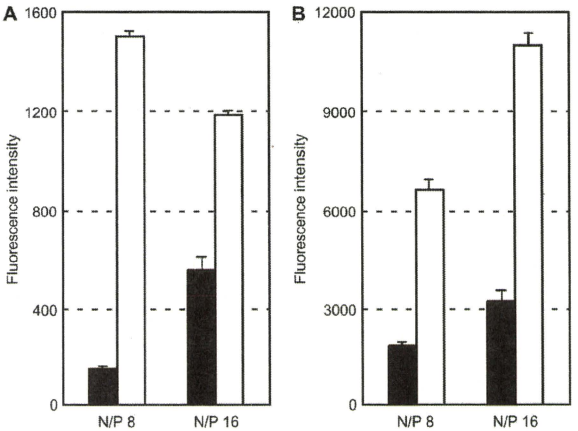

Fig. 8. Cellular uptake of Cy3-labeled pDNA (A) and Alexa680-labeled polymers (B). Closed bars: PEG-PAsp(DET) polyplex micelles. Open bars: PEG-PAsp(DET)-Chole polyplex micelles. Error bars in the graph represent SEM, $n = 4$.

quantified and shown in Fig. 9B. At N/P = 8, more than 80% of pDNA in PEG-PAsp(DET) micelles was localized in the late endosomes/lysosomes, while only 20% of that in PEG-PAsp(DET)-Chole micelles was localized there. These results revealed that PEG-PAsp(DET)-Chole micelles internalized into the cells could achieve effective endosomal escape. Note that increasing N/P ratio appreciably decreased the endosomal/lysosomal entrapment of PEG-PAsp(DET) micelles from more than 80% to less than 60%, consistent with the result of the transfection efficiency (Fig. 6).

3.11. FCS measurement

FCS analysis was performed in order to estimate a change in the association state of polyplex micelles with respect to dilution and pH (Fig. 10). At pH 7.3, the relative diffusion time of PEG-PAsp (DET)-Chole micelle solution was approximately 8-fold higher than that of PEG-PAsp(DET)-Chole polymer solution, and the diffusion time was not significantly changed by 9-fold dilution (Fig. 10A). These results suggest that the association state of PEG-PAsp(DET)-Chole micelles remains constant in this concentration range, which

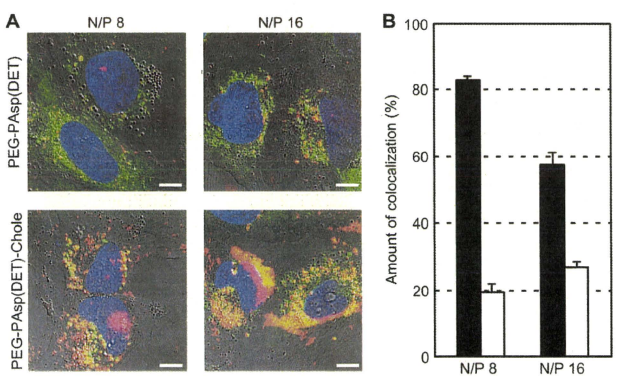

Fig. 9. (A) CLSM observation of the intracellular distribution of polyplex micelles containing Cy5-labeled pDNA (red) with late endosomes/lysosomes (green) and nuclei (blue) stained using Lyso Tracker Green and Hoechst 33342, respectively. Bars represent 10 μm. (B) Quantification of Cy5-labeled pDNA colocalization with Lyso Tracker Green in the Huh-7 cells. Closed bars: PEG-PAsp(DET) polyplex micelles. Open bars: PEG-PAsp(DET)-Chole polyplex micelles. Error bars in the graph represent SEM, $n = 10$.

