of the Persistence Length of Double-Stranded RNA. *Biophys. J.* **2005**, *88*, 2737–2744.
25. Kenausis, G. L.; Voros, J.; Elbert, D. L.; Huang, N.; Hofer, R.; Ruiz-Taylor, L.; Textor, M.; Hubbell, J. A.; Spencer, N. D. Poly(L-lysine)-g-Poly(ethylene glycol) Layers on Metal Oxide Surfaces: Attachment Mechanism and Effects of Polymer Architecture on Resistance to Protein Adsorption. *J. Phys. Chem. B* **2000**, *104*, 3298–3309.
26. Giljohann, D. A.; Seferos, D. S.; Prigodich, A. E.; Patel, P. C.; Mirkin, C. A. Gene Regulation with Polyvalent siRNA-Nanoparticle Conjugates. *J. Am. Chem. Soc.* **2009**, *131*, 2072–2073.
27. Zuckerman, J. E.; Choi, C. H. J.; Han, H.; Davis, M. E.; Polycation-siRNA Nanoparticles, Can Disassemble at the Kidney Glomerular Basement Membrane. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 3137–3142.
28. Lee, J.-S.; Green, J. J.; Love, K. T.; Sunshine, J.; Langer, R.; Anderson, D. G. Gold, Poly(β-amino ester) Nanoparticles for Small Interfering RNA Delivery. *Nano Lett.* **2009**, *9*, 2403–2406.
29. Mislick, K. A.; Baldeschwieler, J. D. Evidence for the Role of Proteoglycans in Cation-Mediated Gene Transfer. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 12349–12354.
30. Chithrani, D. B. Intracellular Uptake, Transport, and Processing of Gold Nanostructures. *Mol. Membr. Biol.* **2010**, *27*, 299–311.
31. Seymour, L. W.; Duncan, R.; Strohalm, J.; Kopecek, J. Effect of Molecular Weight (Mw) of *N*-(2-Hydroxypropyl)-methacrylamide Copolymers on Body Distribution and Rate of Excretion After Subcutaneous, Intraperitoneal, and Intravenous Administration to Rats. *J. Biomed. Mater. Res.* **1987**, *21*, 1341–1358.
32. Matsumoto, Y.; Nomoto, T.; Cabral, H.; Matsumoto, Y.; Watanabe, S.; Christie, R. J.; Miyata, K.; Oba, M.; Ogura, T.; Yamasaki, Y.; *et al.* Direct and Instantaneous Observation of Intravenously Injected Substances Using Intravital Confocal Micro-Videography. *Biomed. Opt. Express* **2010**, *1*, 1209–1216.
33. Kim, H. J.; Oba, M.; Pittella, F.; Nomoto, T.; Cabral, H.; Matsumoto, Y.; Miyata, K.; Nishiyama, N.; Kataoka, K. PEG-Detachable Cationic Polyaspartamide Derivatives Bearing Stearoyl Moieties for Systemic siRNA Delivery toward Subcutaneous BxPC3 Pancreatic Tumor. *J. Drug Targeting* **2012**, *20*, 33–42.
34. Nomoto, T.; Matsumoto, Y.; Miyata, K.; Oba, M.; Fukushima, S.; Nishiyama, N.; Yamasoba, T.; Kataoka, K. *In Situ* Quantitative Monitoring of Polyplexes and Polyplex Micelles in the Blood Circulation Using Intravital Real-Time Confocal Laser Scanning Microscopy. *J. Controlled Release* **2011**, *151*, 104–109.
35. Harada, A.; Kataoka, K. Formation of Polyion Complex Micelles in an Aqueous Milieu from a Pair of Oppositely-Charged Block Copolymers with Poly(ethylene glycol) Segments. *Macromolecules* **1995**, *28*, 5294–5299.
36. Mehrara, E.; Forssell-Aronsson, E.; Ahlman, H.; Bernhardt, P. Quantitative Analysis of Tumor Growth Rate and Changes in Tumor Marker Level: Specific Growth Rate *Versus* Doubling Time. *Acta Oncol.* **2009**, *48*, 591–597.

