsions. The cytotoxicity of the cisplatin-loaded carriers against urothelial carcinoma cells was also determined. The drug release was evaluated using an *in vitro* Franz diffusion assembly. Drug permeation into the bladder wall was examined using the rat as an animal model. Finally, possible mechanisms of the efficiency of intravesical cisplatin delivery by nanoemulsions were explored.

## MATERIALS AND METHODS

### Materials

Cisplatin, carboplatin, fluorescein isothiocyanate (FITC), soybean oil, Span 80,  $\alpha$ -terpineol, and oleic acid were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 80 was obtained from Kanto Chemical (Tokyo, Japan). Brij 98 was provided by Acros Organics (Geel, Belgium).

### Preparation of Nanoemulsions

The oil and aqueous phases were separately prepared. The oil phase consisted of soybean oil and emulsifiers, while the aqueous phase consisted of water and the drug (to achieve a concentration of 1.3 mM in the final product). The two phases were heated separately to 55°C. The oil phase was further mixed using a high-shear homogenizer (Pro250, Pro Scientific, Monroe, CT, USA) for 10 min. Then the oil phase was added to the aqueous phase and sonicated using a probe-type sonicator (VCX600, Sonics and Materials, Newtown, CT, USA) for 15 min at 35 W. Compositions of the nanoemulsions are listed in Table I.

### Determination of the Size and Zeta Potential

The mean vesicle size (z-average) and zeta potential of the nanoemulsions were measured by photon correlation spectroscopy (Malvern Nano ZS<sup>®</sup> 90, Worcestershire, UK) using a helium-neon laser with a wavelength of 633 nm. The formulations were diluted 5-fold with soybean oil before

the measurement. Photon correlations of spectroscopic measurements were carried out at a scattering angle of 90°.

### Determination of Viscosity

The viscosity of the nanoemulsions was measured as the shear stress (Pa) as a function of the shear rate (1/s) using a Carri-Med CSL<sup>2</sup> 100 rheometer (TA Instruments, New Castle, DE, USA). The diameters of both the cone and plate spindle were 60 mm. The determination mode was set to flow-step measurements with shear rates of 0~1000/s. The cone angle used for the measurements was 2°.

### Cytotoxicity Assay

The T24 human urothelial carcinoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were seeded at an initial concentration of  $2 \times 10^4$  cells/well in a 24-well plate and incubated in 1 ml of medium (10% fetal bovine serum, 89% RPMI 1640, and 1% penicillin-streptomycin). Water and the nanoemulsions with or without cisplatin diluted with medium were added at 24 h post-inoculation, and plates were incubated in a 5% CO<sub>2</sub> atmosphere at 37°C. Subsequently, the medium was incubated for 72 h. After washing with phosphate-buffered saline (PBS), cells were incubated with 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) in RPMI 1640 for 2 h at 37°C. Formazan crystals resulting from the reduction of MTT were dissolved by adding 200  $\mu$ l DMSO and gently agitated for 30 min. The absorbance of the supernatant was then measured spectrophotometrically in an enzyme-linked immunosorbent assay (ELISA) reader at 550 nm. Cell viability was calculated as a percentage of the control.

### Partition Coefficient ( $\log P_{\text{oil/water}}$ ) of the Drug between the Oil and Water Phases

The partition coefficient was determined by equilibrating drug partitioning between the oil and water phases. Cisplatin or carboplatin at 0.5 mg was dispersed in a mixture of soybean oil (1 ml) and water (1 ml) and shaken for 30 min at 37°C. The aqueous phase was subsequently filtered through a polyvinylidene difluoride (PVDF) membrane with a pore size of 0.45  $\mu$ m. The drug concentration in the aqueous phase was determined by atomic absorption spectrophotometry (AAS; Z-5000, Hitachi, Tokyo, Japan).

### In Vitro Drug Release from the Nanoemulsions

Cisplatin or carboplatin release from the w/o nanoemulsions was measured using a Franz diffusion cell. A

**Table I.** The compositions and their percentages (% w/w) of water-in-oil nanoemulsions used in this study

Code	Water	Soybean oil	Span 80	Tween 80	Brij 98	$\alpha$ -Terpineol	Oleic acid
N1	8	50	30	12	—	—	—
N2	8	50	30	—	12	—	—
N3	8	45	30	—	12	5	—
N4	8	45	30	—	12	—	5

cellulose membrane (CelluSep® T3, with a molecular weight cutoff of 3500) was mounted between the donor and receptor compartments. The donor medium consisted of 0.5 ml of vehicle containing drugs. The receptor medium (5.5 ml) consisted of pH 7.4 citrate-phosphate buffer. The available diffusion area between the cells was 0.785 cm<sup>2</sup>. The stirring rate and temperature were set to 600 rpm and 37°C, respectively. At appropriate intervals, 300- $\mu$ l aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drug released was determined by AAS. In the study examining the influence of urine on drug release, 0.5 ml of synthetic urine was added to the donor compartment. The synthetic urine solution contained 14.1 g NaCl, 2.8 g KCl, 17.3 g urea, 1.9 ml of a 25% (v/v) ammonia solution, 0.6 g CaCl<sub>2</sub>, and 0.43 g of MgSO<sub>4</sub> made up to 1 L with water (14).

### **In Vivo Intravesical Delivery**

Female Sprague-Dawley rats weighing 225~250 g were used in this study. The animal protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung University (Taoyuan, Taiwan). Animals were subcutaneously anesthetized with urethane (1.2 g/kg) before intravesical instillation. Residual urine was evacuated by pressing the lower abdomen. A polyurethane catheter (BD Angiocath Plus®, Becton Dickinson Korea, Gyeongbuk, Korea) was inserted into the bladder. The bladder was washed with 0.5 ml of normal saline. Subsequently, 0.2 ml of nanoemulsions containing cisplatin or carboplatin was instilled into the bladder. The catheter was removed, and the urethra was ligated with cotton thread. The dwell times for instillation were 1, 2, 3, and 4 h. After the application time, the bladder was excised and washed with saline.