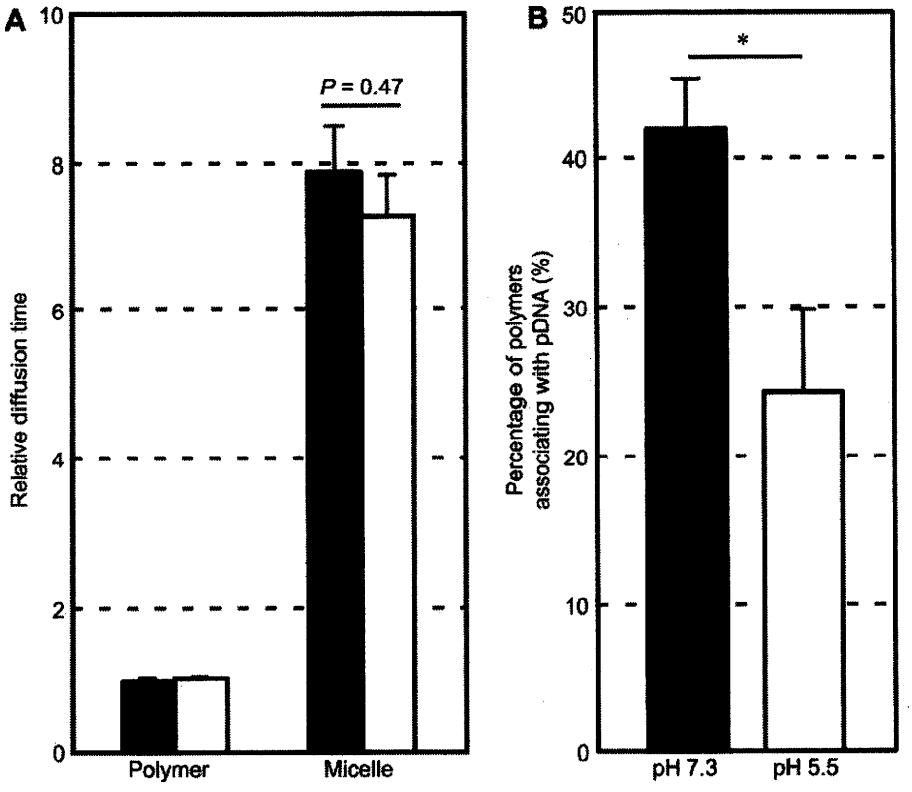

Fig. 10. (A) Relative diffusion time of PEG-PAsp(DET-Alexa680)-Chole block copolymers and their polyplex micelles in the Hepes buffer (pH 7.3). Closed bars: 18.7 μg/mL of block copolymers in the solution. Open bars: 2.08 μg/mL of block copolymers in the solution. (B) Percentage of PEG-PAsp(DET-Alexa680)-Chole block copolymers associating with pDNA in the micelle solutions at different pH. Closed bars: Hepes buffer (pH 7.3). Open bars: MES buffer (pH 5.5). Polyplex micelles were prepared at N/P = 8. Error bars in the graph represent SEM, $n = 7$. $^*P < 0.01$.

corroborates well with the result that PEG-PAsp(DET)-Chole micelles achieved high gene transfer under the diluted conditions (Fig. 7B). The amount of polymer associated with pDNA in PEG-PAsp(DET)-Chole micelles (N/P = 8) at pH 7.3 estimated by FCS (42% in Fig. 10B) was in accordance with that calculated from ultracentrifugation analysis (45% in Fig. 4), where 340 of total 756 block copolymers per pDNA were associated with a pDNA. Furthermore, the percentage of polymers associating with pDNA significantly decreased upon lowering the pH from 7.3 to 5.5 (Fig. 10B). Note that ethanediamine units in the side chain of PAsp (DET) adopt a mono-protonated form at pH 7.4 and then become di-protonated at pH 5.5 [11,12]. Thus, this change in the charge state of PAsp(DET) might lead to electrostatic repulsion among block copolymers in the polyplex micelle, thereby releasing a considerable amount of polymers associating with pDNA at pH corresponding to endosomal compartments.

