

**Fig. 6.** Antiangiogenic effect of expressed sFlt-1 in BxPC3 tumor bearing mice. Tumor microvessels were detected by CD31 (PECAM1) antibody staining of tumor cryosections 20 days after therapy. (A) Representative CD31 immunostaining images. (B) Quantitative analysis of microvessel density in tumor cryosections. The results represent a percentage area of green pixels per image. Seven images were taken from each tumor tissue, from 3 mice, mean  $\pm$  s.d. \* $P$ <0.05 compared to control group.

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# Improving Drug Potency and Efficacy by Nanocarrier-Mediated Subcellular Targeting

Mami Murakami,<sup>1</sup> Horacio Cabral,<sup>1,2</sup> Yu Matsumoto,<sup>3</sup> Shourong Wu,<sup>3</sup> Mitsunobu R. Kano,<sup>4</sup> Takao Yamori,<sup>5</sup> Nobuhiro Nishiyama,<sup>2,3\*</sup> Kazunori Kataoka<sup>1,2,3,6\*</sup>

Nanocarrier-mediated drug targeting is an emerging strategy for cancer therapy and is being used, for example, with chemotherapeutic agents for ovarian cancer. Nanocarriers are selectively accumulated in tumors as a result of their enhanced permeability and retention of macromolecules, thereby enhancing the antitumor activity of the nanocarrier-associated drugs. We investigated the real-time subcellular fate of polymeric micelles incorporating (1,2-diaminocyclohexane) platinum(II) (DACHPt/m), the parent complex of oxaliplatin, in tumor tissues by fluorescence-based assessment of their kinetic stability. These observations revealed that DACHPt/m was extravasated from blood vessels to the tumor tissue and dissociated inside each cell. Furthermore, DACHPt/m selectively dissociated within late endosomes, enhancing drug delivery to the nearby nucleus relative to free oxaliplatin, likely by circumvention of the cytoplasmic detoxification systems such as metallothionein and methionine synthase. Thus, these drug-loaded micelles exhibited higher antitumor activity than did oxaliplatin alone, even against oxaliplatin-resistant tumors. These findings suggest that nanocarriers targeting subcellular compartments may have considerable benefits in clinical applications.

## INTRODUCTION

In 2009, about 10 million people worldwide were newly diagnosed with cancer (1). Application of nanotechnology to cancer therapy may offer therapeutic effects that cannot be achieved with other strategies. The main aim of this approach is to develop nanoscale drug vehicles for targeted cancer therapy (2–5). Nanocarriers selectively accumulate in solid tumors as a result of the enhanced permeability and retention (EPR) effect, which is characterized by microvascular hyperpermeability to circulating macromolecules and impaired lymphatic drainage in tumor tissues (6). At present, several nanocarrier formulations have been approved for clinical use against ovarian cancer and HIV-associated Kaposi's sarcoma (Doxil) and breast cancer (Abraxane). These formulations allow better accumulation of the drugs doxorubicin and paclitaxel in tumors (7).

Polymeric micelles, self-assemblies of block copolymers, have gained increasing popularity as tumor-targetable nanocarriers since they were first used as drug vehicles in the late 1980s (8–12). These micelles, which are several tens of nanometers in size and have a characteristic core-shell structure consisting of a drug-loaded hydrophobic core and poly(ethylene glycol) (PEG) hydrophilic shell, are long-lived in the bloodstream and effectively accumulate in solid tumors after intravenous injection (8). The critical features of polymeric micelles for their function as drug vehicles, including size, drug loading and release, and specific binding to the target cells, can be modulated by engineering the

constituent block copolymers. At present, our micelle formulations incorporating doxorubicin, paclitaxel, SN-38, cisplatin, and (1,2-diaminocyclohexane) platinum(II) (DACHPt) are undergoing clinical trials (development code names NK911, NK105, NK012, NC-6004, and NC-4016, respectively), and four of these have advanced to Phase II studies (13–17). These clinical studies have revealed that polymeric micelles reduce side effects from the incorporated drugs and are effective against various intractable tumors, such as triple-negative breast cancers (18), indicating their clinical potential.

Recently, increasing attention has been paid to another potentially useful property of nanocarriers: to achieve subcellular drug targeting. Subcellular drug targeting of nanocarriers could enhance the pharmacological activity of the loaded drugs through improved subcellular drug distribution (19). Drug vehicles designed to release active drugs in acidic organelles, such as the endosome and lysosome, can circumvent recognition by the drug efflux pump (for example, P-glycoprotein) through internalization by endocytosis, thus overcoming multidrug resistance in cancer cells (20–22). Here, we aimed to investigate the potential of DACHPt-loaded micelles (DACHPt/m) for *in vivo* subcellular targeting. DACHPt/m is formed by the polymer-metal complexation between DACHPt and the carboxylic group of poly(ethylene glycol)-*b*-poly(glutamic acid) [PEG-*b*-P(Glu)] copolymers. DACHPt is the parent complex of the clinically approved drug oxaliplatin. Oxaliplatin has a hydrolyzable oxalate group to increase its solubility in water, which can be removed by nucleophiles in biological media, such as chloride ions. Aqua complexes ( $[(\text{DACH})\text{Pt}(\text{H}_2\text{O})\text{Cl}]^+$  or  $[(\text{DACH})\text{Pt}(\text{H}_2\text{O})_2]^{2+}$ ) of DACHPt exhibit chemotherapeutic activity. DACHPt/m releases DACHPt and the micelle structure dissociates depending on the pH and chloride ion concentrations, a result of ligand substitution of the Pt(II) from the carboxylates in the micelle core with the chloride ions in the medium (Fig. 1A) (23, 24). Moreover, after DACHPt/m is internalized into cancer cells, it would be expected to be exposed to different pH and chloride ion concentrations during subcellular trafficking (25). We hypothesized that DACHPt/m would be selectively released in low-pH cellular compartments, bypassing

<sup>1</sup>Department of Bioengineering, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. <sup>2</sup>Center for NanoBio Integration, University of Tokyo, Tokyo 113-8656, Japan. <sup>3</sup>Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan. <sup>4</sup>Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan. <sup>5</sup>Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan. <sup>6</sup>Department of Materials Engineering, Graduate School of Engineering, University of Tokyo, Tokyo 113-8656, Japan.

\*To whom correspondence should be addressed. E-mail: nishiyama@bmw.t.u-tokyo.ac.jp (N.N.); kataoka@bmw.t.u-tokyo.ac.jp (K.K.)



cytoplasmic detoxification and thereby improving potency and efficacy (Fig. 1B). Indeed, we previously reported that cisplatin-loaded micelles, which are formed in the same manner as DACHPt/m, caused different gene expression patterns than did cisplatin alone because of their different internalization pathways and the facilitated drug release in endosomes and lysosomes (26). To test the above-mentioned hypothesis, we constructed fluorescent-labeled DACHPt/m (F-DACHPt/m) with a dual fluorescent-labeling method so that we could follow the intracellular localization and dissociation of the micelles by using *in vivo* confocal microscopy, and intravitally evaluated the extravasation, penetration, cellular uptake, and subcellular fate of DACHPt/m in tumor tissues and their activity against human colorectal cancers.

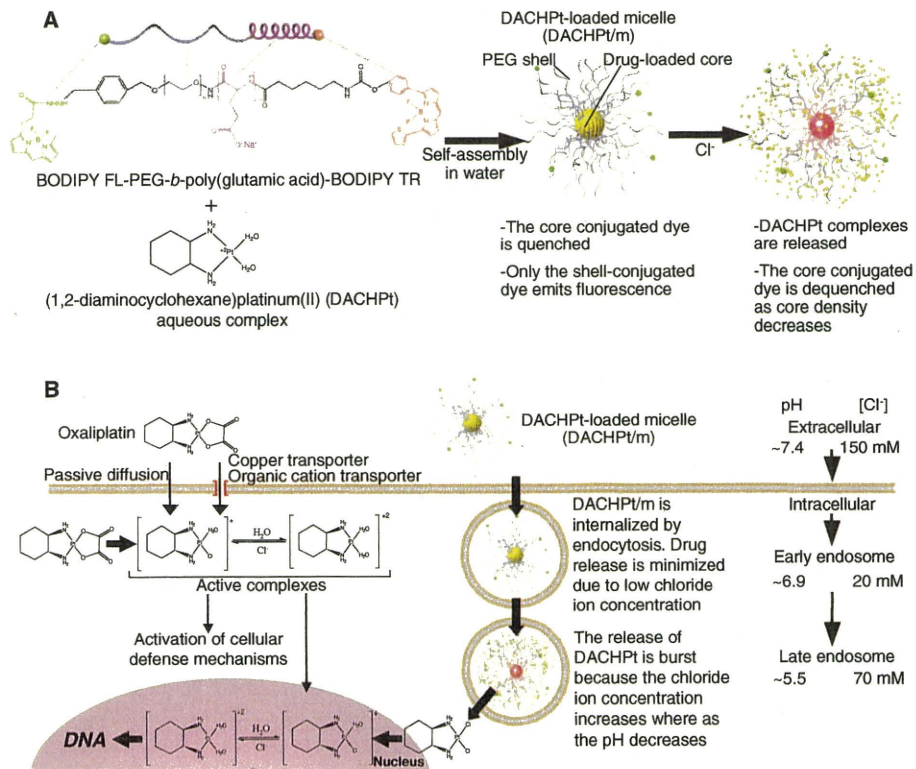
## RESULTS

### Construction and characterization of F-DACHPt/m

To construct the dual fluorescent-labeled block copolymer, we conjugated the fluorescent dyes boron dipyrromethene (BODIPY) FL (excitation wavelength, 503 nm; emission wavelength, 512 nm) and BODIPY TR (excitation wavelength, 588 nm; emission wavelength, 616 nm) to the  $\alpha$ - and  $\omega$ -end groups of  $\alpha$ -4-(diethoxymethyl)benzyl-poly(ethylene glycol)-*b*-poly(L-glutamic acid), respectively, and thus obtained BODIPY FL-PEG-*b*-P(Glu)-BODIPY TR (fig. S1). The conjugation degree for BODIPY FL to the block copolymer was 0.2 mol BODIPY FL per mole of polymer and for BODIPY TR was about 0.8 mol BODIPY TR per mole of polymer. We built F-DACHPt/m by the formation of polymer-metal complexes between DACHPt and the carboxylic groups of

poly(glutamic acid) in BODIPY FL-PEG-*b*-P(Glu)-BODIPY TR (Fig. 1A). F-DACHPt/m had a diameter of 30 nm, similar to that of DACHPt/m (fig. S2). The intact F-DACHPt/m emitted fluorescence only from the shell-conjugated dye (BODIPY FL) because the core-conjugated dye (BODIPY TR) was quenched owing to its high local concentration. The close proximity of BODIPY TR fluorophores in the core of the micelles leads to self-quenching by the formation of nonfluorescent ground-state BODIPY TR dimers (or higher aggregates) (27). When DACHPt is released as a result of the ligand substitution of the Pt(II) from the carboxylates in the micelle core with the chloride in the medium, the density of the micelle core is reduced and the fluorescence of BODIPY TR is dequenched (Fig. 1A). We investigated the release of DACHPt from F-DACHPt/m and the fluorescence of BODIPY FL and BODIPY TR at the surface and core of the micelles, respectively, under conditions that mimicked the extracellular medium (pH 7.4 and 150 mM Cl<sup>-</sup>), early endosomes (pH 6.9 and 20 mM Cl<sup>-</sup>), and late endosomes and lysosomes (pH 5.5 and 70 mM Cl<sup>-</sup>) (25). The fluorescence from BODIPY FL on the shell of the micelles was constant regardless of the pH and salt concentration of the media (Fig. 2, A to C, middle columns), suggesting that the fluorescence from BODIPY FL can be used to trace the position of the micelles in the biological environment. In addition, the drug release profile of F-DACHPt/m was similar to that of unmodified DACHPt/m, suggesting the feasibility of a direct comparison (Fig. 2, A to C, left columns). Under extracellular conditions, BODIPY TR fluorescence increased after an incubation period of 17 hours, simultaneous with the release of DACHPt from F-DACHPt/m (Fig. 2A). The release of DACHPt from F-DACHPt/m in the late endosomal conditions was considerably faster (Fig. 2C) than in the extra-

**Fig. 1.** Design of fluorescent-labeled DACHPt/m (F-DACHPt/m) for visualization of the localization and drug release in the cell. **(A)** F-DACHPt/m self-assembled through polymer-metal complex formation between DACHPt and boron dipyrromethene (BODIPY) FL-poly(ethylene glycol)-*b*-poly(glutamic acid)-BODIPY TR in distilled water. In the micelle state, only BODIPY FL (green) emits fluorescence, whereas BODIPY TR (red) remains quenched. As DACHPt is released from F-DACHPt/m in chloride ion-containing media, BODIPY TR is dequenched and emits fluorescence. **(B)** Schematic representation of hypothetical subcellular pathways and action of DACHPt/m. Oxaliplatin enter cells by passive diffusion or through copper/organic cation transporters. Once oxaliplatin is in the cytoplasm, most of the activated aqua species ( $[(\text{DACH})\text{Pt}(\text{H}_2\text{O})\text{Cl}]^+$  or  $[(\text{DACH})\text{Pt}(\text{H}_2\text{O})_2]^{2+}$ ) are eliminated by cellular detoxification mechanisms, but a small fraction binds to DNA. In contrast, DACHPt/m that enters tumor cells by endocytosis (middle) is exposed to an environment with increasing acidity and chloride ion concentration because early endosomes mature into the late endosomes. Drug release from DACHPt/m is accelerated in the late endosomal environment close to the perinuclear region, resulting in enhanced efficiency of drug delivery to the nucleus.





cellular and early endosomal conditions (Fig. 2, A and B), occurring without any delay. Further, mirroring the DACHPt release, F-DACHPt/m exhibited more robust fluorescence recovery of BODIPY TR under the late endosomal conditions (Fig. 2C) than under the early endosomal conditions (Fig. 2B, right columns). Thus, the fluorescence profiles of BODIPY TR are correlated with the release profiles of DACHPt from the micelles.