The drug in the bladder tissue was extracted by a homogenization method. Two milliliters of 4.31% sulfosalicylic acid was added to the excised tissue in a glass homogenizer and ground for 5 min. The resulting solution was centrifuged for 15 min at 12,000 rpm. The drug content in the supernatant was measured by AAS.

### **Confocal Laser Scanning Microscopy (CLSM)**

Localization of FITC within the bladder wall was determined by CLSM after *in vivo* intravesical delivery. FITC in the vehicles at a concentration of 10  $\mu$ M was used. After *in vivo* instillation for 3 h, the bladder samples were excised to examine the fluorescent images. The thickness of the bladder wall was optically scanned at ~10- $\mu$ m increments through the Z-axis of a Leica TCS SP2 confocal microscope (Wetzlar, Germany). Optical excitation was carried out with a 488-nm argon laser, and the fluorescence emission was detected at 496~559 nm.

### **Histological Examination of the Bladder**

Immediately after treatment with an aqueous solution or the nanoemulsions by intravesical instillation for 3 h, a specimen of the exposed area was taken for histological examination. Each specimen was fixed in a 10% buffered formaldehyde solution (pH 7.4) for at least 48 h and stained

with hematoxylin and eosin. For each sample, three different sites were examined and evaluated under light microscopy (Eclipse 4000, Nikon, Tokyo, Japan).

### **Statistical Analysis**

Statistical analysis of differences between different treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. An analysis of variance (ANOVA) was also used if necessary.

## **RESULTS**

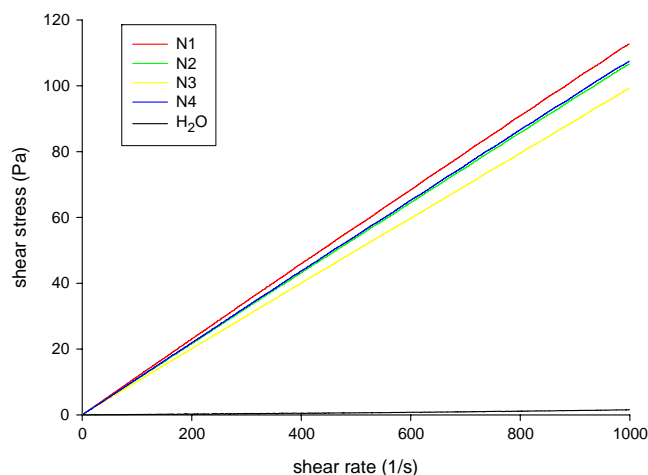
### **Physicochemical Characterization of the Nanoemulsions**

The drug delivery potential of the nanoemulsions may depend upon the droplet characteristics, including their size, charge, hydrophilicity, and viscosity. The present formulation (N1) was stabilized by a combination of two emulsifiers: a sugar ester of sorbitan monooleate (Span 80) and a polyoxyethylene 20 sorbitan monooleate (Tween 80). The ingredients and their percentages of this formulation were designed and modified from the previous study by Wu *et al.* (11) for skin delivery purpose. A pseudo-ternary phase diagram with a stable percentage range of oil, water, and surfactant mixtures is determined by the nanoemulsions developed in this work (11). The substitution of Tween 80 (with a hydrophilic-lipophile balance (HLB) of 15) with another polyoxyethylene surfactant, Brij 98 (with an HLB of 15.3), also formed a stable emulsion system (N2). The size analysis indicated that the nanoemulsions with Span 80 and Tween 80 (N1) had mean droplet sizes of 90.0 nm, while that of the N2 nanoemulsion was 55.6 nm (Table I). The absolute zeta potential of the Tween 80-containing formulation (N1) was -9.3 mV. Replacement of the hydrophilic non-ionic emulsifier of Tween 80 with Brij 98 (N2) led to an initial decrease in the zeta potential ( $p < 0.05$ ). The Brij 98-containing nanoemulsion (N2) was chosen for further formulation design to which the enhancers were added. The addition of  $\alpha$ -terpineol and oleic acid produced nanoemulsions (N3 and N4) with reduced sizes of 27.7 and 45.4 nm, respectively ( $p < 0.05$ ). On the other hand, the zeta potential did not change after  $\alpha$ -terpineol incorporation (N2 vs. N3,  $p > 0.05$ ). The addition of oleic acid (N4) contributed to a slight but significant increase ( $p < 0.05$ ) in the negative zeta potential. A preliminary stability test had demonstrated an insignificant change ( $p \geq 0.05$ ) of mean size and zeta potential within 30 days of storage at 37°C and 65% relative humidity (data not shown).

The apparent viscosity of the nanoemulsions was calculated from values of the shear stress and shear rate on the upward curve shown in Fig. 1. The appearance of the curves confirms the Newtonian behavior of the investigated formulations. Tween 80-containing nanoemulsion (N1) showed the highest viscosity, followed by N4, N2, and N3.

### **Cytotoxicity Assay**

The present work evaluated the efficacy of the nanoemulsions of delivering cisplatin to urothelium cancer cells by examining their cytotoxicity. As shown in Fig. 2, the viability of the urothelium cancer cell line treated with free cisplatin