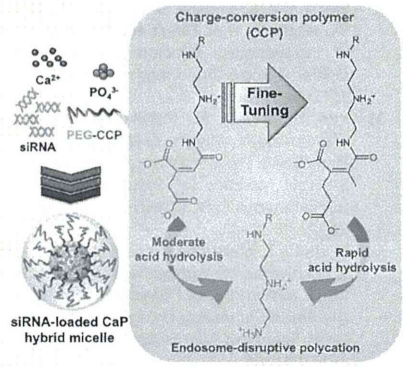
Macromolecular Journals

Macromolecular Rapid Communications

Fine-Tuning of Charge-Conversion Polymer Structure for Efficient Endosomal Escape of siRNA-Loaded Calcium Phosphate Hybrid Micelles

Yoshinori Maeda, Frederico Pittella, Takahiro Nomoto, Hiroyasu Takemoto, Nobuhiro Nishiyama, Kanjiro Miyata,* Kazunori Kataoka*

For efficient delivery of siRNA into the cytoplasm, a smart block copolymer of poly(ethylene glycol) and charge-conversion polymer (PEG-CCP) is developed by introducing 2-propionic-3-methylmaleic (PMM) amide as an anionic protective group into side chains of an endosome-disrupting cationic polyaspartamide derivative. The PMM amide moiety is highly susceptible to acid hydrolysis, generating the parent cationic polyaspartamide derivative at endosomal acidic pH 5.5 more rapidly than a previously synthesized *cis*-aconitic (ACO) amide control. The PMM-based polymer is successfully integrated into a calcium phosphate (CaP) nanoparticle with siRNA, constructing PEGylated hybrid micelles (PMM micelles) having a sub-100 nm size at extracellular neutral pH 7.4. Ultimately, PMM micelles achieve the significantly higher gene silencing efficiency in cultured cancer cells, compared to ACO control micelles, probably due to the efficient endosomal escape of the PMM micelles. Thus, it is demonstrated that fine-tuning of acid-labile structures in CCP improves the delivery performance of siRNA-loaded nanocarriers.



Y. Maeda, T. Nomoto, Prof. K. Kataoka
Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8656, Japan
E-mail: kataoka@bmw.t.u-tokyo.ac.jp
Dr. F. Pittella, Dr. K. Miyata, Prof. K. Kataoka
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
E-mail: miyata@bmw.t.u-tokyo.ac.jp
Dr. H. Takemoto, Prof. N. Nishiyama
Polymer Chemistry Division, Chemical Resources Laboratory, Tokyo Institute of Technology, R1–11, 4259 Nagatsuta, Midori-ku, Yokohama 226–8503, Japan
Prof. K. Kataoka
Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8656, Japan

1. Introduction

Small interfering RNA (siRNA) has been greatly highlighted as a potential therapeutic agent for a variety of intractable diseases, including cancer.[1] To obtain therapeutic benefits, siRNA needs to be transported to the cytoplasm after cellular internalization. However, endocytosed macromolecules are generally entrapped by acidic vesicular compartments, i.e., endosomes, within cells, leading to the lysosomal degradation.[2] Hence, various polymeric materials have been developed to facilitate endosome disruption for smooth endosomal escape of siRNA.[3] Polyethylenimine (PEI) is one of the most widely used polymers for the endosomal escape of nucleic acids. It is believed that the low pK_a amines in PEI can serve as a proton sponge in acidic endosomes (pH ≈ 5.5) to

DOI: 10.1002/marc.201400049

induce endosome disruption,[4,5] due to the increased osmotic pressure within the vesicles and/or the direct interactions of highly charged PEI with oppositely charged endosomal membrane.[6,7] However, significantly protonated PEI even under extracellular conditions (pH 7.4) concurrently induces the considerable cytotoxicity due to the cytoplasmic membrane damage.[8,9] Therefore, further development of endosome-disrupting polymers is still demanded for more efficient, yet less toxic endosomal escape of siRNA.