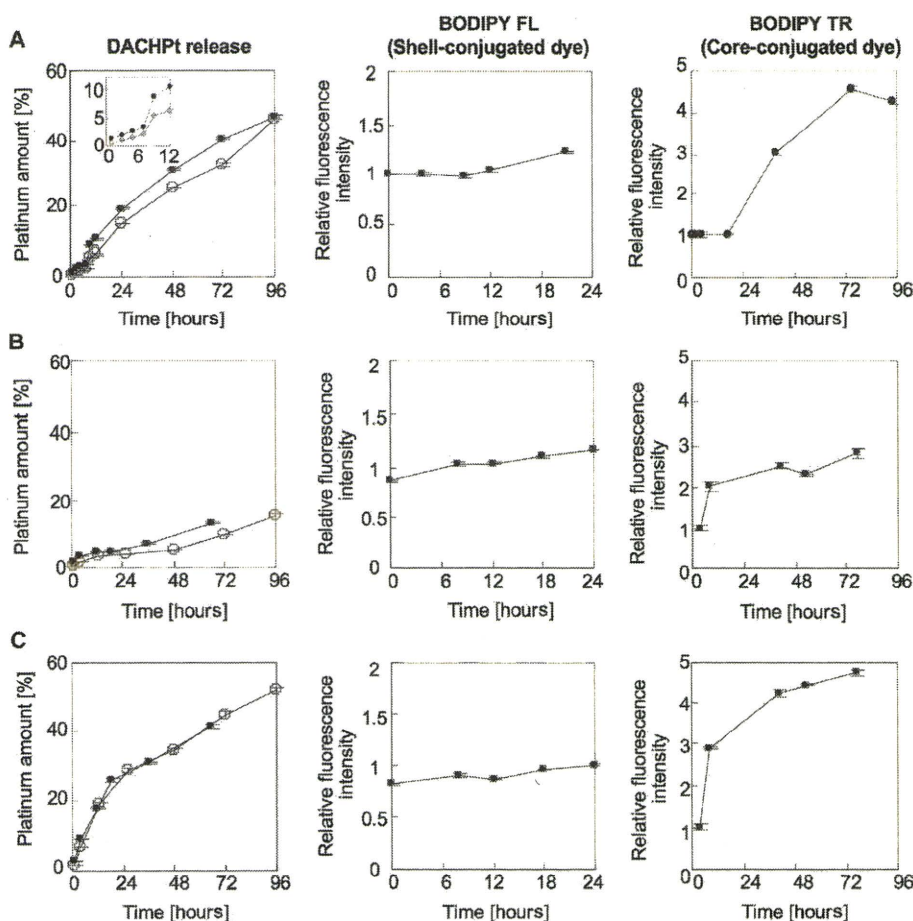
### In vitro subcellular trafficking of F-DACHPt/m

The cellular internalization and fate of F-DACHPt/m in human tumor-derived colorectal cancer (HT29) cells, which are the most frequently used cell lines in oxaliplatin studies (28), were observed with time-lapse confocal laser scanning microscopy (CLSM) (Fig. 3A and video S1). The intensity of BODIPY FL fluorescence increased slightly over time (Fig. 3B), and the BODIPY FL fluorescence in the images was clearly visible within a 6-hour incubation (Fig. 3A). Meanwhile, with time,

the intensity of BODIPY TR fluorescence continuously increased to a greater extent than did that of BODIPY FL fluorescence (Fig. 3B), and the BODIPY TR fluorescence in the images became visible at about 24 hours of incubation (Fig. 3A). This continuous increase in the intensity of BODIPY TR fluorescence corresponds to the dequenching of BODIPY TR fluorescence driven by the release of DACHPt in the cell. These results suggest that F-DACHPt/m enters the cells as a micelle form and then dissociates within the subcellular environments.

Using CLSM with higher magnification, we further evaluated the detailed subcellular trafficking and fate of F-DACHPt/m by focusing on individual cells. We confirmed that the micelles entered the cancer cells via endocytosis by incubating the cells with F-DACHPt/m at 37°C and 4°C. As endocytosis ceases at 4°C, the fluorescent signal of F-DACHPt/m inside the cells was undetectable, whereas at 37°C, the fluorescence from F-DACHPt/m was observed inside the cells (fig. S3). To examine the

subcellular trafficking of the micelles, we determined the colocalization of BODIPY FL fluorescence from F-DACHPt/m with an early endosome marker, Rab5a-RFP, and a late endosome and lysosome marker, LysoTracker, in HT29 cells (Fig. 3C). Note that individual vesicular organelles can be recognized as punctate fluorescence in the images. After a 6-hour incubation at 37°C, BODIPY FL colocalized mainly with Rab5a-RFP (yellow fluorescence in Fig. 3C, upper images) rather than with LysoTracker. After prolonged incubation (24 and 55 hours), BODIPY FL showed decreased colocalization with Rab5a-RFP and increased colocalization with LysoTracker (yellow fluorescence in Fig. 3C, lower images). This observation was confirmed by quantification of colocalized fluorescent intensities of BODIPY FL with Rab5a-RFP or LysoTracker (Fig. 3D). These results suggested that the micelles might localize mainly in the early endosome until 6 hours and then move into the late endosome/lysosome compartment. Furthermore, we studied the timing and location of the micelle dissociation and concomitant drug release by evaluating the colocalization of F-DACHPt/m with LysoTracker (Fig. 3E and fig. S4A) and the quantification of BODIPY FL and BODIPY TR fluorescent intensities (fig. S4B), as well as the colocalization ratio (Fig. 3F). The fluorescence intensity from BODIPY FL gradually increased (fig. S4B). Meanwhile, the fluorescence of BODIPY TR became visible after 24-hour incubation (Fig. 3E and fig. S4A) and then increased over time (fig. S4B). Both BODIPY FL and BODIPY TR colocalized with LysoTracker (Fig. 3E and fig. S4A), and the colocalization ratio between BODIPY FL or BODIPY TR and LysoTracker increased over time (Fig. 3F). These observations sug-

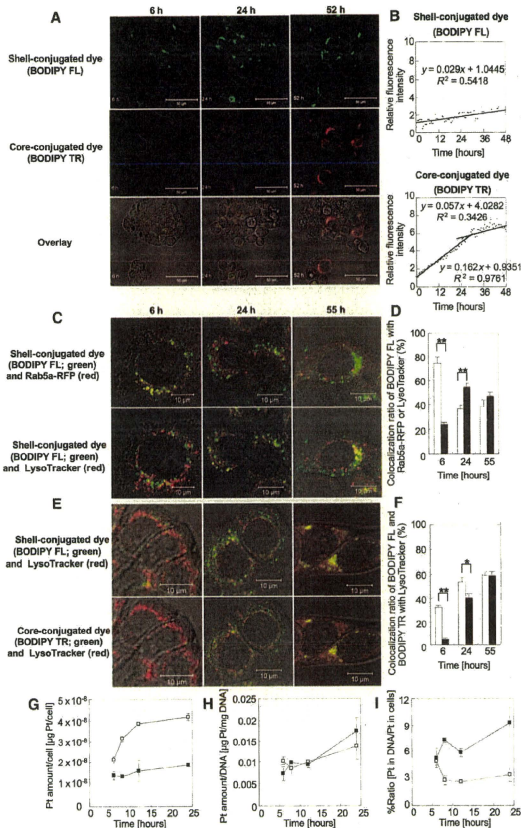


**Fig. 2.** Properties of F-DACHPt/m under conditions mimicking extracellular and subcellular environments. (A to C) Release profiles of DACHPt from DACHPt/m and F-DACHPt/m (left column) and fluorescence profiles of BODIPY FL (middle column) and BODIPY TR (right column) under conditions mimicking (A) the extracellular environment (10 mM PBS, pH 7.4, and 150 mM NaCl), (B) the early endosomal environment (10 mM PBS, pH 6.9, and 20 mM NaCl), and (C) the late endosomal environment (10 mM PBS, pH 5.5, and 70 mM NaCl), all at 37°C. Inset in top left panel is the magnification of the profiles of DACHPt release until 12 hours. The release of DACHPt from the micelles was evaluated by dialysis as described in Materials and Methods. Data for DACHPt/m and F-DACHPt/m in the left column are shown as open and filled circles, respectively. Data are expressed as means  $\pm$  SEM ( $n = 3$ ).

gested that F-DACHP/m progressively dissociated in the late endosome and lysosomal compartments. Because these acidic organelles reside in the perinuclear region, F-DACHP/m would be expected to deliver the active platinum complexes close to the nucleus. Thus, in vitro confocal microscopy revealed that F-DACHP/m appears to exhibit late endosome/lysosome-selective dissociation concomitant with the release of DACHP1, thereby achieving efficient DACHP1 delivery close to the nucleus.

**Effect of subcellular pathway on drug efficiency**

Oxaliplatin enters the cells by passive diffusion or through copper/organic cation transporters (29), and it then changes to active DACHP1 aqua complexes in the cytoplasm, some of which may ultimately cross-link with DNA, disrupting DNA function and exerting therapeutic activity (Fig. 1B). However, 75 to 85% of activated platinum drugs are sequestered by abundant sulfur species that serve as cellular defense mechanisms in the cytoplasm, and only 5 to 10% of oxaliplatin can bind to DNA (Fig. 1B) (30–32). We hypothesized that DACHP1/m facilitates drug delivery close to the nucleus through its perinuclear subcellular localization. Therefore, we studied the pharmacological activity of DACHP1/m. DACHP1/m displayed a value of  $IC_{50}$  (the mean concentration that causes 50% growth inhibition) against HT29 cells that was lower than that of oxaliplatin by a factor of 4.7 (Table 1). It is rare that a nanocarrier-encapsulated drug surpasses the free form of the drug for in vitro cytotoxicity (33). To elucidate the mechanism of DACHP1/m action, we evaluated the subcellular accumulation of platinum and quantity of Pt-DNA adducts. Exposure of HT29 cells to oxaliplatin resulted in twice as much accumulation of platinum than did exposure to DACHP1/m (Fig. 3G). This is probably because oxaliplatin rapidly enters the cells by diffusion and through copper/organic cation transporters (29), whereas DACHP1/m is gradually internalized by endocytosis. Nevertheless, we did not observe a significant difference in the Pt-DNA adducts formed after exposure to oxaliplatin and DACHP1/m (Fig. 3H), indicating that DACHP1/m may efficiently deliver the active platinum drug to DNA (Fig. 3I).



**Fig. 3.** In vitro observation of subcellular trafficking and fate of F-DACHP1/m, cellular platinum accumulation, and formation of platinum-DNA adducts. (A) Representative images of time-lapse CLSM observation of HT29 cells treated with F-DACHP1/m (green, BODIPY FL; red, BODIPY TR; yellow, their colocalization). (B) Relative fluorescence intensity of BODIPY FL (upper) and BODIPY TR (lower) of F-DACHP1/m. (C) Fluorescent images of colocalization of BODIPY FL of DACHP1/m (green) with an early endosome marker, Rab5a-RFP (red in upper images), or a late endosome and lysosome marker, LysoTracker (red in lower images), in HT29 cells after incubation for 6, 24, and 55 hours. (D) Colocalization ratio of BODIPY FL with Rab5a-RFP (open bars) or LysoTracker (closed bars). Data are expressed as means  $\pm$  SEM ( $n = 10$ ).  $^{**}P < 0.01$ . (E) Fluorescent images of colocalization of BODIPY FL (green in upper images) and BODIPY TR (green in lower images) of F-DACHP1/m with LysoTracker (red) in HT29 cells after incubation for 6, 24, and 55 hours. (F) Colocalization ratio of BODIPY FL (open bars) or BODIPY TR (closed bars) with LysoTracker. Data are expressed as means  $\pm$  SEM ( $n = 10$ ).  $^{*}P < 0.05$ ;  $^{**}P < 0.01$ . (G) In vitro cellular accumulation of platinum. (H) DNA platination. (I) Ratio of platinum in DNA to total platinum in cells expressed as a percentage. DNA platination was converted from  $\mu\text{g Pt/mg DNA}$  to  $\mu\text{g Pt/cell}$ , and the ratio was calculated. Open squares, oxaliplatin; filled squares, DACHP1/m. Data are expressed as means  $\pm$  SEM ( $n = 3$ ).



We also evaluated the mean concentration required for 50% growth inhibition ( $GI_{50}$ ), which is defined as in (34, 35), in a human cell panel composed of 37 cancer cell lines (fig. S5), and investigated the correlation between  $GI_{50}$  and expression of 26 genes that we selected on the basis of their potential association with the pharmacological activity of platinum compounds (36). The cytotoxic activity of oxaliplatin was inversely correlated with the expression of metallothionein (MT1Q) and methionine synthase (MTR), which are found in the cytoplasm and inactivate platinum compounds. DACHPt/m cytotoxicity did not exhibit similar correlations (table S1). We conclude that DACHPt/m may bypass cytoplasmic detoxification by MTR and MT1Q and efficiently deliver active platinum complexes to the nucleus, because they are internalized by endocytosis and selectively release the active platinum complexes in the late endosome/lysosome compartment (Fig. 1B).