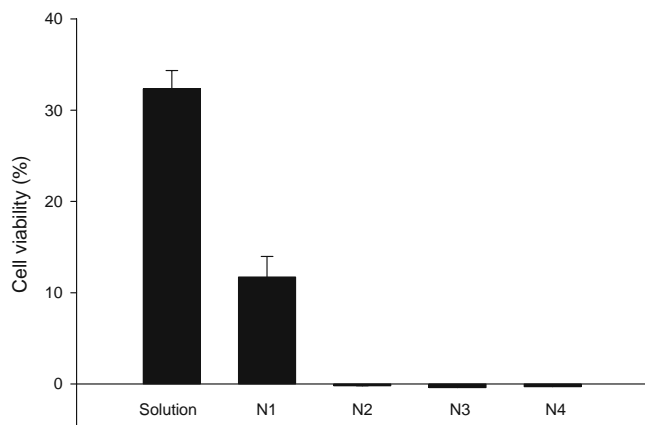


**Fig. 1.** The viscosity of nanoemulsions measured by shear stress (Pa) with increasing shear rates of 0~1000/s.

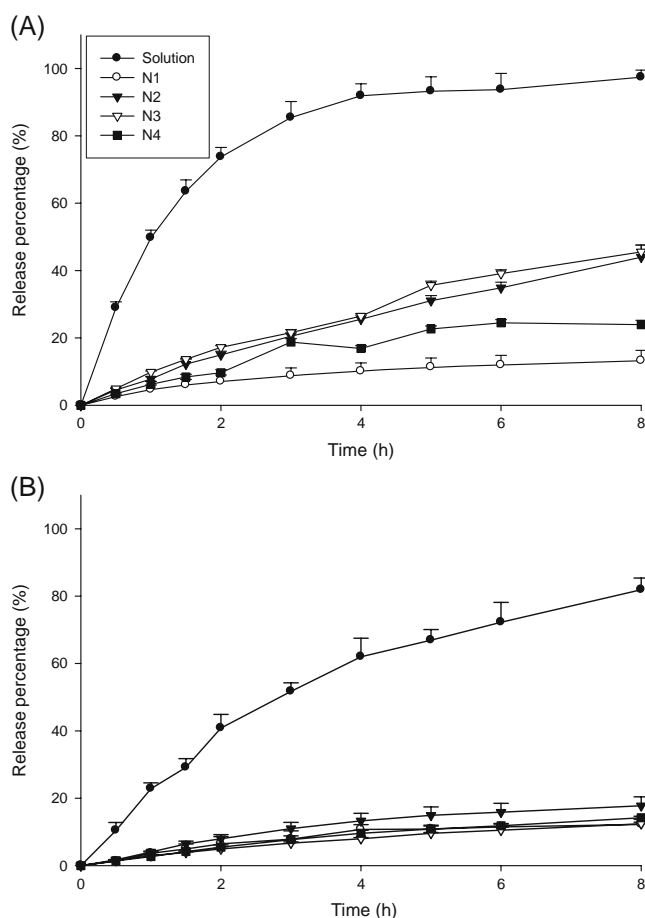
(2  $\mu$ M) was ~32%. Cisplatin loaded in the nanoemulsions showed higher cytotoxic activity ( $p < 0.05$ ) than the free drug in a 72-h incubation experiment. Nanoemulsions with Brij 98 (N2~N4) completely inhibited the growth of urothelial carcinoma cells. The incorporation of enhancers into the nanoemulsions did not influence cisplatin's activity against cancer cells. Empty nanoemulsions without cisplatin were also tested, and it was found that the systems themselves had no effect on the cytotoxicity. This indicates that the cytotoxicity toward the urothelium carcinoma cells was mainly a consequence of the cisplatin molecules.

### In Vitro Cisplatin Release from the Nanoemulsions

In order to develop a prolonged release system with general applicability, it is of great importance to understand the release mechanisms and kinetics. The *in vitro* release behaviors of cisplatin from the aqueous solution and nanoemulsions are shown in Fig. 3A. Release of cisplatin from the solution (free form) was rapid, with 97% released after 8 h at 37°C. The release kinetics of cisplatin from the nanoemulsions exhibited slower, more-continuous release for 8 h. The



**Fig. 2.** Viability (%) of human T24 urothelial carcinoma cells following treatment with free cisplatin in water and cisplatin-loaded nanoemulsions. Each value represents the mean  $\pm$  SD of five experiments.



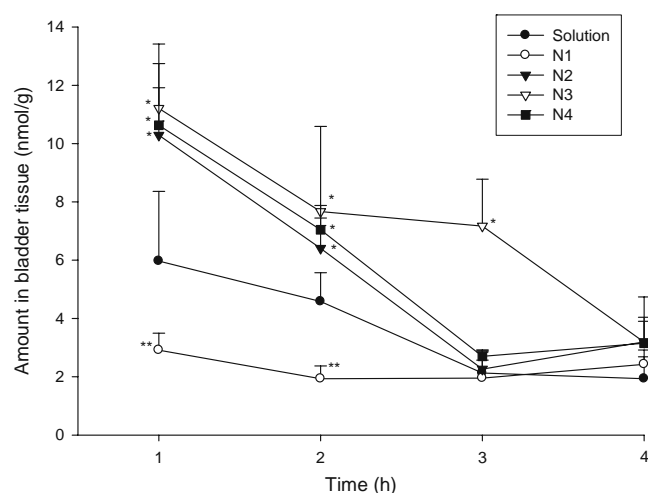
**Fig. 3.** *In vitro* release percentage (%) of cisplatin across a cellulose membrane from double-distilled water (control) and nanoemulsions (A) and in the presence of synthetic urine (0.5 ml) in the donor compartment (B). All data are presented as the mean  $\pm$  SD of four experiments.

release rate was found to change depending on the ingredients added to the nanoemulsions. Only 13% of the drug dose had been released from N1 after application for 8 h. Substitution of Tween 80 (N1) by Brij 98 (N2) further increased the cisplatin release percentage by 3.3-fold ( $p < 0.05$ ).  $\alpha$ -Terpineol incorporation (N3) did not alter cisplatin release. The addition of oleic acid (N4), however, produced a significant decrease ( $p < 0.05$ ) in drug release.

Synthetic urine was added to the donor phase and mixed with nanoemulsions in order to simulate *in vivo* conditions when urine is infused in the bladder. Cisplatin release in the presence of urine is depicted in Fig. 3B. Free cisplatin from the solution still showed greater release during 8 h. The release profile of cisplatin was not influenced by different ingredients in the systems ( $p > 0.05$ ) in the presence of urine.