3.12. Stability of polyplex micelles in the blood stream

In order to evaluate the stability of polyplex micelles in the blood, the concentration of Cy5-pDNA in the plasma was measured at various times after intravenous injection of polyplex micelles (N/P = 8) containing Cy5-pDNA via the tail vein of mice (Fig. 11). Almost all the fluorescence from Cy5-pDNA incorporated into PEG-PAsp(DET) micelles disappeared from the blood 30 min after injection. On the other hand, PEG-PAsp(DET)-Chole micelles retained more than 15% and 2% of the injected dose of pDNA in the blood at 30 min and at 60 min after

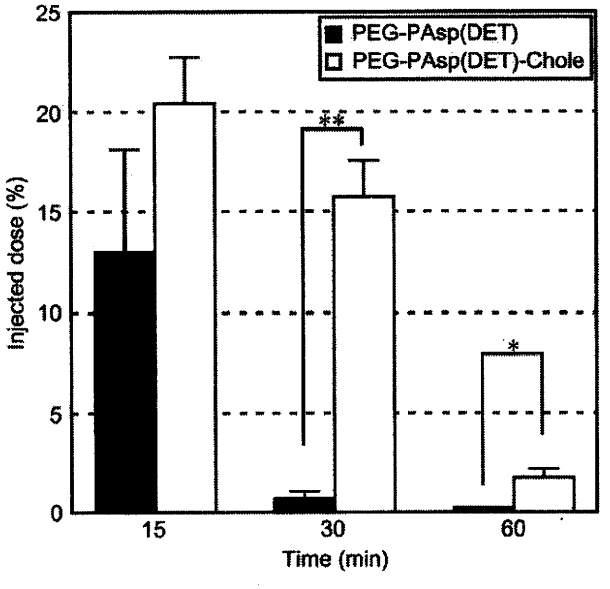

Fig. 11. Cy5-labeled pDNA concentration in the blood after intravenous injection of PEG-PAsp(DET) (closed bars) and PEG-PAsp(DET)-Chole (open bars) polyplex micelles (N/P = 8, 20 μg pDNA/mouse). Error bars in the graph represent SEM, $n = 4$. $^*P < 0.05$ and $^{**}P < 0.01$.

injection, respectively. PEG-PAsp(DET)-Chole micelles could hold pDNA more stably in the blood compared to PEG-PAsp(DET) micelles, probably due to their higher stability in proteinous medium (Fig. 5).

3.13. Anti-tumor activity

Polyplex micelles containing sFlt-1 pDNA were injected intravenously into mice-bearing pancreatic adenocarcinoma BxPC3, followed by evaluation of tumor volume (Fig. 12). sFlt-1, which is a soluble form of VEGF receptor-1, is a well-known anti-angiogenic protein [21,22]. We recently reported that systemic injection of polyplex micelles containing sFlt-1 pDNA into mice significantly decreased subcutaneously inoculated BxPC3 growth [16,23], and therefore, this subcutaneous BxPC3 model is appropriate to evaluate the performance of systemic gene delivery vectors. PEG-PAsp (DET) (N/P = 10 and 20) and PEG-PAsp(DET)-Chole (N/P = 15) micelles were administrated every four days for three total doses, i.e. on days 0, 4, and 8. Only the PEG-PAsp(DET)-Chole micelle significantly suppressed tumor growth compared to Hepes buffer (control) ($P < 0.05$).

4. Discussion

PEG-PAsp(DET) micelles are promising gene delivery vectors due to their high transfection ability with low cytotoxicity, however, they must be prepared at high N/P ratio to achieve high transfection efficiency [11,13–15,24]. In general, gene vectors internalized into the cells must escape from the endosome prior to enzymatic degradation in lysosome vesicles for efficient transfection. PAsp(DET) polycations enabled effective escape from the endosome into cytoplasm due to their pH-selective membrane destabilization [12], and thus, a certain amount of PAsp(DET) should be contained within the same endosomal compartment with pDNA to facilitate release. PEG-PAsp(DET) micelles are needed to be prepared at N/P > 20 for effective *in vitro* transfection [11]. In this regard, we showed that almost all the PEG-PAsp(DET) polymers added at N/P ≥ 4, where they may form stoichiometric charged polyplex micelles with pDNA, existed as free polymers by ultracentrifugation analysis (Fig. 1A). Therefore, a large fraction of PEG-PAsp(DET) polymers present in micelle solutions prepared at high N/P ratios are not associated with pDNA in the culture medium but still assist in the endosomal escape of polyplex micelles. Indeed, the transfection efficiency of pDNA micelles prepared with PEG-PAsp(DET) increased with simultaneous addition of free polymer with micelle solution prepared at N/P = 4 to the cell culture medium (open circles in Fig. 1B), whereas similar transfection efficiency (closed circles in Fig. 1B) was observed with PEG-PAsp (DET) micelle solutions prepared at higher N/P ratios (which corresponded to the same amount of free polymer added to culture medium in the experiments with micelle solutions prepared at a constant N/P value of 4). This result indicates that the amount of free polymer in the culture medium is important for improved transfection efficiency, which is consistent with the above-mentioned hypothesis.