**Effect of DACHPt/m on oxaliplatin resistance in vitro**

Our proposed mechanism of action of DACHPt/m led us to investigate their efficacy in oxaliplatin-resistant cancer cells, because MTR and MT1Q are overexpressed in these cells (37–39). We developed oxaliplatin-resistant HT29 cells (HT29/ox) by chronic exposure of HT29 cells to oxaliplatin with gradual dose escalation. Relative to the parental HT29 cells, HT29/ox cells were 10 times as resistant to oxaliplatin (Table 1). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blotting revealed that the HT29/ox cells showed up-regulated messenger RNA (mRNA) as well as protein for MTR and MT1Q compared with HT29 cells (Fig. 4, A and B). Moreover, the down-regulation of MT1Q and MTR with small interfering RNA (siRNA) restored the sensitivity of HT29/ox cells to oxaliplatin (fig. S6). In vitro cytotoxicity studies showed that DACHPt/m was 120 times as cytotoxic as oxaliplatin in HT29/ox cells (Table 1). These results suggested that DACHPt/m may overcome acquired resistance to oxaliplatin.

**In vivo intratumoral imaging of F-DACHPt/m in a human colon cancer model**

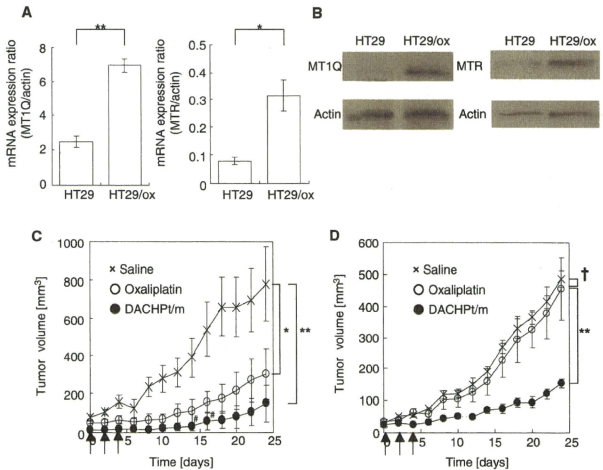
DACHPt/m would need to extravasate, penetrate into the interstitial tissue, and be internalized by cancer cells after systemic administration to exert the in vivo antitumor activity predicted from the results above. We performed real-time intravital observation of the accumulation and subcellular fate of F-DACHPt/m in HT29 xenografts by using in vivo CLSM equipped with a high-speed resonant scanner developed to acquire live tissue images of experimental animals (fig. S7). Immediately after intravenous injection, F-DACHPt/m was observed in the blood vessels of solid tumors (Fig. 5A and video S2). The fluorescence from F-DACHPt/m in the blood vessels corresponded only to that of BODIPY

FL. Even 12 hours later, only BODIPY FL fluorescence was observed flowing in the blood vessels (Fig. 5B and video S3). These observations indicate that F-DACHPt/m stably circulates in the bloodstream while maintaining their micellar structure. Images of tumor tissue revealed the accumulation and dissociation behaviors of F-DACHPt/m (Fig. 5, C and D, and video S3). Two hours after injection, F-DACHPt/m accumulated within the tumor tissue because of the EPR effect and was identified within the cells, whereas BODIPY TR fluorescence remained quenched. BODIPY TR fluorescence gradually appeared inside the cells 4 hours after injection and was clearly visible after 12 hours, indicating the release of DACHPt

**Table 1.** In vitro cytotoxicity of free oxaliplatin and DACHPt/m against HT29 and HT29/ox cells after a 48-hour incubation. Data are expressed as means  $\pm$  SEM (n = 4).

Cells	$IC_{50}$ ( $\mu$ M)*		
	Free oxaliplatin	DACHPt/m	Oxaliplatin/DACHPt/m
HT29	2.2 $\pm$ 2.2	0.47 $\pm$ 0.05	4.7
HT29/ox	22.8 $\pm$ 2.6	0.19 $\pm$ 0.11	120
Ratio of HT29/ox/HT29	10.4	0.4	—

\* $IC_{50}$  values obtained from the MTT assay.



**Fig. 4.** Expression of MT1Q and MTR in HT29/ox cells and effects of DACHPt/m on HT29 and HT29/ox tumors in vivo. (A) Relative mRNA expression of metallothionein (MT1Q) and methionine synthase (MTR) in parent HT29 and HT29/ox cell lines. Data are expressed as means  $\pm$  SEM (n = 3). \* $P$  < 0.05; \*\* $P$  < 0.01. (B) Western blots of MT1Q and MTR in HT29 and HT29/ox cell lines. (C and D) In vivo effect of DACHPt/m on subcutaneous HT29 (C) and HT29/ox (D) tumor cells. Crosses, saline; open circles, oxaliplatin (8 mg/kg); filled circles, DACHPt/m (4 mg/kg); arrows, injection of oxaliplatin and DACHPt/m; †, tumor regression; ‡  $P$  > 0.1; \* $P$  < 0.05; \*\* $P$  < 0.01. Data are expressed as means  $\pm$  SEM (n = 4).

from the micelles inside the cells in the tumor tissue. The cell membrane, stained with CellMask, and cell nuclei were substantially free of F-DACHPt/m (Fig. 5, C and D, and video S3). These results were consistent with our *in vitro* results (Fig. 3A) and suggested that F-DACHPt/m percolated into interstitial tissues and was efficiently internalized to endosomal compartments of cells in a micelle form, followed by dissociation of the multimolecular structure of the micelles in the late endosomal and lysosomal compartments.

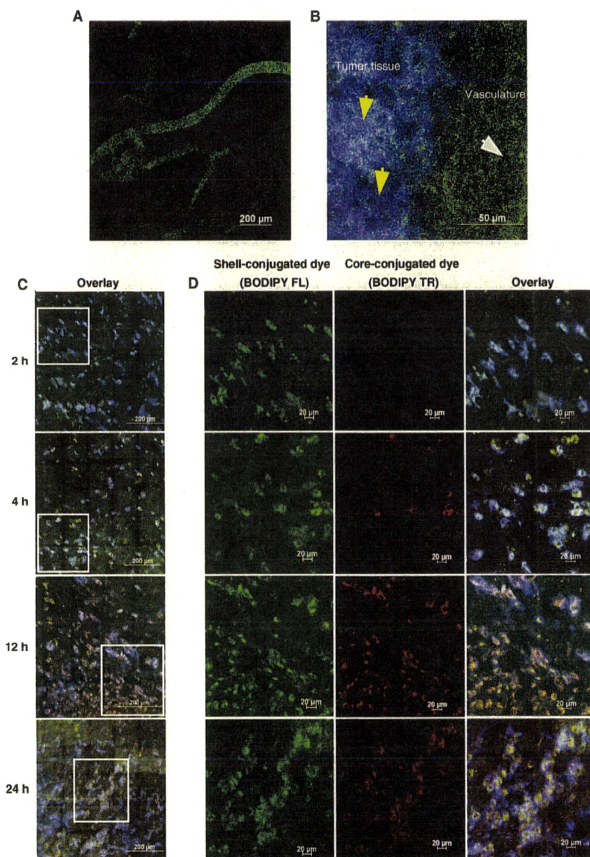
### DACHPt/m enhances antitumor activity and overcomes oxaliplatin resistance *in vivo*

On the basis of our observations of the *in vivo* behavior of F-DACHPt/m, we hypothesized that DACHPt/m may also overcome oxaliplatin resistance *in vivo*. Thus, we evaluated DACHPt/m *in vivo* antitumor activity against subcutaneous HT29 and HT29/ox tumors (Fig. 4, C and D). Although free oxaliplatin failed to inhibit the growth of HT29/ox tumors, DACHPt/m exhibited substantial antitumor activity in the oxaliplatin-resistant xenograft model and successfully overcame the oxaliplatin resistance of HT29/ox cells *in vivo* (Fig. 4D). Note that DACHPt/m also achieved higher antitumor activity than oxaliplatin against the HT29 tumors (Fig. 4C). Thus, our micelle-based drug delivery vehicle was able to circumvent the detoxification mechanisms against platinum drugs in tumor cell cytoplasm through selective subcellular drug release and hence overcome acquired resistance.

### DISCUSSION

Colorectal cancer is a major cause of morbidity and mortality worldwide (1). Oxaliplatin is currently the standard therapy for colorectal cancer, and acquired resistance to oxaliplatin is a major clinical drawback in the treatment of colorectal cancer [virtually all metastatic colorectal cancer becomes resistant to oxaliplatin, with a median time to progression of 8.7 months (40)]. The major cellular processes by which oxaliplatin enters and attacks cancer cells include uptake and transport, formation of DNA adducts and their recognition by damage response proteins, and signal transduction leading to apoptosis. Any factors that interfere with these pathways can lead to drug resistance (41). Here, we showed

that DACHPt/m can overcome drug resistance by circumventing recognition by MTR and MT1Q, and achieve subcellular drug delivery both *in vitro* and *in vivo* to the perinuclear region of cells. It has been reported that drug delivery systems can overcome multidrug resistance



**Fig. 5.** *In vivo* CLSM observation of F-DACHPt/m in blood vessels and tumors after intravenous administration. (A and B) CLSM observation of F-DACHPt/m in the blood vessels of solid tumors (A) immediately after injection and (B) in the tumor tissue at 12 hours after injection. Yellow arrows, tumor tissue; white arrow, blood vessel. (C) Time-dependent CLSM observation of F-DACHPt/m in the tumor tissues at 2, 4, 12, and 24 hours after injection. Green, fluorescence from the shell-conjugated dyes (BODIPY FL); red, core-conjugated dyes (BODIPY TR); blue, cell surfaces stained by CellMask. (D) Magnification of selected areas [square regions in (C)] by channel.



by avoiding the drug efflux mechanism of P-glycoprotein (20–22). Because P-glycoprotein is not associated with platinum drug resistance (42), we used a different approach. Our data indicate that cytoplasmic detoxification mechanisms against platinum drugs can potentially be avoided by using the appropriate drug delivery system.

Nanocarriers encounter numerous barriers *in vivo* en route to their target during the processes of blood circulation, extravasation, penetration, and cellular uptake. It is therefore difficult to extrapolate *in vivo* outcomes of a drug-nanocarrier combination from its *in vitro* behavior, although real-time observation of *in vivo* behaviors such as we have used here can ascertain critical barriers residing in a living body and facilitate the design of a nanocarrier optimized for *in vivo* delivery. The *in vivo* CLSM technique that we used in this study enabled spatiotemporal and quantitative analyses of extravasation, tissue penetration, and cellular internalization of nanocarriers in living animals. Using dual fluorescent labeling of DACHPT/m, we elucidated the real-time intratumoral behavior of DACHPT/m. The dual fluorescent label of DACHPT micelles allowed us to trace the micelles' position by the ever-present fluorescent signal from the surface of the micelles, and the drug release and dissociation of the multimolecular structure of the micelles by the quenching and fluorescence recovery of the core-conjugated dye. *In vitro* cellular experiments, these micelles were internalized intact, and then they were disassembled and the drug was released in late endosomes. In our *in vivo* microscopy, we observed that DACHPT/m maintained their micelle form during circulation in the blood, probably because of the stable inner core structure formed by the polymer-metal complexes, and extravasated into solid tumors. DACHPT/m was able to deeply penetrate cancerous tissue after extravasation and was internalized by different cell populations that were distant from the blood vessels. Such efficient penetration of tissue is a requirement of successful drug delivery and a prerequisite for effective subcellular targeting. It has been reported that PEG-modified liposomes with 100-nm diameter accumulated at perivascular regions of solid tumors and failed to penetrate the tumor interstitium deeply (43). Although this characteristic may depend on the cancer type, it is possible that the deep tumor penetration of DACHPT/m is a result of their smaller 30-nm size. Finally, the DACHPT/m structure dissociated at the perinuclear regions of the cell after internalization, based on the pH and chloride ion concentration-selective release of DACHPT. This observation is also consistent with our hypothesis that DACHPT/m can overcome oxaliplatin resistance in tumors by bypassing the cytoplasmic detoxification mechanisms of MTR and MT1Q. There are, however, several limitations to the type of study that we have performed in a subcutaneous tumor model. Compared with subcutaneous tumors, orthotopic and spontaneously forming tumors may have characteristic differences such as vascular density and degree of fibrosis, which may affect the transport of nanocarriers (44).

Our research provides one approach for subcellular targeting of cytoplasmic drugs. Such nanocarriers have the potential to enhance the drug efficacy and overcome drug resistance.

## MATERIALS AND METHODS

### Materials

$\gamma$ -Benzyl L-glutamate and bis(trichloromethyl) carbonate (triphosgene) were purchased from Sigma Chemical and Tokyo Kasei Kogyo, respec-

tively. *N,N*-Dimethylformamide (DMF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemicals. Oxaliplatin and  $\text{NaCH}_2\text{CN}$  were purchased from Sigma-Aldrich Inc. Dichloro(1,2-diamino cyclohexane) platinum(II) was purchased from W. C. Heraeus GmbH.  $\alpha$ -Methoxy- $\omega$ -amino-poly(ethylene glycol) [ $\text{CH}_3\text{O}$ -PEG- $\text{NH}_2$ ; molecular weight (MW), 12,000] was purchased from Nippon Oil and Fats. BODIPY TR-succinimidyl ester, BODIPY FL-hydrazide, LysoTracker Blue, CellLight Early Endosome-RFP (Rab5a-RFP), CellMask, and Lipofectamine RNAiMAX were purchased from Invitrogen.

### Cell lines and animals

HT29 cells were purchased from the American Type Culture Collection. HT29 cells were maintained in McCoy's 5A modified medium (Invitrogen) containing 10% fetal bovine serum (Gibco) as well as 1% penicillin and streptomycin (Sigma) and were cultured at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ . To develop HT29/ox cells (45), we treated HT29 cells with oxaliplatin at  $\text{IC}_{50}$  doses for 1 hour. After 24 hours, cells were subcultured into new flasks and oxaliplatin was added to a culture of 80% confluent cells. The concentration was incrementally increased by factors of 1.2 to 2. The process was continued until the cells were resistant to drug concentrations at least 10 times as great. BALB/c-nu/nu mice (female; body weight, 18 to 20 g, age, 6 weeks old) were purchased from Charles River Japan. All animal experiments were carried out in accordance with the guidelines for animal experiments at the University of Tokyo, Japan.