### In Vivo Intravesical Cisplatin Delivery

Rats received an intravesical dose of cisplatin to examine the amounts in the bladder wall during 4 h of administration. Fig. 4 shows the time profile of cisplatin concentration in the tissue. A steeper decrease of the cisplatin concentration was observed in tissue segments following an increase in the application time. The results indicate that higher cisplatin



**Fig. 4.** *In vivo* cisplatin accumulation (nmol/g) in bladder tissues after intravesical instillation of water (free control) and nanoemulsions with cisplatin for 1~4 h. All data are presented as the mean  $\pm$  SD of six experiments. \* Significantly higher ( $p < 0.05$ ) compared to the control at the same time point; \*\* significantly lower ( $p < 0.05$ ) compared to the control at the same time point.

concentrations were obtained in the bladder wall when administering nanoemulsions with Brij 98 (N2 to N4) compared to the free control solution. On the other hand, systems with Tween 80 (N1) showed the lowest drug accumulation ( $p < 0.05$ ) in tissues among all formulations examined.  $\alpha$ -Terpineol incorporation increased the drug penetration ( $p < 0.05$ ) at 3 h, whereas oleic acid had no measurable effect ( $p > 0.05$ ) at this time point. All formulations exhibited comparable cisplatin accumulations at the last time point (4 h).

### Carboplatin as a Model Drug for Nanoemulsions

Specific groups of patients, such as the elderly and patients with renal dysfunction or a poor performance status or co-morbidities who cannot tolerate cisplatin therapy, should receive carboplatin-based treatment (8). Carboplatin is selected mainly because of its lower non-hematological toxicity compared to cisplatin. The delivery of carboplatin from the nanoemulsions was also tested. As shown in Fig. 5A, release of carboplatin from the nanoemulsions was sustained with trends similar to those of cisplatin. The addition of urine to the carriers produced an approximately equivalent release of carboplatin for all nanoemulsions (Fig. 5B). In the *in vivo* drug permeation study, the free carboplatin solution showed higher accumulation ( $p < 0.05$ ) in the tissue compared to free cisplatin as observed in Fig. 6. N3 was selected as the nanoemulsion to examine carboplatin absorption because of its good performance of cisplatin deposition. The tissue level of carboplatin after nanoemulsion application was significantly higher ( $p < 0.05$ ) than that with free carboplatin application at 2~4 h. The system yielded a bladder tissue concentration of 12~16 nmol/g during 4 h of administration. Steady-state carboplatin accumulation was thus achieved.

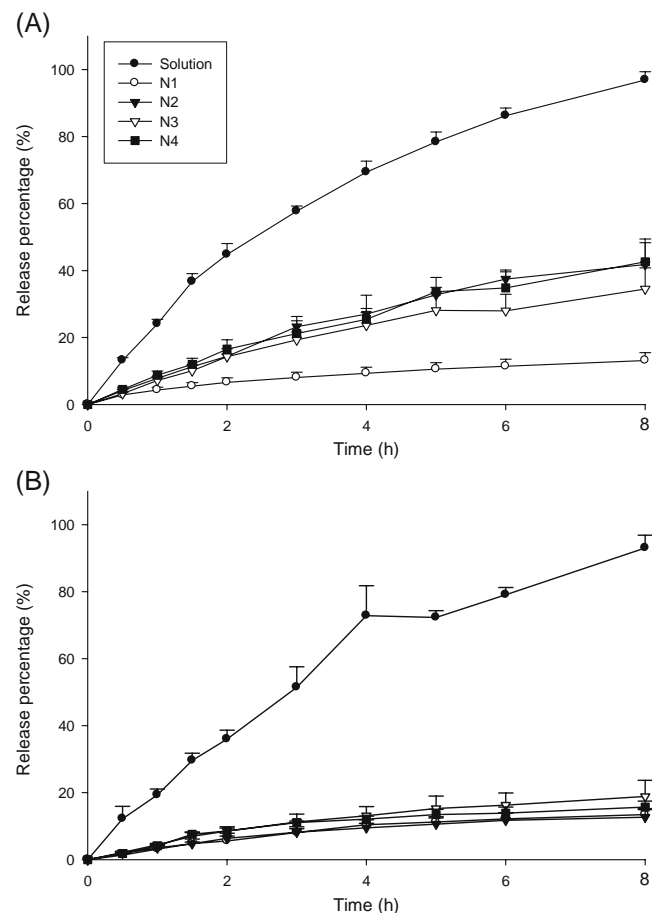
### Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to observe the superficial FITC distribution in the bladder wall. Because of the high hydro-

philicity of FITC, it likely resided in the inner phase. The superficial layer of nine fragments was optically scanned at  $\sim 10$ - $\mu\text{m}$  increments from the surface of the mucosa as shown in Fig. 7. The total  $\sim 70$   $\mu\text{m}$  comprises the urothelium and a part of the lamina propria. Fig. 7A illustrates the results obtained from CLSM following intravesical delivery of free FITC in an aqueous solution for 3 h. There was no or negligible green fluorescence in the images of the free control. It can be seen that the intensity of the green fluorescence was much greater with the N2 formulation (Fig. 7B). The fluorescence in the horizontal section at 27~46  $\mu\text{m}$  was strong for the N2 system, then the intensity gradually decreased with increasing depth.  $\alpha$ -Terpineol in the nanoemulsion (N3) further enhanced the signals to a more intense level (Fig. 7C). FITC loaded in the N3 formulation permeated the deeper layers. The signals were even stronger in deeper regions of the tissue.