In this study, a hydrophobic cholesterol moiety was introduced into the ω-terminus of the PAsp(DET) segment of PEG-PAsp(DET) block copolymer for the purpose of achieving sufficient gene transfer at low N/P ratio and under dilute conditions, thus further developing PEG-PAsp(DET) micelles towards *in vivo* systemic vectors (Scheme 1). PEG-PAsp(DET)-Chole was designed to increase the association number of block copolymers with polyplex micelles by exploiting the hydrophobic nature of cholesterol, which possesses high self-associating ability, to form polyplex micelles over the stoichiometric charge ratio. Indeed, quantification of free polymer in micelle solutions by ultracentrifugation revealed that the number of PEG-PAsp(DET) associating with a pDNA did not change at N/P ≥ 4 ($N^+/P \geq 2$) and that polymer added over N/P = 4 existed as free polymers unassociated with pDNA. On the other hand, PEG-PAsp(DET)-Chole micelles prepared at N/P ≥ 2 ($N^+/P \geq 1$) showed an increase in the number of polymers associated with pDNA with increased N/P ratio (Fig. 4A). Furthermore, the introduction of cholesterol contributed not only to the enhancement of associating ability of polymers to pDNA, but also increased the stability of polyplex micelles. PEG-PAsp(DET)-Chole micelles maintained their structure for 12 h in the presence of BSA with no change in their initial size and PDI (Fig. 5). Note that a stability of a gene delivery vector against serum proteins is an important factor for *in vitro* transfection in the presence of serum and also for *in vivo* transfection via systemic administration and exposure to complex biological milieu in blood. Uptake of pDNA incorporating micelles in culture cells was drastically increased by the introduction of cholesterol (Fig. 8A), likely due to the increased stability of polyplex micelles in the culture medium containing serum. With respect to block copolymer uptake (in experiments performed with micelle solutions prepared with fluorescent-labeled block copolymer), PEG-PAsp(DET)-Chole was internalized into Huh-7 cells significantly more than PEG-PAsp(DET), implying that their uptake is enhanced when associated with polyplex micelles. CLSM observation of the intracellular distribution of polyplex micelles in culture cells revealed that PEG-PAsp(DET)-Chole micelles could more effectively escape from the late endosome/lysosome