### Methods

**Synthesis of block copolymers.**  $\alpha$ -(4-Diethoxymethyl)benzyl- $\omega$ -amino-poly(ethylene glycol) (Ac-Bz-PEG- $\text{NH}_2$ ) was previously synthesized in our laboratory (46). Poly(ethylene glycol)-*b*-poly(L-glutamic acid) [PEG-*b*-P(Glu)] [MW<sub>PEG</sub>, 12,000; polymerization degree of P(Glu), 20] and Ac-Bz-PEG-*b*-poly(L-glutamic acid) [Ac-Bz-PEG-*b*-P(Glu)] [MW<sub>PEG</sub>, 12,000; polymerization degree of P(Glu), 20] were synthesized according to the previously described synthetic method (47). Briefly, *N*-carboxyanhydride of  $\gamma$ -benzyl L-glutamate (BLG-NCA) was synthesized by the Fuchs-Farthing method with triphosgene (48). BLG-NCA was polymerized in DMF initiated by the amino group of  $\text{CH}_3\text{O}$ -PEG- $\text{NH}_2$  or Ac-Bz-PEG- $\text{NH}_2$  to obtain PEG-*b*-poly( $\gamma$ -benzyl L-glutamate) (PEG-*b*-PBLG) or Ac-Bz-PEG-*b*-PBLG, respectively. The MW distribution of PEG-*b*-PBLG and Ac-Bz-PEG-*b*-PBLG was determined by gel permeation chromatography (GPC) [column, TSK-gel G3000HHR, G4000HHR (Tosoh); eluent, DMF containing 10 mM LiCl; flow rate, 0.8 ml/min; detector, refractive index; temperature, 25°C]. PEG-*b*-PBLG and Ac-Bz-PEG-*b*-PBLG showed narrow MW distributions ( $M_w/M_n$ : 1.09 and 1.16, respectively) in GPC. The degrees of polymerization of PBLG in PEG-*b*-PBLG and Ac-Bz-PEG-*b*-PBLG were determined to be 20 by comparing the proton ratios of methylene units in PEG (-OCH<sub>2</sub>CH<sub>2</sub>-;  $\delta$  = 3.7 ppm) and phenyl groups of PBLG (-CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>-;  $\delta$  = 7.3 ppm) in <sup>1</sup>H-NMR (nuclear magnetic resonance) measurement. Both PEG-*b*-PBLG and Ac-Bz-PEG-*b*-PBLG were deprotected by mixing with 0.5 N NaOH at room temperature to obtain PEG-*b*-P(Glu) and Ac-Bz-PEG-*b*-P(Glu), respectively. Complete deprotection was confirmed by <sup>1</sup>H NMR measurement.

**Preparation of micelles.** For conjugation of BODIPY TR to a polymer, a solution of BODIPY TR-succinimidyl ester in DMSO (1 mg/ml) was mixed with Ac-Bz-PEG-*b*-P(Glu). The reaction was carried out overnight at room temperature with stirring. Unreacted

BODIPY TR was removed by dialysis [MW cutoff (MWCO) size, 2000 daltons] against DMSO and water. To conjugate BODIPY FL to the Ac-Bz-PEG-*b*-P(Glu)-BODIPY TR polymer, we mixed a solution of BODIPY FL-hydrazide in DMSO with the polymer, followed by the addition of 1 N HCl to deprotect the acetal group. Samples were stirred overnight at room temperature and treated with NaBH<sub>3</sub>CN to reduce the link between BODIPY FL and the polymer. For purification, unbound BODIPY FL was removed by dialysis (MWCO, 2000 daltons) against DMSO and water. The prepared BODIPY FL-PEG-*b*-P(Glu)-BODIPY TR copolymer was freeze-dried overnight and stored at -20°C. DACHPt/m and F-DACHPt/m were prepared according to the previously described method (23). DACHPt (5 mM) was suspended in distilled water and mixed with silver nitrate ([AgNO<sub>3</sub>]/[DACHPt] = 1) to form aqueous complexes. The solution was kept in the dark at 25°C for 24 hours. AgCl precipitates were eliminated by centrifugation. The supernatant was purified by passage through a 0.22- $\mu$ m filter. DACHPt aqueous complex solution was then mixed with PEG-*b*-P(Glu) or BODIPY FL-PEG-*b*-P(Glu)-BODIPY TR ([Glu] = 5 mM; [DACHPt]/[Glu] = 1.0) and reacted for 120 hours to obtain DACHPt/m or F-DACHPt/m, respectively. DACHPt/m and F-DACHPt/m were purified by ultrafiltration (MWCO, 30,000 daltons; Fig. 1A). The size distributions of DACHPt/m and F-DACHPt/m were evaluated by dynamic light scattering at 25°C with a Zetasizer Nano ZS90 (Malvern Instruments). The platinum content of DACHPt/m was determined by ion-coupled plasma mass spectrometry (4500 ICP-MS; Hewlett Packard).

**Drug release and fluorescence profiles of micelles under different conditions.** The release of platinum from DACHPt/m and F-DACHPt/m in phosphate-buffered saline (PBS) at 37°C was evaluated as described (23). Briefly, a micelle solution of known platinum concentration was placed inside a dialysis bag (MWCO, 2000 daltons). The solution was then dialyzed against PBS under different conditions mimicking the extracellular environment (10 mM PBS, pH 7.4, and 150 mM NaCl), early endosomes (10 mM PBS, pH 6.9, and 20 mM NaCl), and late endosomes (10 mM PBS, pH 5.5, and 70 mM NaCl) at 37°C (25). The concentration of platinum present in the dialysate was determined with ICP-MS. The fluorescence profiles of F-DACHPt/m were also evaluated under the same conditions with a spectrofluorometer (FP6600, Jasco) or NanoDrop (ND3300, Scrum). Changes in fluorescence intensity were measured at a defined time period.

**In vitro observation of subcellular localization and the fate of F-DACHPt/m by CLSM.** HT29 cells were cultured at  $1 \times 10^5$  cells in 35-mm glass-based dishes (Asahi Techno Glass). After overnight incubation in a fresh medium, the cells were washed twice with PBS. The medium was then replaced by 1 ml of fresh medium containing F-DACHPt/m (100  $\mu$ M on Pt base). Live-cell CLSM imaging was performed with a Zeiss LSM 510 META nonlinear optics scan head attached to an inverted Axiovert 200 M SP equipped with a 63  $\times$  1.4 numerical aperture Plan Apochromat oil immersion objective (Carl Zeiss). For long-term time-lapse imaging, culture dishes were wrapped with an optically clear foil cover (Carl Zeiss) to avoid evaporation and mounted onto the microscope stage incubator (37°C, 5% CO<sub>2</sub>, 90% relative humidity). Bright-field DIC (differential interference contrast) images and fluorescent sequences were taken every 30 min for 72 hours. BODIPY FL was excited at 488 nm with an Ar laser and fluorescence was detected at 500 to 530 nm, whereas BODIPY TR was excited at 543 nm with a He-Ne laser, and fluorescence was detected at 565 to 615 nm. Laser power was kept low at 0.36 mW for 488 nm and

at 0.018 mW for 543 nm so that photobleaching was negligible. To determine whether DACHPt/m was taken up by endocytosis, we treated HT29 cells with F-DACHPt/m at 37°C or 4°C for 6 hours and then observed by CLSM. For the colocalization studies, we used micelles prepared from BODIPY FL-PEG-P(Glu) that only emit fluorescence from the shell. HT29 cells, which had been preincubated with CellLight Early Endosome-RFP to express an early endosome marker, Rab5a-RFP, were treated with BODIPY FL-conjugated DACHPt/m, and images were taken at indicated time points after staining with LysoTracker Blue. Rab5a-RFP was excited at 543 nm with a He-Ne laser, and fluorescence was detected at 565 to 615 nm. LysoTracker Blue was excited in multiphoton mode at 710 nm with a Mai Tai tunable broadband laser (Spectra-Physics), and fluorescence was detected at 390 to 465 nm. Colocalization was quantified as follows:

$$\text{amount of colocalization (\%)} = \text{BODIPY FL pixels}_{\text{colocalization}} / \text{BODIPY FL pixels}_{\text{total}} \times 100$$

where BODIPY FL pixels<sub>colocalization</sub> represents the number of BODIPY FL pixels colocalizing with Rab5a-RFP or LysoTracker pixels in the cytoplasm, and BODIPY FL pixels<sub>total</sub> represents the number of all BODIPY FL pixels in the cytoplasm. The timing and location of the micelle dissociation and concomitant drug release were studied by evaluating the colocalization of BODIPY FL and BODIPY TR signals from F-DACHPt/m with the late endosomes/lysosomes (Fig. 3, E and F). Cells were treated with F-DACHPt/m, and images were taken at indicated time points after staining with LysoTracker Blue. Colocalization was quantified as follows:

$$\text{amount of colocalization (\%)} = \text{BODIPY FL or BODIPY TR pixels}_{\text{colocalization}} / \text{BODIPY FL or BODIPY TR pixels}_{\text{total}} \times 100$$

where BODIPY FL or BODIPY TR pixels<sub>colocalization</sub> represents the number of BODIPY FL or BODIPY TR pixels colocalizing with LysoTracker pixels in the cytoplasm, and BODIPY FL or BODIPY TR pixels<sub>total</sub> represents the number of all BODIPY FL or BODIPY TR pixels in the cytoplasm.

**Determination of subcellular Pt accumulation and amount of Pt-DNA adducts.** HT29 cells ( $6 \times 10^6$ ) were seeded in 100-mm tissue culture dishes. After 24 hours, cells were treated with 10  $\mu$ M oxaliplatin or DACHPt/m on a platinum base. After 6, 8, 12, and 24 hours of drug exposure, the medium was removed and the cells were washed three times with PBS, scraped, and harvested. Samples were freeze-dried overnight, dissolved in heated nitric acid, and evaporated to dryness. The samples were redissolved in water and the Pt content was determined by ICP-MS. For the quantification of Pt-DNA adducts, DNA was extracted with a DNA purification kit (Promega) according to the manufacturer's protocol. The amount and purity of DNA were determined by measuring absorption at 260 and 280 nm with NanoDrop (ND3300). The DNA was dissolved in nitric acid, dried, and redissolved in water. The Pt content was determined by ICP-MS, and the DNA platination levels were expressed as micrograms of Pt per milligram of DNA.

**In vitro cytotoxicity study against human cancer cells.** The in vitro cytotoxicity of oxaliplatin and DACHPt/m was examined against a panel of 37 human cancer cells as described (34, 35). Cancer cells were plated into flat-bottomed 96-well plates at  $5 \times 10^3$  per well. Cells were treated by continuous exposure to oxaliplatin or DACHPt/m in a final volume of 100  $\mu$ l. Plates were incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, and cell viability was determined



by MTT assay. To determine the relationship between cellular sensitivity to oxaliplatin or DACHPt/m and the expression of genes involved in the sensitivity or resistance of cells to platinum compounds, we assessed the gene expression profile of 21 human cancer cell lines for 26 genes selected on the basis of previous studies on cisplatin- or oxaliplatin-resistant cells (36). Expression of these individual genes was determined by searching the National Cancer Institute database. The coefficient of correlation between the  $GI_{50}$ s of free oxaliplatin and DACHPt/m as well as the level of gene expression was calculated for each gene.

**Quantitative real-time RT-PCR.** The expression of the metallothionein (MT1Q) [human MT1B (same as MT1Q), NM\_005947, 4MQ-012725-01-0002] and methionine synthase (MTR) (human MTR, NM\_000254, 4LQ-009896-00-0002) was confirmed by quantitative real-time RT-PCR. After 24 hours of treatment, cells were washed with PBS and harvested. Total RNA was prepared with TRIzol (Invitrogen), and complementary DNA (cDNA) was reverse-transcribed with a QuantiTect reverse transcription kit (Qiagen). PCR primer sequences were as follows: MT1Q, 5'-GAACTCCAGGCTTGTCTGG-3' (forward) and 5'-CATTTGCACTCTTTGCACTTG-3' (reverse); MTR, 5'-ACCCAACCTCCAGGGAGACT-3' (forward) and 5'-GGCACATGATCTTGGACTT-3' (reverse); actin, 5'-AGATGTGGATCAGCAAGCAG-3' (forward) and 5'-GCGCAAGTTAGGTTTTGTCA-3' (reverse); and 18S, 5'-CGGCGACGACCCATTCGAAC-3' (forward) and 5'-GAATCGAACCCTGATTCCCGTC-3' (reverse). cDNA from HT29 cells was amplified with specific primers with a SYBR Green Core Reagent Kit (Qiagen) and a real-time PCR instrument (Applied Biosystems). Expression of each gene was standardized with endogenous actin or 18S as a control, and its relative levels in HT29 or HT29/ox cells were quantified by calculating  $2^{-\Delta\Delta C_T}$ , where  $\Delta\Delta C_T$  is the difference in  $C_T$  (cycle number at which the amount of amplified target reaches a fixed threshold) between target and reference.