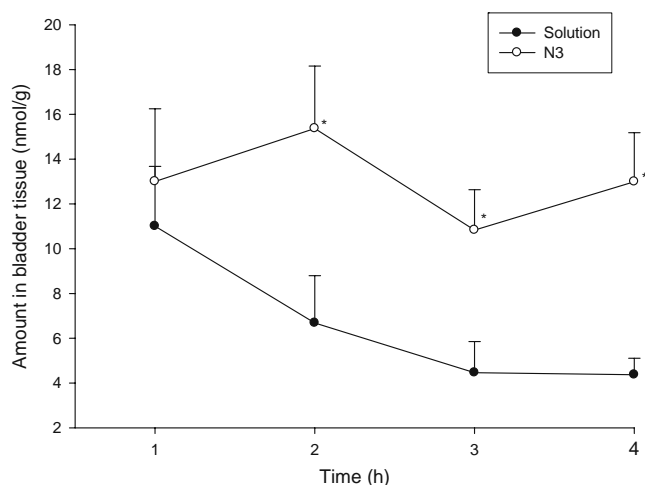
### Histological Examination of the Bladder

Light microscopy was applied to evaluate the influence of the nanoemulsions on the morphological properties of the bladder wall. As shown in Fig. 8A, the bladder tissue remained intact after exposure to water for 3 h (control).



**Fig. 5.** *In vitro* release percentage (%) of carboplatin across a cellulose membrane from double-distilled water (control) and nanoemulsions (A) and in the presence of synthetic urine (0.5 ml) in the donor compartment (B). All data are presented as the mean  $\pm$  SD of four experiments.





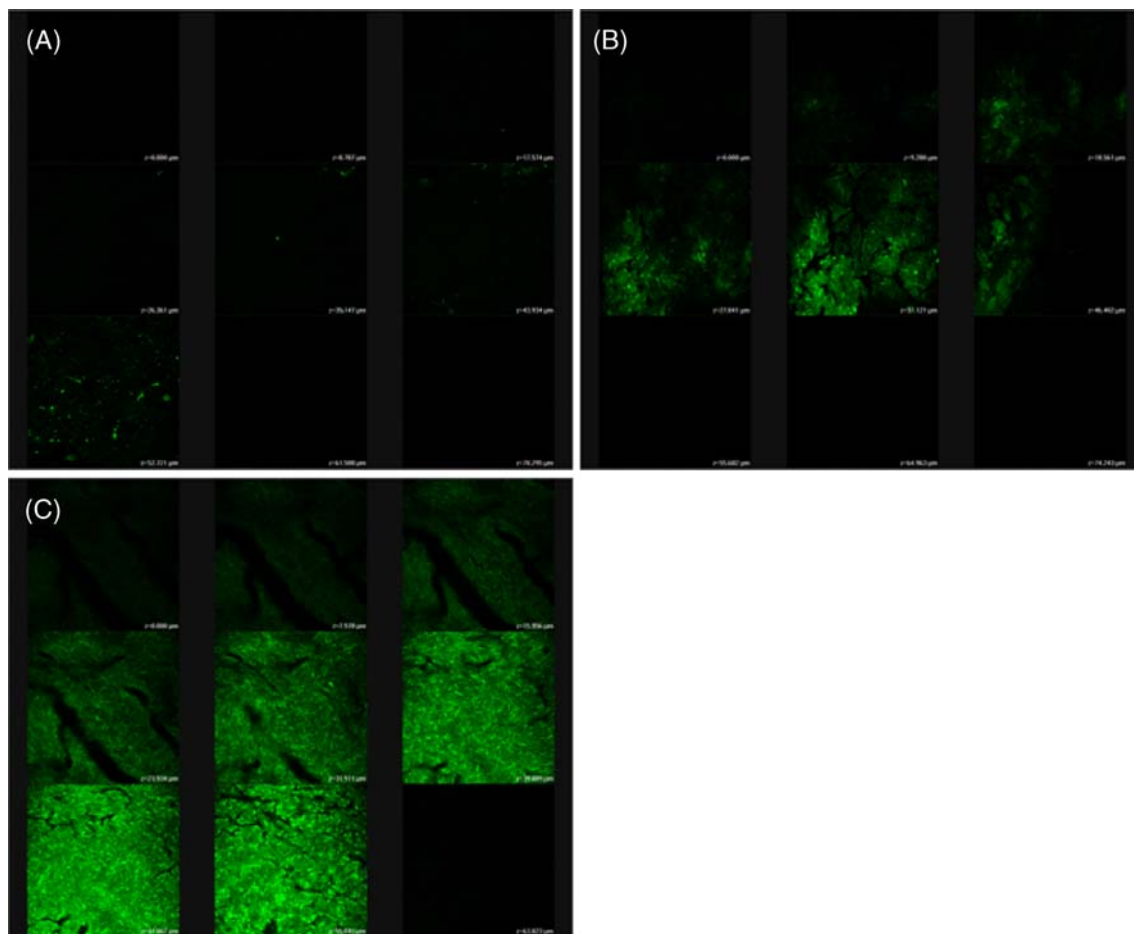
**Fig. 6.** *In vivo* carboplatin accumulation (nmol/g) in bladder tissues after intravesical instillation of water (free control) and  $\alpha$ -terpineol-containing nanoemulsion (N3) with carboplatin for 1~4 h. All data are presented as the mean  $\pm$  SD of six experiments. \* Significantly higher ( $p < 0.05$ ) compared to the control at the same time point; \*\* significantly lower ( $p < 0.05$ ) compared to the control at the same time point.

The results of microscopy revealed that the morphology of tissue exposed to N2 showed no changes (Fig. 8B). Intravesical instillation of the nanoemulsion with  $\alpha$ -terpineol (N3) revealed histological changes as shown in Fig. 8C. Some detached superficial urothelium was noted (arrows), which may have resulted from the desquamation of umbrella cells. Some congestion was also observed in the blood vessels. These changes were due to early, mild disruption of the bladder structure.