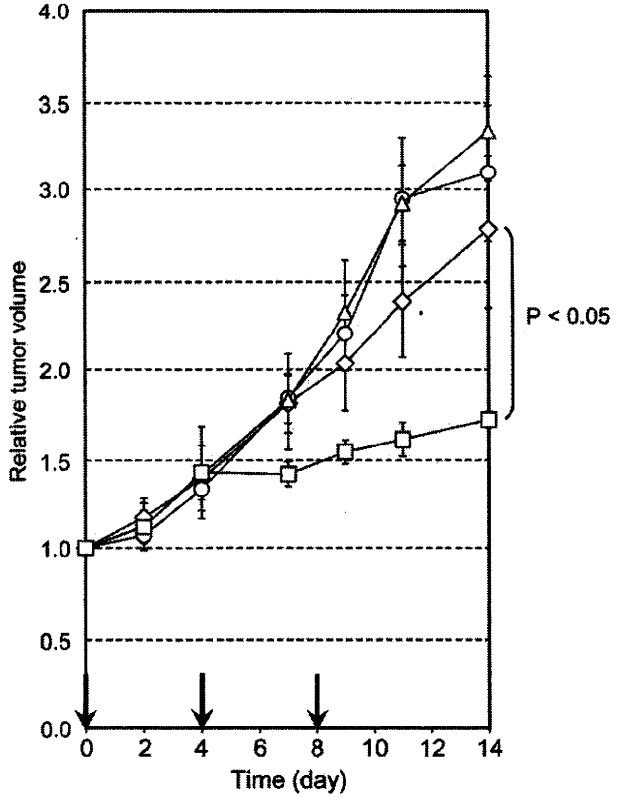

Fig. 12. Anti-tumor activity after intravenous injection of Hepes (diamonds), PEG-PAsp (DET) polyplex micelles at N/P = 10 (circles), at N/P = 20 (triangles), and PEG-PAsp (DET)-Chole polyplex micelles at N/P = 15 (squares). Error bars in the graph represent SEM, $n = 4$.

compartments compared to PEG-PAsp(DET) micelles prepared at the same N/P ratio (Fig. 9). In order for polyplex micelles based on the PEG-PAsp(DET) to be effective gene delivery vectors, block copolymer should be released from polyplex micelles in the endosome and the directly associate with the endosomal membrane to disrupt the vesicle structure and facilitate escape of polyplex micelles into the cytoplasm and allow pDNA to further transport into the nucleus. The relative number of PEG-PAsp(DET)-Chole polymers associating with pDNA estimated by FCS measurement was significantly reduced with decreasing pH, from 42% (pH 7.3) to 24% (pH 5.5) (Fig. 10B). These results suggest that formation of PEG-PAsp(DET)-Chole micelles over stoichiometric charge ratio facilitated effective detachment of block copolymers from polyplex micelles in the acidic endosome by electrostatic repulsion among block copolymers, resulting in efficient endosomal escape based on destabilization of the endosomal membrane. Transfection experiments performed *in vitro* revealed that PEG-PAsp(DET)-Chole micelles achieved high transfection efficiency at lower N/P ratios compared to PEG-PAsp(DET) micelles (Fig. 6A). This enhanced transfection ability is likely due to a synergistic effect between effective uptake of pDNA by increased micelle stability, the ability to form micelles with high polymer association above the stoichiometric N/P value, and efficient endosomal escape by polymers released from the micellar structure upon acidification without increased cytotoxicity, even at high N/P ratios (Fig. 6B).

Gene delivery vectors administered systemically are diluted instantly upon injection, and cannot always reach target sites in high concentration. Therefore, systemic gene vectors must transfect efficiently even under dilute conditions. In this regard, PEG-PAsp(DET)-Chole micelles were confirmed to overcome this issue (Fig. 7). Furthermore, whereas the *in vitro* transfection efficiency of PEG-PAsp(DET) micelles dropped dramatically with decreasing pDNA concentration contained in the culture medium, a decrease in transfection efficiency of PEG-PAsp(DET)-Chole micelles was well prevented. This result corroborates well with the results of FCS measurement of micelle solutions (Fig. 10A), which revealed that the diffusion time of PEG-PAsp(DET)-Chole micelles was not changed by dilution, suggesting that their association state was no altered. The inherent characteristics of PEG-PAsp(DET)-Chole micelles, i.e., high transfection efficiency both at low N/P ratios and under dilute conditions, should be suitable for their use as systemic gene delivery vectors.

The increased stability of polyplex micelles was also confirmed by evaluation of blood circulation after systemic injection into mice via the tail vein (Fig. 11). Naked pDNA is not stable in blood and is reported to be degraded within 5 min after intravenous injection [25]. Although pDNA loaded PEG-PAsp(DET) micelles retained more than 20% of injected dose after 15 min, almost all the pDNA were cleared from the blood after 30 min (Fig. 11). PEG-PAsp(DET) micelles are known to easily decondense in the presence of serum [20], thus, PEG-PAsp(DET) micelles injected directly into the blood stream are likely to decondense and release pDNA, which is subsequently degraded and removed from circulation. On the other hand, PEG-PAsp(DET)-Chole micelles showed significantly prolonged blood circulation compared to PEG-PAsp(DET) micelles (Fig. 11). PEG-PAsp(DET)-Chole micelles, which were stable in the presence of BSA (Fig. 5), likely resist rapid decondensation, leading to longer circulation time.