**MT1Q and MTR gene knockdown.** The siRNAs against MT1Q and MTR and the control siRNA were purchased from Thermo Fisher Scientific Inc. The siRNA target sequences against MT1Q are the following: siMT1Q, GCAAAGGCUCAUCAGAGAA. The siRNA sequences against MTR are the following: siMTR, CUGAGAAGCU-CUUACGUUA. The siRNAs were transfected into the cell with Lipofectamine RNAiMAX (Invitrogen) according to the instructions of the manufacturer. Briefly, HT29/ox cells ( $4 \times 10^5$ ) were seeded in six-well plates. Twenty-four hours later, the mixture of siMT1Q (50 nM) and siMTR (50 nM) was transfected into the cells with Lipofectamine RNAiMAX reagent. Knockdown of MT1Q and MTR was separately confirmed by real-time PCR (fig. S6, A and B, respectively). To investigate the role of MT1Q and MTR in the oxaliplatin resistance in HT29/ox cells, we seeded HT29/ox cells ( $5 \times 10^3$ ) in 96-well plates, and 24 hours later, the mixture of siMT1Q (50 nM) and siMTR (50 nM) or the control siRNA was transfected into the cells with Lipofectamine RNAiMAX reagent. Twenty-four hours after transfection, the transfected HT29/ox cells were treated by continuous exposure to oxaliplatin in a final volume of 100  $\mu$ l. Plates were further incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, and their cytotoxicity was determined by MTT assay.

**Western blotting.** HT29 or HT29/ox cells ( $5 \times 10^6$ ) were seeded in 100-mm<sup>2</sup> plates and washed with PBS (100  $\mu$ l). Cell extracts were resolved in TNE buffer [1% NP-40, 150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, aprotinin (10  $\mu$ g/ml), 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF]. The cell suspension was centrifuged for 20 min at 15,000g. Sampling buffer (4 $\times$ ) was added to the aliquots, followed by incubation

for 5 min at 100°C. Transfer to a polyvinylidene difluoride membrane (Invitrogen) was performed by electrophoresis for 90 min at 125 V. Membranes were blocked with 6% nonfat milk or tris-buffered saline (TBS) with 0.1% Tween 20 (MT1Q) for 1 hour. They were then probed at room temperature with the following antibodies: anti-metallothionein (ab12228, 1:1000, Abcam), anti-methionine synthase (ab66039, 1:2000, Abcam), and anti- $\beta$ -actin (#4967, 1:1000, Cell Signaling). Membranes were washed three times with washing buffer (TBS with 0.1% Tween 20) and then probed with the secondary anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) (W401B, 1:10,000, Promega) or anti-mouse IgG HRP (W402B, 1:10,000, Promega) conjugate for 1 hour. The secondary antibody was washed three times with washing buffer and then evenly coated with enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare) for 30 s. The membrane was immediately exposed to Fuji Medical X-ray film (Fujifilm) at room temperature for various periods in a film cassette. Protein levels were standardized with the signal from the  $\beta$ -actin probe.

**In vivo antitumor activity studies.** BALB/c-nu/nu mice (female,  $n = 4$ ) were inoculated subcutaneously with HT29 or HT29/ox cells ( $1 \times 10^7$ /ml). Tumors were allowed to grow for 1 week (tumor size at this point was about 40 mm<sup>3</sup>). Mice were then treated intravenously three times at 2-day intervals with oxaliplatin (8 mg/kg) or DACHPt/m (4 mg/kg) on a platinum base. Antitumor activity was evaluated in terms of tumor size ( $V$ ) with the following equation:

$$V = a \times b^2 / 2$$

Here,  $a$  and  $b$  are the major and minor axes, respectively, of the tumor as measured by a caliper.

**Intravital observation of the in vivo behavior of F-DACHPt/m.** Intravital observation of F-DACHPt/m was performed as described (49). Female BALB/c mice (6 to 8 weeks old) were inoculated subcutaneously with HT29 cells ( $1 \times 10^7$ /ml). After 5 days, when the tumor volumes reached 70 mm<sup>3</sup>, F-DACHPt/m (10 mg/kg) was administered intravenously. At 2, 4, 12, and 24 hours after treatment, mice were anesthetized with 2.5% isoflurane (Abbott Japan) with a Univentor 400 Anesthesia Unit (Univentor). An arc-shaped incision was made around the subcutaneous tumor, and the skin flap was elevated without injuring the feeding vessels. The mouse was placed directly onto a thermoplate (Tokai Hit), and the skin flap was everted and stretched with several bent 30-gauge needles. The plasma membrane stain, CellMask Deep Red, was directly applied to the subcutaneous tumor, and a coverslip (Muto Pure Chemicals) was attached with just enough pressure to flatten the tumor surface. All in vivo images were acquired with a Nikon A1R CLSM attached to an upright Eclipse FN1 (Nikon). The A1R incorporates a conventional galvano scanner and a high-speed resonant scanner. The latter allows an acquisition speed of 30 frames per second while maintaining a relatively high resolution of 512  $\times$  512 scanned points. BODIPY FL, BODIPY TR, and CellMask were excited with three lasers (488-nm Ar, 560-nm He-Ne, and 640-nm He-Ne lasers), and the fluorescent signals were detected. Laser powers were kept at 19.5 mW for 488-nm Ar, 7.5 mW for 561-nm He-Ne, and 1 mW for 640-nm He-Ne.

**Statistical analysis.** Data are presented as means  $\pm$  SEM. The significant differences between the groups were analyzed by a Student's  $t$  test, and a  $P$  value of <0.05 was considered significant.



## SUPPLEMENTARY MATERIAL

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Fig. S1. Synthetic scheme of BODIPY FL-PEG-b-P(Glu)-BODIPY TR.

Fig. S2. Size distribution of DACHPT/m and F-DACHPT/m as determined by dynamic light scattering.

Fig. S3. Fluorescent images of HT29 cells after 6-hour incubation with F-DACHPT/m at 37°C and 4°C.

Fig. S4. In vitro CLSM observation of dissociation of F-DACHPT/m in the late endosomes/lysosomes.

Fig. S5. In vitro cytotoxicity of oxaliplatin and DACHPT/m against a human cancer cell panel.

Fig. S6. Knockdown of MT1Q and MTR restores the sensitivity of HT29/ox to oxaliplatin.

Fig. S7. Schematic illustration of experimental settings of in vivo CLSM.

Table S1. Coefficient of correlation between the  $G_{150S}$  of free oxaliplatin or DACHPT/m and the expression levels of genes involved in the sensitivity or resistance of cells to platinum compounds.

Video S1. In vitro live imaging.

Video S2. In vivo live imaging (immediately after injection).

Video S3. In vivo live imaging (12 hours after injection).

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## Polyplex micelles prepared from $\omega$ -cholesteryl PEG-polycation block copolymers for systemic gene delivery

Makoto Oba<sup>a</sup>, Kanjiro Miyata<sup>b</sup>, Kensuke Osada<sup>c</sup>, R. James Christie<sup>c</sup>, Mai Sanjoh<sup>d</sup>, Weidong Li<sup>c</sup>, Shigeto Fukushima<sup>c</sup>, Takehiko Ishii<sup>d</sup>, Mitsunobu R. Kano<sup>b,e</sup>, Nobuhiro Nishiyama<sup>b</sup>, Hiroyuki Koyama<sup>a</sup>, Kazunori Kataoka<sup>b,c,d,\*</sup>

<sup>a</sup> Department of Clinical Vascular Regeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

<sup>b</sup> Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

<sup>c</sup> Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

<sup>d</sup> Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

<sup>e</sup> Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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### ABSTRACT

Polyplex micelles formed with plasmid DNA (pDNA) and poly(ethylene glycol) (PEG)-*block*-poly(*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide) [PAsp(DET)] exhibit effective endosomal escaping properties based on di-protonation of diamine side chains with decreasing pH, which improves their transfection efficiency and thus are promising candidates for local *in vivo* gene transfer. Here, PEG-PAsp(DET) polyplex micelles were further improved as *in vivo* systemic vectors by introduction of cholesterol (Chole) into the  $\omega$ -terminus of PEG-PAsp(DET) to obtain PEG-PAsp(DET)-Chole. Introduction of the cholesterol resulted in enhanced association of block copolymers with pDNA, which led to increased stability in proteinous medium and also in the blood stream after systemic injection compared to PEG-PAsp(DET) micelles. The synergistic effect between enhanced polymer association with pDNA and increased micelle stability of PEG-PAsp(DET)-Chole polyplex micelles led to high *in vitro* gene transfer even at relatively low concentrations, due to efficient cellular uptake and effective endosomal escape of block copolymers and pDNA. Finally, PEG-PAsp(DET)-Chole micelles achieved significant suppression of tumor growth following intravenous injection into mice bearing a subcutaneous pancreatic tumor using therapeutic pDNA encoding an anti-angiogenic protein. These results suggest that PEG-PAsp(DET)-Chole micelles can be effective systemic gene vectors for treatment of solid tumors.

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### 1. Introduction

As expectations for gene therapy increase, so have efforts to develop non-viral vectors with high transfection ability and low toxicity [1,2]. Polyplexes, which are composed of polycations and plasmid DNA (pDNA), are expected as alternatives to viral vectors due to the fine-tuned properties for specific applications by altering the structure of the polycation used for polyplex formation [3–5]. Polyplex micelles formed with poly(ethylene glycol) (PEG)-*block*-polycation block copolymers and pDNA are particularly promising candidates [6–8], due to their excellent

characteristics as *in vivo* gene vectors [9,10]. The biocompatible PEG shell layer surrounding the polyplex core contributes to high colloidal stability, allows micelles to maintain their initial size of approximately 100 nm, and reduces non-specific interactions with blood components, which are all desirable properties for systemic administration.

Recently, we reported that polyplex micelles prepared with pDNA and PEG-*block*-poly(*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide) [PEG-PAsp(DET)] [11] achieved successful *in vitro* transfection of primary cells due to effective endosomal escape of pDNA contained in the micelle core. The PAsp(DET), polycationic segment of the block copolymer is characterized by a distinctive two-step protonation behavior in response to pH and possessed endosomal membrane-selective destabilizing capacity upon acidification [12]. Furthermore, PEG-PAsp(DET) polyplex micelles have shown successful *in vivo* gene transfer by local administration in

\* Corresponding author. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan. Tel.: +81 3 5841 7138; fax: +81 3 5841 7139.

E-mail address: [kataoka@bmv.t.u-tokyo.ac.jp](mailto:kataoka@bmv.t.u-tokyo.ac.jp) (K. Kataoka).



several animal models including: a clamped rabbit carotid artery with neointima without vessel occlusion by thrombus [13], a mouse skull by regulated release from a calcium phosphate cement scaffold to induce bone regeneration through the osteogenic factors [14], and a rat lung pulmonary arterial hypertension model via intratracheal administration [15]. In these cases, however, excess block copolymers relative to pDNA (high N/P ratio) were required to achieve high transfection efficiency, suggesting the existence of free polymer. If free polymer plays a significant role for gene transfer with polyplex micelles prepared from PEG-PAsp (DET) and pDNA, the transfection efficiency under highly diluted conditions, such as systemic application, could be drastically decreased.

The aim of this study was to further develop PEG-PAsp (DET) polyplex micelles towards *in vivo* systemic pDNA delivery vectors. In order to enhance the association of PEG-PAsp (DET) polymers with pDNA and thus increase the efficiency of cellular internalization of polymer necessary for improved endosome escaping, we utilized both electrostatic interaction between polycations and pDNA and hydrophobic interaction by cholesterol to form micelles with improved stability. Specifically, cholesterol was introduced onto the  $\omega$ -terminus of the PAsp (DET) segment in PEG-PAsp (DET) block copolymer. Cholesterol introduction significantly increased the number of block copolymers associating with a pDNA. *In vitro* experiments were done to demonstrate improved transfection efficiency of PEG-PAsp (DET)-Chole polyplex micelles at low N/P ratios and under the diluted conditions compared to the control micelles formed without cholesterol modified block copolymer. Then, the enhanced stability by the cholesterol introduction in blood was shown, thus allowing successful treatment of a subcutaneous tumor by systemic administration of micelles prepared with PEG-PAsp (DET)-Chole and therapeutic pDNA encoding for an anti-angiogenic protein.

## 2. Materials and methods

### 2.1. Materials

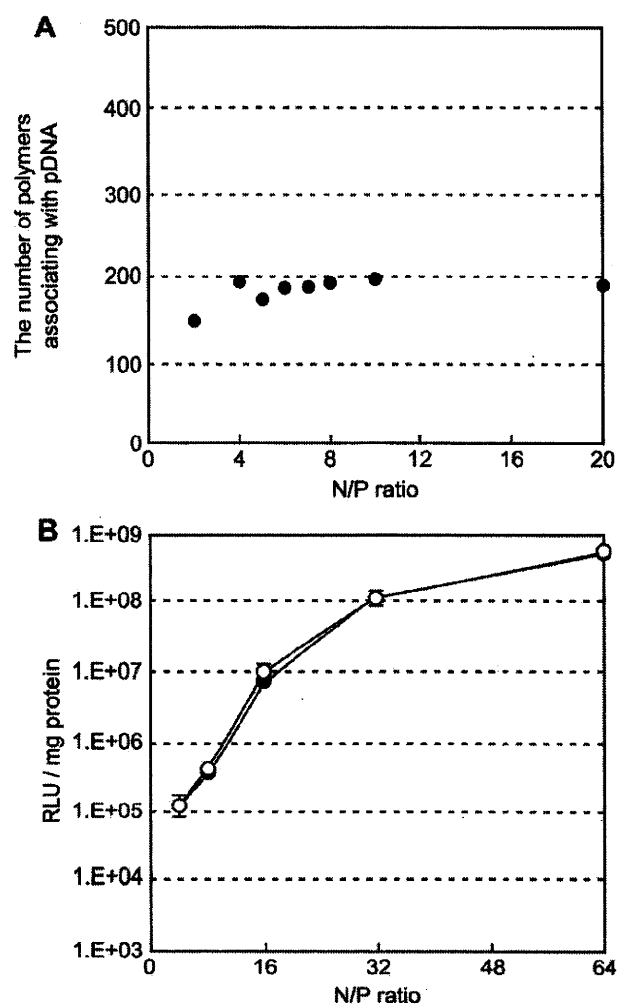
Dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), *N,N*-dimethylformamide (DMF), triethylamine (TEA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Wako Pure Chem. Co. Ltd. (Osaka, Japan). Cholesterol chloroformate was purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). Diethylenetriamine (DET) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and distilled over  $\text{CaH}_2$  under reduced pressure. DMF was dehydrated using activated molecular sieves (4A) and distilled under reduced pressure. PEG-PAsp (DET) block copolymer (PEG: 12,000 g/mol, polymerization degree of PAsp (DET) segment: 68) was synthesized as previously reported [11]. Alexa Fluor 680 (Alexa680) succinimidyl ester was a product of Invitrogen (Carlsbad, CA). A Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). The Luciferase assay kit was a product of Promega (Madison, WI). Plasmid pCacc+Luc coding for firefly luciferase under the control of the CAG promoter was provided by RIKEN Gene Bank (Tsukuba, Japan), amplified in competent DH5a *Escherichia coli*, and then purified using a HiSpeed Plasmid MaxiKit purchased from Qjagen Sciences (Germantown, MD). pDNA encoding for a soluble form of VEGF receptor-1 (sFlt-1) was prepared as previously reported [16].