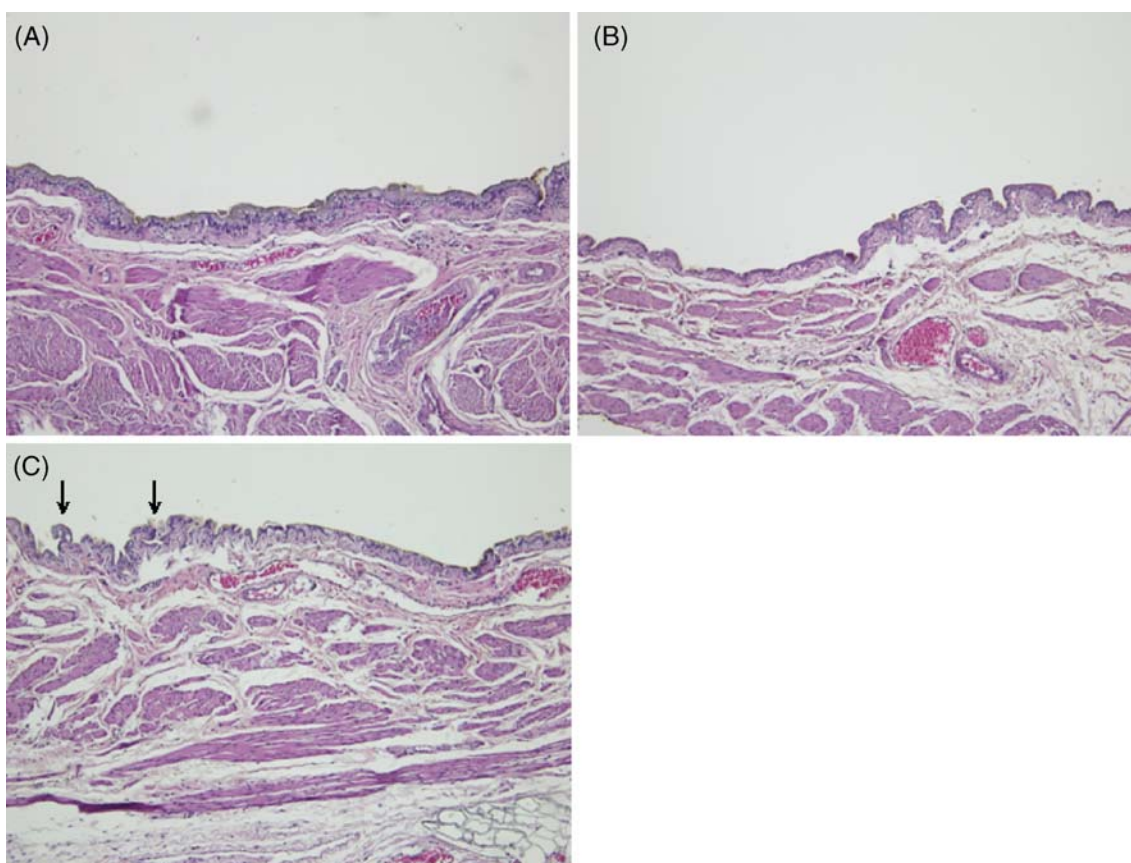
## DISCUSSION

Despite intravesical chemotherapy being used after TUR of superficial bladder tumors, the recurrence rate is still 36%~44% (15). The incomplete response often seen with the use of conventional formulations for intravesical therapy can be partly attributed to resistant drug targets or unsuccessful drug delivery to diseased bladder tissue (7). In the current study, w/o nanoemulsions with an optimal formulation design provided enhanced and controlled cisplatin and carboplatin delivery to the bladder. Sustained release of the drug was observed after nanoemulsion encapsulation.

Droplet sizes of the present formulations ranged 30~90 nm, which fall in the range of so-called nanoemulsions



**Fig. 7.** Confocal laser scanning microscopic (CLSM) micrographs of rat bladder tissues after the *in vivo* intravesical instillation of FITC for 3 h from (A) water (free control), (B) nanoemulsion with Brij 98 (N1), and (C) the  $\alpha$ -terpineol-containing nanoemulsion (N3) (original magnification, 20x). The specimen was viewed by CLSM at  $\sim 10\text{-}\mu\text{m}$  increments through the Z-axis from the surface to the deeper layers (top to bottom).



**Fig. 8.** Histological examination of rat bladder tissues after *in vivo* intravesical instillation for 3 h with (A) water (control), (B) nanoemulsion with Brij 98 (N1), and (C)  $\alpha$ -terpineol-containing nanoemulsion (N3) (original magnification, 100x).

(20~200 nm). All formulations were <100 nm, which is advantageous since the emulsions can be sterilized simply by passing them through a sterile syringe-driven filter with no need for thermal treatment (16,17). The incorporation of Brij 98 (N2) instead of Tween 80 (N1) led to a significant reduction in the droplet size. Brij 98 may have the ability to strengthen droplet integrity (18), resulting in strong cohesion of the droplets and the avoidance of aggregation. All emulsifiers used here were non-ionic species. The negative charge in the interface is believed to be the result of the ionization of the free fatty acids derived from the hydrolysis of triglycerides in soybean oil. The larger surface area in the larger inner phases may provide a higher surface potential (17). This can explain the higher zeta potential of the Tween 80-containing nanoemulsion (N1) compared to the Brij 98-containing one (N2). The first barrier to drug diffusion in bladder tissue is the highly negatively charged glycosaminoglycan layer and membrane plaque, which prevent drugs from reaching the urothelium (19). The N1 system may have been unfavorable for interactions with the bladder wall because of its higher negative charge. On the other hand, interfacial tension tends to be lower for emulsions with smaller droplet sizes (20), rendering their permeation easier. Thereby, the N2 system was selected for further formulation design.

The addition of enhancers reduced the mean size of the nanoemulsions, especially that with  $\alpha$ -terpineol (N3). The  $\log P_{\text{octanol/water}}$  values of  $\alpha$ -terpineol and oleic acid are 3.1~3.3 and 7, respectively (21). The discrepancy between the polarity of the enhancer-containing oil phase and the aqueous

phase was smaller for  $\alpha$ -terpineol. Stabilization and mixing of the two phases with these emulsifiers were easier with the  $\alpha$ -terpineol-containing system, resulting in the production of smaller droplet sizes. Alkali fatty acids, such as oleic acid, are thought to yield mixed films with a higher packing density of interfacial film-forming components (22). Adding oleic acid to the systems may increase the negative zeta potential as observed in the present study.