Our previous studies revealed that diaminoethane unit (—$NHCH_2CH_2NH$—) showed a distinctive change in the protonated state between pH 7.4 and 5.5, i.e., the monoprotonated state at pH 7.4 and the diprotonated state at pH 5.5.[10,11] Accordingly, a polyaspartamide derivative bearing the diaminoethane unit in the side chain, poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), successfully induced acidic pH-responsive membrane destabilization because of the change in its protonated state.[11–14] PAsp(DET) allowed efficient gene expression of plasmid DNA in cultured cells, associated with significantly lower cytotoxicity compared to PEI.[10–14] Meanwhile, primary amines in PAsp(DET) could be further modified with *cis*-aconitic (ACO) anhydride to generate an endosome-disrupting polyanion PAsp(DET-ACO), where ACO amide was subjected to acid hydrolysis for regeneration of the parent polycation PAsp(DET).[15] Thus, this polyanion was termed the charge-conversion polymer (CCP) based on its charge-conversion from the negative to the positive at acidic pH.

This CCP-based strategy was quite useful for siRNA delivery, when a block copolymer of poly(ethylene glycol) and CCP (PEG–CCP) was applied for construction of hybrid micelles with calcium phosphate (CaP) precipitates (Figure S1, Supporting Information).[16–18] CaP precipitates have been extensively used as a conventional transfection reagent of nucleic acids, because of their extremely low-cost and simple preparation scheme. However, the rapid growth of CaP crystal has substantially hampered the utilization of CaP precipitates for systemic nucleic acid delivery.[19] In this regard, PEG–CCP provided CaP precipitates with a PEG shell for size-controlling as well as biocompatibility to form monodispersive hybrid polymeric micelles. Indeed, siRNA-loaded hybrid micelles were prepared with CaP and PEG–CCP, having a size of sub-100 nm with a narrow size distribution, and induced efficient gene silencing in various cultured cancer cells.[16–18] Ultimately, systemically administered hybrid micelles showed the significant antitumor activity in a subcutaneous pancreatic cancer model by delivering the siRNA targeted for vascular endothelial growth factor.[17]

In our previous studies, the ACO amide, which is a maleic acid derivative bearing 2-acetic acid moiety, has been utilized as an acid-labile bond (Figure 1). It is known in this regard that the sensitivity of maleic acid amides to acid hydrolysis can be altered by functional groups substituted at the 2- and 3-positions of the maleic acid amide.[20] Indeed, 2-propionic-3-methyl maleic (PMM) amide was demonstrated to be more susceptible to acid hydrolysis compared to ACO amide.[21] This fact motivated us to finely tune the acid-labile amide structure in PEG–CCP for improving the endosome-escaping functionality. In the present study, a second generation of PEG–CCP was newly synthesized by introducing the PMM moieties into primary amines in PEG–PAsp(DET). The obtained block copolymer PEG–PAsp(DET-PMM) was compared with PEG-PAsp(DET-ACO) in terms of the sensitivity to acid hydrolysis and the delivery efficacy based on the hybrid micelle formulation.

2. Experimental Section

All experimental details are described in Supporting Information.

3. Results and Discussion

siRNA and its nanocarriers, once internalized into cells, are transported to the late endosomes (or the lysosomes),

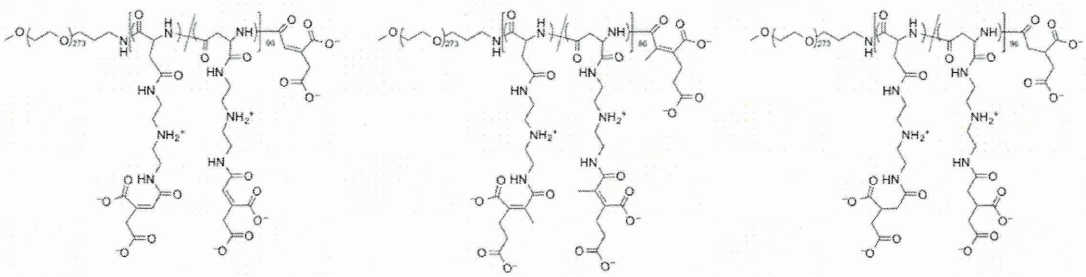


Figure 1. Chemical structures of PEG-PAsp(DET-ACO) and PEG-PAsp(DET-PMM) as PEG-CCPs, and PEG-PAsp(DET-CAR) as a PEG-nonCCP. The carballylic (CAR) moiety is a succinic acid derivative, and thus, its amide is much less sensitive to acid hydrolysis, compared to the maleic acid derivatives, PMM and ACO moieties.