### 2.2. Animals

Balb/c mice (female, 8 weeks old) and balb/c nude mice (female, 5 weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animals were treated in accordance with the guideline of the Animal Ethics Committee of The University of Tokyo.

### 2.3. Synthesis of $\alpha$ -methoxy- $\omega$ -cholesteryl carbamate poly(ethylene glycol)-block-poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide] [PEG-PAsp (DET)]

PEG-*b*-poly( $\beta$ -benzyl L-aspartate) (PEG-PBLA) (PEG: 12,000 g/mol, polymerization degree of PBLA segment: 68) was prepared as previously reported [11]. PEG-PBLA (210 mg) was dissolved in  $\text{CH}_2\text{Cl}_2$  (4 mL), followed by the addition of 11 v/v% TEA/ $\text{CH}_2\text{Cl}_2$  (200  $\mu\text{L}$ ) and cholesterol chloroformate (344 mg) in  $\text{CH}_2\text{Cl}_2$  (1 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The reactant polymer was isolated by precipitation into diethylether and lyophilized from



**Fig. 1.** (A) The number of PEG-PAsp (DET)-Alexa680 block copolymers associating with a pDNA. (B) Transfection efficiency of PEG-PAsp (DET) polyplex micelles. Closed circles: addition of polyplex micelle solutions prepared at various N/P ratios into the cell culture medium. Open circles: addition of polyplex micelle solutions prepared at a constant N/P value of 4, with separate addition of PEG-PAsp (DET) free polymer solution into the cell culture medium to obtain the same N/P values shown in the experiment with open circles.

benzene, to obtain PEG-PBLA-Chole (197 mg). PEG-PBLA-Chole (100 mg) was dissolved in DMF (4 mL), followed by reaction with DET (50 equiv. to benzyl group of PBLA segment, 1.43 g) at 40 °C. After 1 h, the reactant mixture was slowly added to a 20% acetic acid (13.8 mL) solution, and subsequently dialyzed against 0.01 N HCl and finally distilled water. The final solution was lyophilized to obtain PEG-PAsp (DET)-Chole (98 mg).

The  $^1\text{H}$  NMR spectrum of each polymer was obtained with an EX300 spectrometer (JEOL, Tokyo, Japan). Chemical shifts were reported in ppm relative to the residual protonated solvent peak.

### 2.4. Introduction of Alexa680 into block copolymers

Alexa680 was introduced into the side chains of both PEG-PAsp (DET) and PEG-PAsp (DET)-Chole polymers. The typical synthetic procedure of PEG-PAsp (DET)-Alexa680 is described as follows: Alexa680 succinimidyl ester (1 mg) in 100  $\mu\text{L}$  of DMF was added to PEG-PAsp (DET) (30 mg) in 1.5 mL of 0.1 N  $\text{NaHCO}_3$  (pH 9.3) and stirred at 4 °C for 1 h. The reacted polymer was purified by dialysis against distilled water and lyophilized to obtain PEG-PAsp (DET)-Alexa680 (22 mg). Introduction of Alexa680 into PEG-PAsp (DET)-Chole was completed similarly using Alexa680 succinimidyl ester (1 mg) and PEG-PAsp (DET)-Chole (30 mg) to obtain PEG-PAsp (DET)-Alexa680-Chole (23 mg). The number of Alexa680 introduced into the strand of

PEG-PAsp(DET) and PEG-PAsp(DET)-Chole was estimated to be 0.6 and 0.7, respectively, using a spectrofluorometer (ND-3300, NanoDrop, Wilmington, DE).

### 2.5. Preparation of polyplex micelles

Each block copolymer and pDNA was dissolved separately in 10 mM Tris-HCl buffer (pH 7.4) or 10 mM Hepes buffer (pH 7.3). Polymer solutions of various concentrations were added to a two-fold excess volume of pDNA solution to form polyplex micelles with different compositions. The final pDNA concentration was adjusted to 33.3 µg/mL for *in vitro* experiments and 100 µg/mL for *in vivo* experiments and micelle solutions were stored at 4 °C overnight prior to use. The N/P ratio was defined as the residual molar ratio of the amino groups of PAsp(DET) units to the phosphate groups of pDNA. The N<sup>+</sup>/P ratio was defined as the molar ratio of protonated amino groups of PAsp(DET) units to the phosphate groups of pDNA.

### 2.6. Ultracentrifugation

In order to evaluate the amount of free polymer in the polyplex micelle solution, ultracentrifugation analysis of polyplex micelles, composed of Alexa680-labeled block copolymers and pDNA, was carried out by a Beckman XL-I ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) using an An-60 Ti 4-hole rotor and standard double-sectors (Epon centerpieces) equipped with quartz windows. The concentration of polyplex micelle solutions prepared at various N/P ratios were adjusted to 33.3 µg pDNA/mL in 10 mM Hepes buffer (pH 7.3). Sedimentation of polyplex micelles was confirmed by UV absorbance measurement at 260 nm, while that of Alexa680-labeled polymers was confirmed by visible absorbance at 680 nm. Polyplex micelle solutions prepared at various N/P ratios were ultracentrifuged at 49,000 g for 1 h in order to sediment only the polyplex micelles. The concentration of free polymers contained in the supernatant was calculated using a calibration curve prepared from Alexa680-labeled polymer solutions. The number of block copolymers associating with pDNA was then estimated from the calculated concentration of free polymers.

### 2.7. Dynamic light scattering (DLS) measurement

The size of the polyplex micelles was evaluated by DLS using Nano ZS (ZEN3600, Malvern Instruments, Ltd., UK). A He-Ne ion laser (633 nm) was used as the incident beam. The concentration of polyplex micelle solutions prepared at various N/P ratios were adjusted to 33.3 µg pDNA/mL in 10 mM Tris-HCl buffer (pH 7.4). Light scattering data was obtained at a detection angle of 173° and a temperature of 37 °C and was subsequently analyzed by the cumulant method to obtain the hydrodynamic diameters and polydispersity indices (PDI) ( $\mu\text{T}^2$ ) of the micelles.

### 2.8. Stability of polyplex micelles against bovine serum albumin (BSA)

Polyplex micelle solution (33.3 µg pDNA/mL) prepared at N/P ratio = 2 was adjusted to 10 µg pDNA/mL in 10 mM Tris-HCl buffer (pH 7.4) with 150 mM NaCl and 0.1 mg/mL BSA. DLS measurements of the polyplex micelle solution were then carried out every 30 min at 37 °C using Nano ZS.

### 2.9. *In vitro* transfection

Huh-7 and HeLa cells were separately seeded onto 24-well culture plates (10,000 cells/well) and incubated overnight in 500 µL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). The medium was exchanged and the pDNA micelle solutions (33.3 µg pDNA/mL) prepared at various N/P ratios were applied to each well at the desired concentrations. The amount of micelle solution added was as follows: 30 µL for 2 µg pDNA/mL, 10 µL for 2/3 µg pDNA/mL, or 3.3 µL for 2/9 µg pDNA/mL. For experiments shown in Fig. 1B (open circles), polymer solution was added to cultured Huh-7 cells simultaneously with PEG-PAsp(DET) micelle (N/P = 4), in order to elucidate the effect of free polymer on transfection efficiency. After 24-h incubation, the medium was replaced with 500 µL of fresh medium, followed by 24-h further incubation. Luciferase gene expression was then evaluated based on photoluminescence intensity using the Luciferase assay kit and a Luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany). The amount of protein in each well was concomitantly determined using a Micro BCA protein assay kit. One nanogram of luciferase corresponded to  $9.1 \times 10^7$  RLU in our experiments according to a standard curve calibrated with recombinant luciferase (Quantilum, Promega).

### 2.10. Cytotoxicity of polyplex micelles

Huh-7 and HeLa cells were separately seeded onto 96-well culture plates (2500 cells/well) and incubated overnight in 100 µL of DMEM containing 10% FBS. After the medium was replaced with fresh medium, 7.5 µL of polyplex micelle solution (33.3 µg pDNA/mL) prepared at various N/P ratios was applied to each well (0.25 µg pDNA/well). After 24-h incubation, the medium was replaced with 100 µL of fresh medium, followed by 24-h incubation. Cell viability was evaluated using the MTT assay. Briefly, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well,

followed by 3-h incubation at 37 °C. Then, 100 µL of sodium dodecyl sulfate (SDS) solution (20 w/v% in PBS) was added to dissolve the formed formazan. After 15-min incubation at room temperature, the absorbance from each well was measured at 570 nm. Results were expressed as percentage relative to non-treated controls.

### 2.11. Cellular uptake of polymers and pDNA

Polyplex micelles were prepared with non-labeled polymer and Cy3-labeled pDNA (Fig. 8A), or with Alexa680-labeled polymer and non-labeled pDNA (Fig. 8B) for these experiments. pDNA was labeled with Cy3 using a Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI) according to the manufacturer's protocol. Huh-7 cells were seeded on 24-well culture plates (10,000 cells/well) and incubated overnight in 500 µL of DMEM containing 10% FBS. The medium was replaced with fresh medium and then 30 µL of polyplex micelle solution (33.3 µg pDNA/mL) was applied to each well. After 24-h incubation, the medium was removed and the cells were washed 3 times with PBS and detached with trypsin. Harvested cells were re-suspended in PBS and analyzed using the flow cytometer (BD LSR II, BD, Franklin Lakes, NJ).

### 2.12. Confocal laser scanning microscope (CLSM) observation

pDNA was labeled with Cy5 according to manufacturer's protocol using a Label IT Nucleic Acid Labeling Kit. Huh-7 cells (30,000) were seeded on a 35-mm glass base dish (Iwaki, Tokyo, Japan) and incubated overnight in 1.5 mL of DMEM containing 10% FBS. After the medium was exchanged, 90 µL of polyplex micelle solution (33.3 µg pDNA/mL) was applied to each sample. After 24-h incubation, the medium was removed and the cells were washed three times with PBS. The intracellular distribution of the polyplex micelles was observed by CLSM after staining acidic late endosomes/lysosomes with Lyso Tracker Green (Molecular Probes, Eugene, OR), and nuclei with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan). CLSM observation was performed using an LSM 510 (Carl Zeiss, Oberkochen, Germany) equipped with a 63× objective (C-Apochromat, Carl Zeiss) at the excitation wavelengths of 488 nm (Ar laser) for Lyso Tracker Green, 633 nm (He-Ne laser) for Cy5, and 710 nm (MaiTai laser, 2 photon excitation; Spectra-Physics, Mountain View, CA) for Hoechst 33342. To evaluate the endosomal escaping behavior of polyplex micelles, the rate of colocalization of Cy5-labeled pDNA with Lyso Tracker Green was quantified [17]. Colocalization was quantified as follows:

$$\text{Amount of colocalization (\%)} = \frac{\text{Cy5 pixels}_{\text{colocalization}}}{\text{Cy5 pixels}_{\text{total}}} \times 100$$

where  $\text{Cy5 pixels}_{\text{colocalization}}$  represents the number of Cy5 pixels colocalizing with Lyso Tracker Green in the cell, and  $\text{Cy5 pixels}_{\text{total}}$  represents the number of all the Cy5 pixels in the cell.