Local retention conditions in tissues are an important parameter for drug delivery by emulsions (23). The viscosity/rheology properties are factors affecting the retention ability. Larger droplets have higher viscosities (20). This phenomenon is identical to the present results in which the Tween 80-containing formulation (N1) showed a greater viscosity than the Brij 98-containing one (N2). Another possible reason is the potency of the water swelling of Tween 80 to increase the apparent viscosity (24).

The cytotoxicity results showed that free cisplatin induced proliferation inhibition in urothelium cancer cells. Cisplatin was slowly released from the nanoemulsion droplets. Hence the high activity against cultured cells was not totally due to the direct penetration of free drug into the cytoplasm. One possibility is the advantage of the oily external phase of the nanoemulsions increasing drug absorption into tumor cells (25). Another possibility is that cellular uptake of the drug can occur by an endocytotic pathway of droplets or by fusion of the surfaces with the cell membrane, leading to increased internalization of the droplets and drug release inside the tumor (26,27). A detailed investigation of

the nanosystem's effect on cytotoxicity was not the main focus of this study. Further elucidation of the mechanism will be attempted in future studies. Assuming that cisplatin released from the nanosystems still plays an essential role in cytotoxicity (28), the lower cytotoxicity of cisplatin in nanoemulsions with Tween 80 (N1) than the other carriers may be consistent with the relatively slower release profile of the drug.

Cisplatin is a platinum-chelated complex with four ligands: two ammonias and two chlorides. The two chloride ligands are gradually substituted with H<sub>2</sub>O molecules in water. Both Pt(NH<sub>3</sub>)<sub>2</sub>Cl(OH<sub>2</sub>)<sup>+</sup> and Pt(NH<sub>3</sub>)<sub>2</sub>Cl(OH<sub>2</sub>)<sub>2</sub><sup>2+</sup> can be formed by the aqueous complex (29,30). The partition coefficient of cisplatin between soybean oil and water was determined. The log $P_{oil/water}$  value was  $-0.62 \pm 0.06$  for cisplatin. This suggests that the ionic cisplatin complex has a notable affinity for the aqueous phase of hydrophilic environments. It is important for cisplatin to be included in the inner phase of the w/o nanoemulsions. On the other hand, carboplatin showed a log $P_{oil/water}$  value of  $-0.23 \pm 0.07$ . Carboplatin is produced by substituting the chlorides in cisplatin with the 1,1-cyclobutane dicarboxylate ligand. Hence, carboplatin cannot form an aqueous complex. This drug demonstrated less partitioning in the aqueous phase, resulting in less encapsulation in the inner phase of the nanoemulsions. The closeness of the release behavior of the two drugs indicates the negligible influence of this lower level of encapsulation.

The drug released from the inner phase supplements drug depleted in the external phase, supplying sustained and controlled release as was observed by the *in vitro* cisplatin release behavior. This is reasonable since nanoemulsions are considered to be prolonged drug release systems with the oil and emulsifier layers acting as release barriers. Sustained delivery via an intravesical route can ensure the continuous presence of drug in the bladder. It is plausible to expect an increase in efficacy with increased duration of direct contact between the drug and urothelium (7).

The release rate generally increases with smaller droplets, since a small-droplet system has a larger total surface area through which drug diffusion can occur (12,31). This may have contributed to the higher release rate of Brij 98-containing nanoemulsions (N2 to N4) than the Tween 80-containing system (N1). The viscosity of the nanoemulsions is another important factor in the drug release characteristics. It is recognized that increasing the viscosity of w/o emulsions causes a more rigid structure and decreases the rate of drug release (11,32). There was a correlation between the viscosity and the amount of drug released by the four nanoemulsions tested, suggesting a role of viscosity in cisplatin release from these systems. The addition of oleic acid (N3) retarded drug release from the carriers. This may have been due to the ability of oleic acid to be firmly anchored at the level of the emulsifier film (33), thus strengthening the interfaces among droplets.

It was apparent that the urine reduced cisplatin release for most carriers tested. Since intravesical transport can be adversely affected by dilution of the instilled drug vehicle by urine in the bladder (7), the dilution by urine led to a reduction in the concentration of cisplatin and subsequent drug release. Nanoemulsions with Brij 98 (N2 to N4) showed a greater reduction in cisplatin release compared to the

carrier with Tween 80 (N1). It is possible that w/o emulsions can be converted into water-in-oil-in-water (w/o/w) multiple emulsions upon dilution with excess water (25). In w/o emulsions, the oil phase can be regarded as a semipermeable membrane allowing water transport. Water flux into the inner phase of the systems may then be induced by an osmotic gradient (34). The release of the drug from the droplets is reduced when water enters the aqueous phase and produces an osmotic (swelling) effect. The downregulation due to swelling can be explained by a reverse solvent drag or by a reduced drug concentration gradient after swelling occurs. Emulsions with small droplets are more responsive to osmotic pressure than those with larger droplets (34,35). Our results are in accordance with this theory.

The antitumor effect of cisplatin depends on the concentration and exposure time (36). Cisplatin and carboplatin in aqueous solution produced a burst of accumulation in bladder tissues after the first sampling (1 h). The drug concentration was further reduced following an increase in the administration duration. This may have been due to dilution of the drug by urine. The concentration gradient for promoting drug diffusion into the bladder was thus decreased (6). This result was expected, since the duration of drug instillation during intravesical bladder therapy is typically limited to 2 h (15). The urothelium is the toughest known barrier to drug delivery (37). The main site of bladder permeability is located at the superficial cells in the epithelia called umbrella cells. These barrier functions may prevent drug permeation into the bladder wall, especially hydrophilic drugs. Free carboplatin showed higher permeation compared to free cisplatin because of the less hydrophilic characteristic of carboplatin.