Polyplex micelles were further evaluated for anti-tumor activity against a murine solid tumor model after intravenous injection in order to evaluate their performance as systemic gene delivery vectors (Fig. 12). Specifically, mice bearing a subcutaneously xenografted BxPC3 human pancreatic adenocarcinoma tumor and therapeutic pDNA encoding the anti-angiogenic protein sFlt-1 were used. As shown in Fig. 12, PEG-PAsp(DET) micelles showed no significant effect, however, PEG-PAsp(DET)-Chole micelles significantly suppressed tumor growth compared to the Hepes buffer control. Important factors affecting the anti-tumor effect of systemically injected gene delivery vectors are stability in the blood and high transfection efficiency within cells at the target site [16]. PEG-PAsp(DET)-Chole micelles exhibited longer blood circulations (Fig. 11) and also maintained high transfection ability even under dilute conditions (Fig. 7) compared to PEG-PAsp(DET) micelles, which correlated to higher therapeutic effect *in vivo*.

5. Conclusion

PEG-PAsp(DET) micelles achieve high transfection efficiency with low cytotoxicity at high N/P ratios, however, the results of this work showed that block copolymer added over the stoichiometric charge ratio exists as free polymers in the micelles solution. In this study, we further improved the design of PEG-PAsp(DET)-based synthetic gene delivery vectors by incorporating a cholesterol moiety into the terminus of PAsp(DET) segment in the block copolymer. PEG-PAsp(DET)-Chole micelles could be formed over the stoichiometric charge ratio due to self-association of cholesterol, and achieved effective endosomal escape due to the efficient delivery of block copolymers and pDNA into target cells and which increased transfection efficiency at low N/P ratios and under the dilute conditions. Furthermore, cholesterol introduction led to increased stability of polyplex micelles in the blood, which resulted in significant suppression of subcutaneous pancreatic tumor growth by intravenous injection of polyplex micelles loading sFlt-1 pDNA. Conventional polyplexes formed with polyethyleneimine or cationic polypeptides have similar issues regarding the impact of free polycations on transfection efficiency as observed with PEG-PAsp(DET) micelles [26,27]. These polyplexes must be used at high N/P ratio or high concentration of pDNA to achieve effective endosomal escape and high transfection efficiency. Thus, the large amount of free polymer can result in increased cytotoxicity *in vitro* and also adverse side effects *in vivo* after intravenous injection. To circumvent the issue of excess polycations not associating with pDNA, polyplexes utilizing hydrophobic groups such as cholesterol have been reported [28,29] and such systems show promise due to excellent transfection efficiency. Nevertheless, those studies focused primarily on increased stability of polyplexes by introduction of cholesterol, with less attention paid to the association number of polymers with polyplexes. In this study, we showed that PEG-PAsp(DET)-Chole micelles with high polymer association could be formed over the stoichiometric charge ratio by detailed evaluation of micelle solutions using ultracentrifugation analysis. Enhanced stability as well as complex formation over the stoichiometric charge ratio contributed to effective gene transfection both *in vitro* and *in vivo*. These findings are extremely helpful for design of non-viral gene vectors and represent a significant improvement towards the use of synthetic polyplex micelle gene delivery vectors as a practical therapeutic modality.

Acknowledgements

This work was financially supported by the Core Research Program for Evolutional Science and Technology (CREST) from Japan Science and Technology Agency (JST) as well as by Grants-in-Aid for Young Scientists (A) (No. 20689024 to M.O.). We express our appreciation to Prof. Masabumi Shibuya (Tokyo Medical and Dental University) for providing pVL 1393 baculovirus vector pDNA encoding human sFlt-1. We thank Ms. Junko Kawakita and Ms. Satomi Ogura (The University of Tokyo) for technical assistance.

Appendix

Figures with essential color discrimination. Fig. 9 in this article may be difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.09.022.

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