### 2.13. Fluorescence correlation spectroscopy (FCS) measurement

PEG-PAsp(DET-Alexa680)-Chole free polymer, polyplex micelles composed of PEG-PAsp(DET-Alexa680)-Chole and pDNA, and polyplex micelles composed of PEG-PAsp(DET) and Cy5-labeled pDNA, were used in this experiment. Block copolymers and polyplex micelles (N/P = 8) were adjusted to a polymer concentration of 187 µg/mL (33.3 µg pDNA/mL) in 10 mM Hepes buffer (pH 7.3), and then diluted to a concentration of 18.7 µg/mL using 10 mM Hepes buffer (pH 7.3) (Fig. 10A) or 10 mM MES buffer (pH 5.5) (Fig. 10B), or 2.08 µg/mL using 10 mM Hepes buffer (pH 7.3) (Fig. 10A). FCS measurements were carried out using an LSM 510 (Carl Zeiss) equipped with a 40× objective (C-Apochromat, Carl Zeiss) and the ConfoCor3 module. Excitation of Alexa680 and Cy5 was achieved with a He-Ne laser (633 nm). The relative diffusion times of polymer and micelles (Fig. 10A) were determined using PEG-PAsp(DET-Alexa680)-Chole solution and its polyplex micelle solution, respectively. Furthermore, the percentage of polymers associating with pDNA in the micelle solution was calculated as follows:

Percentage of polymers associating with pDNA =

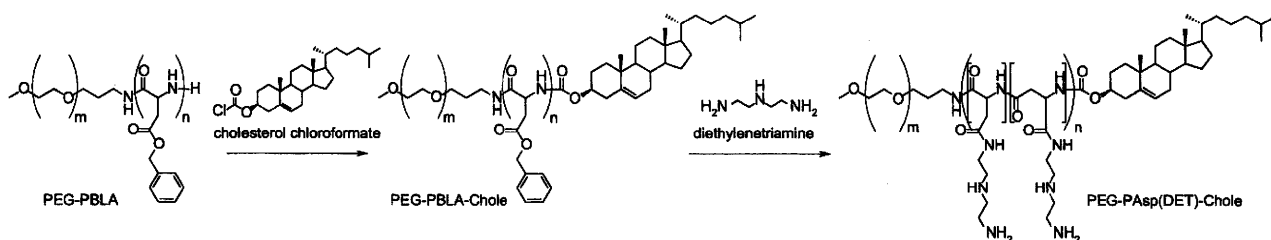
$$\frac{\left[ \text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole}} - \left( \frac{\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole/pDNA}}}{\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole}} - \text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole/pDNA}}} \right) \cdot \text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole}} \right]}{\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole}}} \times 100$$

where  $\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole}}$  represents the number of fluorescent particles (free polymers) in PEG-PAsp(DET-Alexa680)-Chole solution,  $\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole/pDNA}}$  represents the number of fluorescent species (free polymers and micelles) in PEG-PAsp(DET-Alexa680)/pDNA micelle solution, and  $\text{Nfp}_{\text{PEG-PAsp(DET-Alexa680)-Chole/Cy5-pDNA}}$  represents the number of fluorescent species (micelles) in PEG-PAsp(DET)-Chole/Cy5-pDNA micelle solution.

### 2.14. Stability of polyplex micelles in the blood stream

Polyplex micelles (N/P = 8) incorporating Cy5-labeled pDNA (100 µg pDNA/mL, 200 µL) in 10 mM Hepes buffer (pH 7.3) with 150 mM NaCl were intravenously injected into the tail vein of balb/c mice at a dose of 20 µg pDNA/mouse. Blood





Scheme 1. Synthesis of PEG-PAsp(DET)-Chole block copolymer.

was collected from the postcaval vein under anesthesia at appointed times after injection, followed by centrifugation to obtain the plasma. Two microliters of  $10\times$  trypsin-EDTA were added to  $20\ \mu\text{L}$  of the obtained plasma and incubated at  $37\ ^\circ\text{C}$  overnight. The fluorescence intensity of the sample solution was measured using a spectrofluorometer (ND-3300, NanoDrop). The injected dose (%) was calculated using a standard curve.

#### 2.15. Anti-tumor activity assay

Balb/c nude mice were inoculated subcutaneously with human pancreatic adenocarcinoma BxPC3 cells ( $5 \times 10^6$  cells in  $100\ \mu\text{L}$  of PBS). Tumors were allowed to grow for 2–3 weeks to reach proliferative phase (approximately  $45\ \text{mm}^3$ ). Subsequently, polyplex micelles loading pDNA encoding sFlt-1 ( $20\ \mu\text{g}$  pDNA/mouse) in  $10\ \text{mM}$  Hepes buffer (pH 7.3) with  $150\ \text{mM}$  NaCl were injected into the tail vein 3 times at 4-day intervals. Tumor volume ( $V$ ) was calculated as the following equation:

$$V = a \times b^2 / 2$$

where  $a$  and  $b$  denote the long and short diameters of the tumor tissue, respectively.

### 3. Results

#### 3.1. Ultracentrifugation analysis of PEG-PAsp(DET) polyplex micelles

The amount of free block copolymer in PEG-PAsp(DET) micelle solutions was quantified by ultracentrifugation analysis of polyplex micelles prepared with fluorescent-labeled block copolymer [PEG-PAsp(DET-Alexa680)]. Polyplex micelles were confirmed to precipitate after 1 h of ultracentrifugation at  $49,000\ \text{g}$ , whereas free block copolymers could not sediment (data not shown). Thus, the amount of free block copolymers was estimated by visible absorbance at  $680\ \text{nm}$ . Fig. 1A shows the number of block copolymers associating with a pDNA in the polyplex micelle solution prepared at each N/P ratio. Note that a stoichiometric charge ratio of PEG-

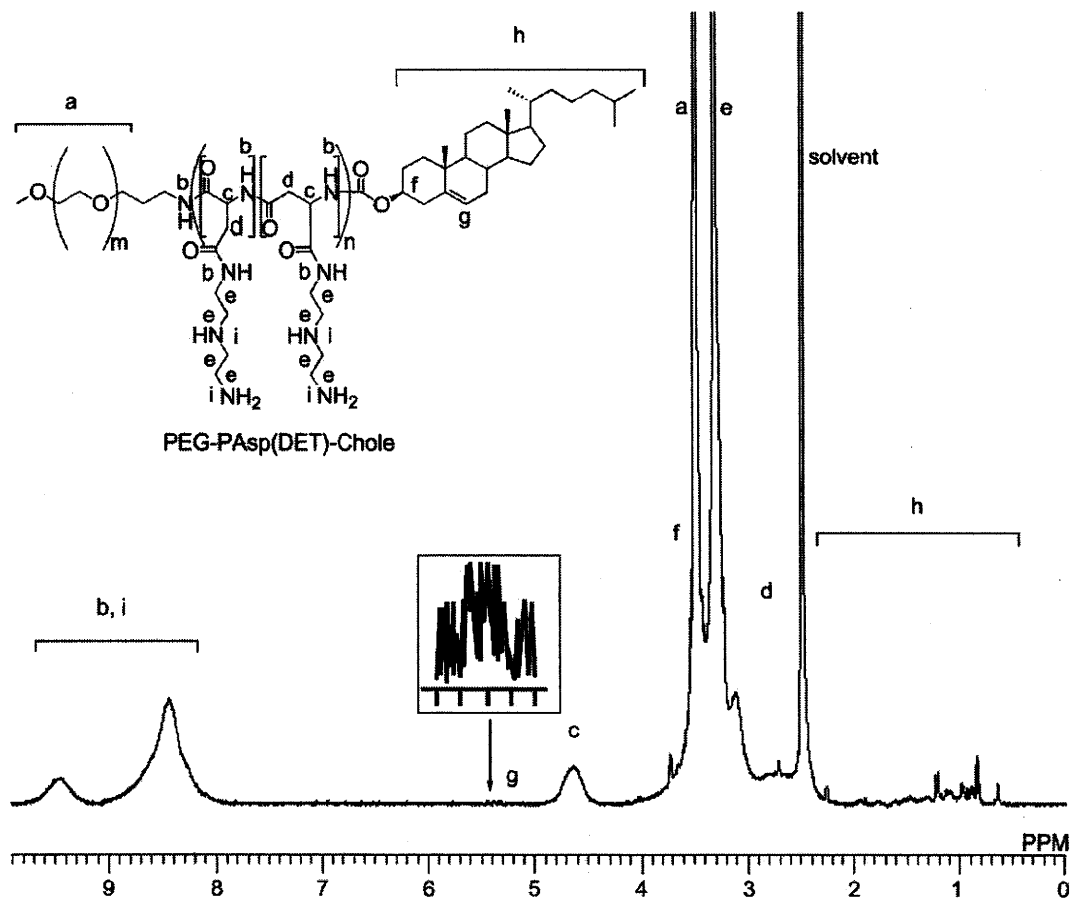


Fig. 2.  $^1\text{H}$  NMR spectrum of PEG-PAsp(DET)-Chole block copolymer in DMSO at  $25\ ^\circ\text{C}$ .

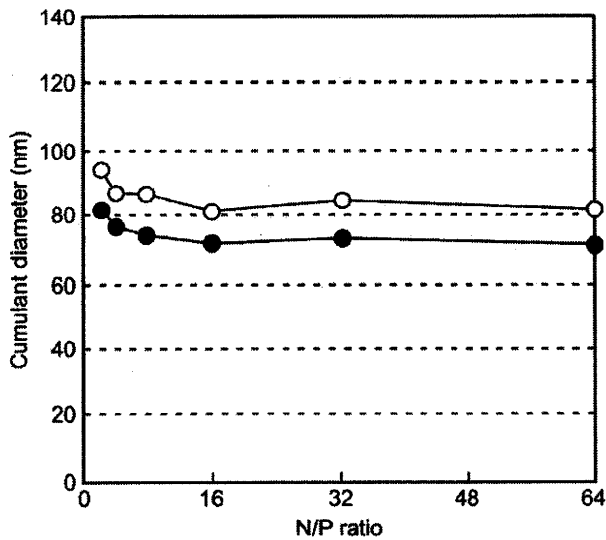


Fig. 3. Cumulant diameters of PEG-PAsp(DET) (closed circles) and PEG-PAsp(DET)-Chole (open circles) polyplex micelles.

PAsp(DET) micelle should be  $N/P = 2$  ( $N^+/P = 1$ ) because half of the amino groups in PAsp(DET) are protonated at pH 7.4 [11]. The number of block copolymers associating with a pDNA at  $N/P = 2$  and at  $N/P \geq 4$  ( $N^+/P \geq 2$ ) was estimated to be 150 and approximately 190, respectively by the results obtained by ultracentrifugation experiments. The theoretical number of block copolymers associating with a pDNA at stoichiometric charge ratio was calculated to be 189 based on complete neutralization of PAsp(DET) with a polymerization degree = 68 and pDNA with 6411 bp long. This result indicated that PEG-PAsp(DET)/pDNA polyplex micelles at  $N/P \geq 4$  ( $N^+/P \geq 2$ ) were formed at polymer/pDNA charge ratio = 1/1 (polymer/pDNA molar ratio = 190/1), and that the block copolymers present in solution in excess of that ratio should exist as free polymers at this concentration.

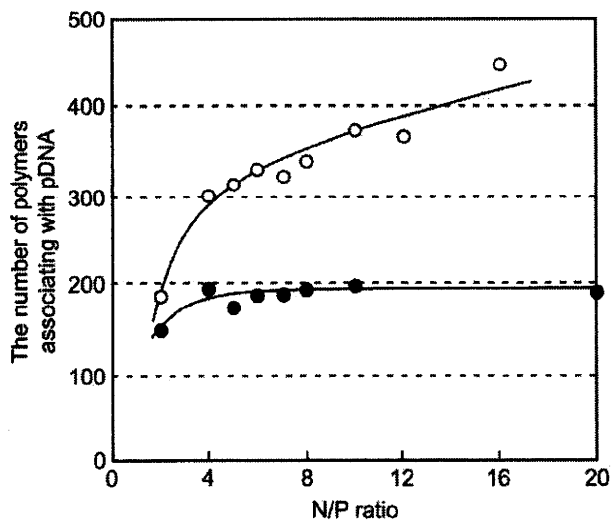


Fig. 4. The number of PEG-PAsp(DET-Alexa680) (closed circles) and PEG-PAsp(DET)-Alexa680-Chole (open circles) block copolymers associating with a pDNA.

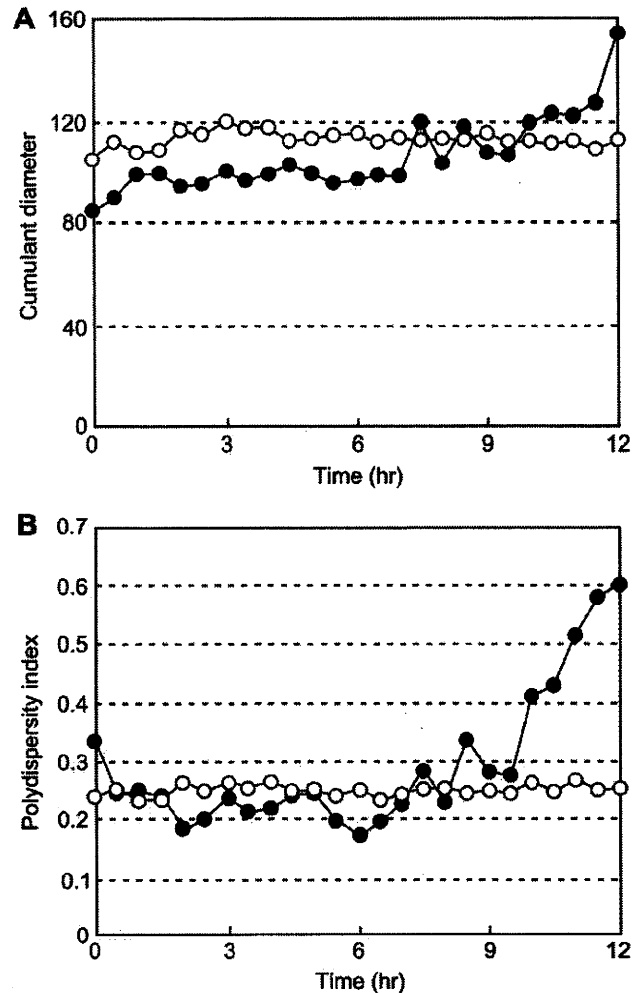


Fig. 5. Time-dependent change of cumulant diameters (A) and polydispersity index (PDI:  $\mu/I^2$ ) (B) of polyplex micelles ( $N/P = 2$ ) in the presence of bovine serum albumin (0.1 mg/mL). Closed circles: PEG-PAsp(DET) polyplex micelles. Open circles: PEG-PAsp(DET)-Chole polyplex micelles.