The *in vivo* results indicate that nanoemulsions increased cisplatin deposition in bladder tissue, except the formulation with Tween 80 (N1). Factors contributing to the increased permeation by the nanoemulsions may be the large surface area due to the small droplet size. The availability of the drug is enhanced because the small size improves contact with the surface. Wetting and spreading can be enhanced by the low surface tension of the nanoemulsions (38). Due to the higher cisplatin concentration gradient on the bladder surface, the drug molecules are efficiently transported towards the urothelium from the nanoemulsions. Another possible mechanism is that the acid pH can increase the ion permeability of the urothelium (13). The pH of the nanoemulsions was around 5, as summarized in Table II. However, this cannot explain the low drug transport of the Tween 80-containing formulation (N1).

The efficiency of drug absorption is increased with formulations possessing bioadhesive characteristics (7). Viscosity can be an index of the adherence properties. Since the

**Table II.** The characterization of water-in-oil nanoemulsions by droplet size, zeta potential, and pH

Code	Size (nm)	Zeta potential (mV)	pH
N1	90.0±0.9	-9.3±0.8	5.8
N2	55.6±1.3	-3.4±0.4	5.6
N3	27.2±0.2	-3.5±0.3	5.2
N4	45.4±1.3	-4.7±0.3	5.4

Each value represents the mean±SD ( $n=3$ ).



N1 formulation showed the highest viscosity, the mucoadhesive property might not have been the controlling factor in intravesical cisplatin delivery. Nanoemulsions with Tween 80 (N1) exhibited slower drug release. Although the carriers with sustained-release activity can serve as drug depots, thereby extending the drug exposure in the bladder cavity after the voiding of urine, the extremely slow drug release may lead to a failure to provide sufficient drug molecules to the target tissues. This suggests the importance of the formulation design for intravesical delivery after completely considering the physicochemical characteristics and release behavior.

Although N2 and N4 showed increased cisplatin uptake in the bladder wall compared to the free control, this enhancement only persisted for 2 h. After that time, a large quantity of urine may have infused into the cavity. The loading of  $\alpha$ -terpineol (N3) could overcome this limitation to prolong a high cisplatin accumulation for a 3-h duration. This formulation even provided a greater carboplatin permeation than the free control until the end of the experiment (4 h). Approaches to enhance urothelium permeability by enhancers were investigated in previous research. Chitosan, polycarboxyl, protamine sulphate, and hyaluronidase are examples of permeation enhancers which can promote intravesical delivery (5,13,39,40). Enhancers cause desquamation of the urothelium, which results in disruption of the diffusion barriers, including the glycosaminoglycan layer, membrane plaques, and tight junctions of umbrella cells. Consequently, increased amounts of the drug can permeate into the wall.  $\alpha$ -Terpineol has long been used as an enhancer for skin delivery because of its ability to disrupt intercellular lipid bilayers and its non-irritative nature toward the skin structure (41,42). The lipophilic  $\alpha$ -terpineol can easily partition into the oil phase of nanoemulsions. The CLSM results confirmed the enhanced permeability of the nanoemulsion with  $\alpha$ -terpineol (N3). This incorporation not only enhanced the absorption amount but also increased the depth of permeation as observed in the fluorescence images. Some detachment of the bladder surface observed in the histological examination may have reduced the barrier function, thus making the urothelium more permeable to cisplatin and carboplatin. Sustained intravesical delivery of drugs at a high level can guarantee the continuous presence of the drug in the bladder without requiring intermittent catheterization. Hence, increased efficacy of antitumor activity would be expected. This enhancement was not detected by adding oleic acid, which is also a skin permeation enhancer.

One immediate instillation after TUR is recommended in all patients. In low-risk patients, no further treatment is needed before recurrence (43). One instillation of the nanoemulsions for a 3-h dwell time was performed to examine the preliminary safety to bladder tissues. The safety of the Brij 98-containing system without enhancers (N2) was confirmed because of the evidence from histological observations. Some detachment of the superficial tissues treated with the nanoemulsion containing  $\alpha$ -terpineol (N3) may have reduced the barrier property of the urothelium. This is an important mechanism of the enhancing potential of N3 to deliver the drug into the urothelium and the underlying tissue. The disrupted urothelium was more permeable than normal urothelium. This disruption was partial and possibly rever-

sible in the clinical status because of the revivification of the membrane. Further investigation, such as recovery duration of the bladder wall, is needed to elucidate the safety concern of the nanoemulsions.

## CONCLUSIONS

With increasing interest in intravesical chemotherapy, w/o nanoemulsions may prove to be promising new carriers for cisplatin and carboplatin. Cisplatin nanoemulsions showed significant activity against bladder cancer cells. The *in vitro* study showed that the nanoemulsions produced sustained release of the drugs. The *in vivo* study using the intravesical delivery of nanoemulsions with Brij 98 indicated that a high concentration of the drug could be maintained in bladder tissue for at least 2 h. Among all nanoemulsions examined, the  $\alpha$ -terpineol-containing system may be the most suited for future development because it provided controlled drug release, high cytotoxicity, and high drug permeation into bladder tissues. The high drug accumulation within the bladder wall could be prolonged for as long as 3~4 h with this formulation. This is especially important for intravesical drug instillation since the dwell time is generally limited to 2 h because of urine production. The continuous presence of drug in the bladder by sustained release, the increased availability of the drug contacting with urothelium by the small size of the systems, wetting and spreading because of the low surface tension, acidic pH environment of nanoemulsions, and desquamation by the enhancer may contribute to the high retention ability of cisplatin/carboplatin in the bladder wall. The great cytotoxicity against cancer cells by cisplatin-loaded nanoemulsions further encourages the use of the nanoemulsions for intravesical instillation to treat tumors. Nanoemulsions hold promise for the future of intravesical delivery of drugs into the bladder.

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