### 3.2. In vitro transfection efficiency of PEG-PAsp(DET) polyplex micelles

In order to confirm the effects of free polymer on *in vitro* transfection, we evaluated the transfection efficiency of PEG-PAsp(DET) micelles in Huh-7 cells under the following conditions: (i) polyplex micelles prepared at each  $N/P$  ratio; (ii) polyplex micelles prepared with PEG-PAsp(DET) at  $N/P = 4$  with addition of free polymer to achieve the same polymer concentration achieved at each  $N/P$  ratio used in condition (i). Under the conventional transfection conditions used in (i) above, the transfection efficiency increased with  $N/P$  ratio as previously reported [11]. Surprisingly, the profile of transfection efficiency under the condition (ii) showed the same behavior as that under the condition (i) with the addition of free polymer as shown in Fig. 1B. These results imply that increased transfection efficiency of PEG-PAsp(DET) micelles prepared at high  $N/P$  ratios might involve the effect of non-associating polymers with pDNA in the culture medium.



### 3.3. Synthesis of PEG-PAsp(DET)-Chole block copolymer

PEG-PAsp(DET)-Chole was synthesized using PEG-PBLA (PEG: 12,000 g/mol, polymerization degree of PBLA segment: 68) [11] as a starting material (Scheme 1). Addition of excessive cholesterol chloroformate and TEA to PEG-PBLA, which possessed a primary amino group in the terminus of PBLA, in  $\text{CH}_2\text{Cl}_2$  afforded PEG-PBLA-Chole with  $\omega$ -terminus of cholesterol. Conversion of PBLA segment into PAsp(DET) segment was achieved by aminolysis reaction [18]. Introduction rates of cholesterol and ethanediamine were determined by  $^1\text{H}$  NMR analysis (Fig. 2) based on the peak intensity ratio of the methylene protons of PEG ( $\text{OCH}_2\text{CH}_2$ ,  $\delta = 3.6$  ppm) to the vinyl proton of cholesterol ( $\text{C}=\text{CHCH}_2$ ,  $\delta = 5.4$  ppm), or to the methylene protons of DET ( $\text{NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NH}_2$ ,  $\delta = 3.1$ – $3.5$  ppm), respectively. Cholesterol introduction and the aminolysis reaction were confirmed to proceed quantitatively.

### 3.4. Formation of PEG-PAsp(DET)-Chole polyplex micelles

Agarose gel electrophoresis showed that free pDNA was not detected in both PEG-PAsp(DET) and PEG-PAsp(DET)-Chole micelles at  $\text{N}/\text{P} > 1.75$  ( $\text{N}^+/\text{P} > 0.875$ ) (data not shown), confirming that all of the pDNA were entrapped in polyplex micelles. Complex formation of pDNA with PEG-PAsp(DET) at pH 7.4 was previously reported to reach completion at  $\text{N}/\text{P} \approx 2$  ( $\text{N}^+/\text{P} \approx 1$ ) [11,19], which is consistent with the result obtained in this work. Complex formation was not hindered by cholesterol introduction into the

PAsp(DET) segment of the block copolymer as the  $\text{N}^+/\text{P}$  for complete pDNA entrapment did not change compared to the parent polymer. The size of the polyplex micelles was evaluated by DLS (Fig. 3). The cumulant diameters of polyplex micelles prepared with both block copolymers were found to be approximately 70–80 nm by DLS measurement.

### 3.5. Ultracentrifugation analysis of PEG-PAsp(DET)-Chole polyplex micelles

The amount of free polymer in PEG-PAsp(DET)-Chole micelle solution was also quantified by the same method as described for micelles prepared with PEG-PAsp(DET). As shown in Fig. 4, the number of polymers associating with a pDNA at  $\text{N}/\text{P} = 2$  ( $\text{N}^+/\text{P} = 1$ ) was approximately 190, which corresponds to the stoichiometric value. However, at  $\text{N}/\text{P} > 2$  ( $\text{N}^+/\text{P} > 1$ ), micelles formed with PEG-PAsp(DET)-Chole exhibited an increased number of polymers in the polyplex (in excess of the stoichiometric charge ratio), whereas micelles prepared with PEG-PAsp(DET) showed a constant number of associated polymers.

### 3.6. Stability of polyplex micelles against bovine serum albumin (BSA)

PEG-PAsp(DET) micelles have high transfection ability with low cytotoxicity at high  $\text{N}/\text{P}$  ratios, however, they are unstable and easily decondensed in the medium containing serum [20], probably

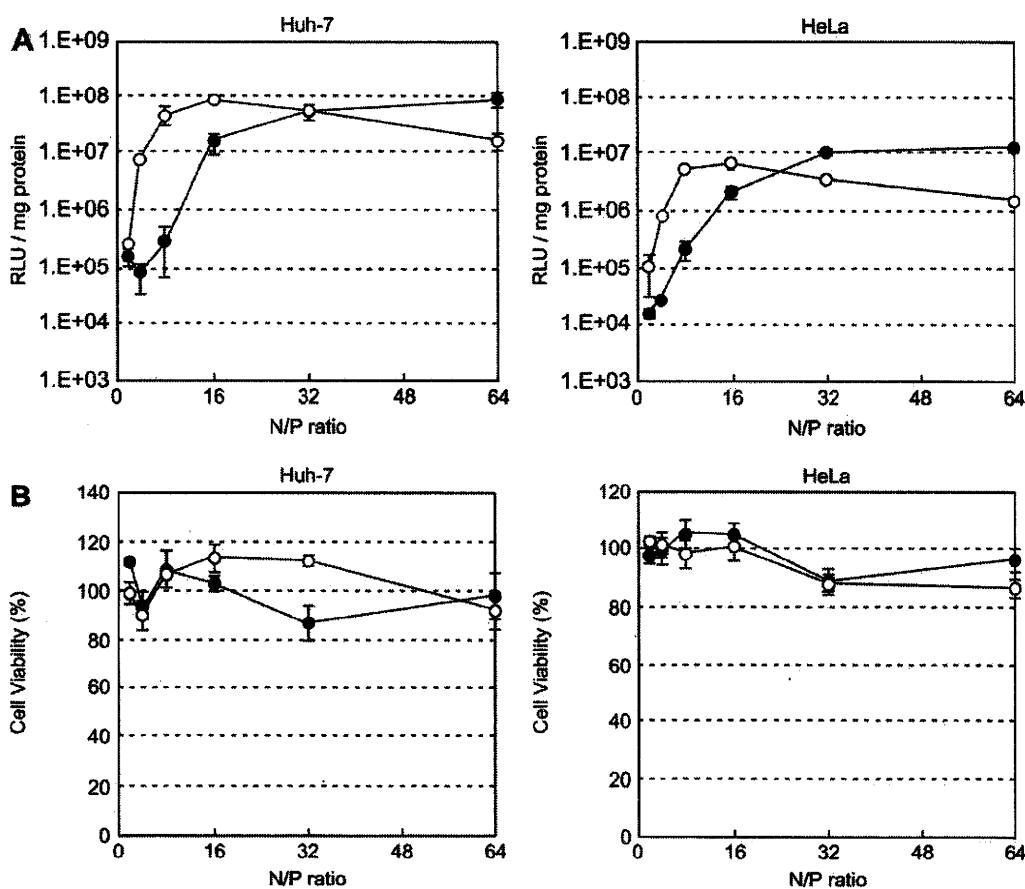


Fig. 6. Transfection efficiency (A) and cytotoxicity (B) of polyplex micelles prepared at various  $\text{N}/\text{P}$  ratios against Huh-7 cells and HeLa cells. Closed circles: PEG-PAsp(DET) polyplex micelles. Open circles: PEG-PAsp(DET)-Chole polyplex micelles. Error bars in the graph represent SEM,  $n = 4$ .

due to the weak association power of PAsp(DET) segment with pDNA. Therefore, the stability of polyplex micelles containing cholesterol was compared to micelles without cholesterol by monitoring the time-dependent change of cumulant diameter and PDI in the presence of BSA (Fig. 5). After adding BSA (0.1 mg/mL) to polyplex micelle solution (prepared at N/P = 2), the cumulant diameter and PDI were measured every 30 min. PEG-PAsp(DET)-Chole micelles maintained their initial size and PDI for 12 h (Fig. 5A). On the other hand, the size and PDI of micelles prepared with PEG-PAsp(DET) remained constant for only ~6 h and then gradually increased, and monodispersity was not maintained (Fig. 5B). These results imply that cholesterol introduction resulted in increased stability of polyplex micelles in BSA solution.

### 3.7. *In vitro* transfection efficiency and cytotoxicity of polyplex micelles

The *in vitro* transfection efficiency of PEG-PAsp(DET) and PEG-PAsp(DET)-Chole micelles prepared at various N/P ratios was evaluated against Huh-7 and HeLa cells by the luciferase assay (Fig. 6A). Transfection efficiencies of PEG-PAsp(DET) micelles increased with N/P ratio, and reached a maximum at N/P 32–64. However, PEG-PAsp(DET)-Chole micelles showed the highest transfection efficiencies at N/P = 8–16, with maximum transfection levels comparable to PEG-PAsp(DET). Cholesterol introduction clearly enhanced transfection efficiency at lower N/P ratios, especially at N/P 4 and 8. Fig. 6B shows the results of cytotoxicity analysis performed under the same condition as the luciferase assay. Increased cytotoxicity as a result of cholesterol introduction was not detected in Huh-7 and HeLa cells.

### 3.8. Effect of pDNA concentration on transfection efficiency

All transfection experiments in the preceding section were carried out at a constant pDNA concentration of 2  $\mu$ g pDNA/mL (1  $\mu$ g pDNA/well, 24-well plate) (Fig. 6). Here additional transfection experiments were repeated against Huh-7 cells at the diluted concentration of 2, 2/3, and 2/9  $\mu$ g pDNA/mL in order to confirm the influence of dilution (Fig. 7). The transfection efficiency of PEG-PAsp(DET) micelles markedly decreased with reduced pDNA concentration (Fig. 7A), while of the transfection ability of PEG-PAsp(DET)-Chole micelles was maintained without a severe decrease (Fig. 7B). PEG-PAsp(DET) micelles at N/P = 16 showed approximately 1/1000 of transfection efficiency in response to the concentration change from 2  $\mu$ g to 2/9  $\mu$ g pDNA/mL. In contrast, the decrease in the transfection with PEG-PAsp(DET)-Chole micelles was less than 1/10. Thus, transfection efficiency of PEG-PAsp(DET)-Chole micelles was confirmed to be more tolerable in dilution compared to PEG-PAsp(DET) micelles, suggesting their feasibility for *in vivo* gene delivery, which requires high transfection ability under highly diluted conditions.

### 3.9. Cellular uptake of micelles

Flow cytometric analysis was used to quantify the cellular uptake of micelles with respect to pDNA (Fig. 8A) and polymer (Fig. 8B) by using Cy3-labeled pDNA and Alexa680-labeled polymer, respectively. PEG-PAsp(DET)-Chole micelles showed higher pDNA uptake than PEG-PAsp(DET) micelles at both N/P = 8 and 16 (Fig. 8A). Cholesterol introduction enhanced the stability of polyplex micelles against BSA (shown in Fig. 5), thus, PEG-PAsp(DET)-Chole micelles likely maintained their structures without dissociation or aggregation in the cell culture medium, which likely increased the uptake of pDNA. The cellular uptake of polymer was

also higher for PEG-PAsp(DET)-Chole than PEG-PAsp(DET) (Fig. 8B). PEG-PAsp(DET)-Chole polymers might be more effectively internalized into the cells due to their strong association power towards pDNA.

### 3.10. CLSM observation and evaluation of endosomal escape

The intracellular distribution of polyplex micelles was investigated by CLSM using Cy5-labeled pDNA (red) incorporated micelles (Fig. 9A). Lyso Tracker Green (green) and Hoechst 33342 (blue) were used to label late endosomes/lysosomes and nuclei, respectively. The amount of Cy5-pDNA observed in the cells was much higher for PEG-PAsp(DET)-Chole micelles than PEG-PAsp(DET) micelles at both N/P = 8 and 16, which was consistent with the results obtained by flow cytometric analysis (Fig. 8A). Colocalization of pDNA with the late endosomes/lysosomes was

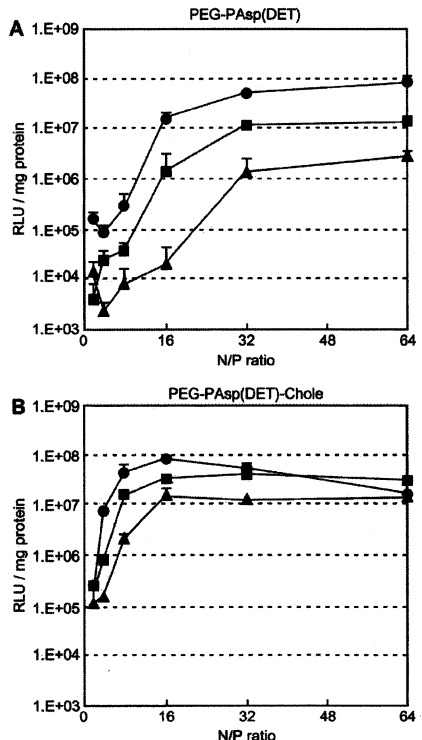


Fig. 7. Effect of pDNA concentration on transfection efficiency of PEG-PAsp(DET) (A) and PEG-PAsp(DET)-Chole (B) polyplex micelles against Huh-7 cells. Closed circles, squares, and triangles represent 2  $\mu$ g/mL, 2/3  $\mu$ g/mL, and 2/9  $\mu$ g/mL of pDNA concentration in the culture medium, respectively. Error bars in the graph represent SEM,  $n = 4$ .