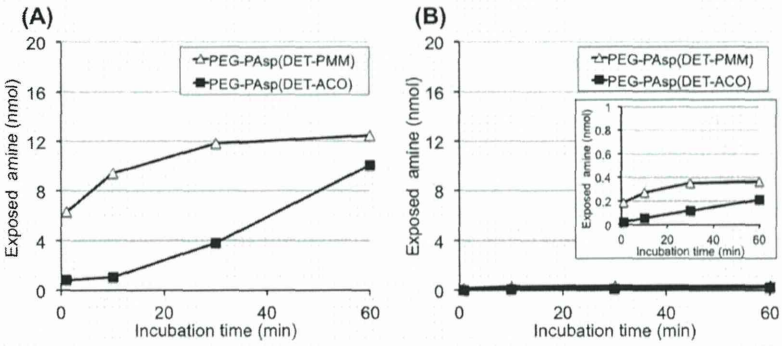


Figure 2. Amount of exposed amine in PEG–PAsp(DET–PMM) and PEG–PAsp(DET–ACO) after incubation at A) pH 5.5 and B) pH 7.4. The amount of exposed amine was determined from standard curves prepared with glycine solutions in an acetate buffer (pH 5.5) and a phosphate buffer (pH 7.4).

followed by lysosomal degradation.[2,22] Thus, they need to escape from those vesicular compartments to the cytoplasm before degrading for exerting gene silencing effect. Here, a smart block copolymer of biocompatible PEG and endosome-disrupting PAsp(DET–PMM) was synthesized by introducing a PMM moiety into primary amines in PAsp(DET) side chains through the amide bond formation (Figure 1 and Scheme S1, Supporting Information). It is reported that PMM amide is cleaved more rapidly than ACO amide in acidic conditions, probably because the pK_a value of carboxylates in dimethylmaleamylate derivatives is higher than that in citraconylate derivatives.[21] Thus, PAsp(DET–PMM) is expected to be more rapidly converted to the parent polycation PAsp(DET) in acidic endosomes compared with PAsp(DET–ACO), for facilitating the endosome disruption (Figure S1, Supporting Information). The successful synthesis of PEG–PAsp(DET–PMM) was confirmed from the size exclusion chromatogram (Figure S2, Supporting Information) and ^{1}H NMR spectrum (Figure S3 and Table S1, Supporting Information).

The charge-conversion functionality of PEG–PAsp(DET–PMM) was compared with that of PEG–PAsp(DET–ACO) by determining the amount of amines generated from the CCP segments at pH 7.4 and 5.5 (Figure 2). Obviously, PEG–PAsp(DET–PMM) exerted higher conversion rate than PEG–PAsp(DET–ACO) over time at both pHs of 5.5 and 7.4, demonstrating more rapid hydrolysis of the PMM amide bonds. Also, the accelerated hydrolysis at the lower pH of 5.5 was demonstrated for both CCP segments. Nevertheless, the considerable increase in the conversion ratios even at pH 7.4 is likely to induce destabilization of hybrid micelles under extracellular neutral conditions. Thus, the stability of hybrid micelles in serum-containing medium was further examined as described below.

The newly synthesized PEG–CCP, PEG–PAsp(DET–PMM), was applied for the preparation of CaP hybrid micelles loaded with siRNA and then characterized by DLS. The obtained DLS (volume-weighted) histogram of siRNA-loaded hybrid micelles prepared with PEG–PAsp(DET–PMM) (PMM micelles) displays a hydrodynamic diameter of ≈70 nm (Figure S4, Supporting Information), associated with a narrow size distribution (polydispersity index = 0.1), similar to hybrid micelles prepared with PEG–PAsp(DET–ACO) (ACO micelles). Note that the micelle formation was not observed in the absence of calcium and phosphate ions under the similar condition because of the hydrophilic nature of PEG–CCPs. In sharp contrast, non-PEGylated CaP precipitates showed much larger size (≈1 μm) (data not shown). These results demonstrate that the block copolymers with PEG were essential for the sub-100 nm nanoparticle formation due to enhanced colloidal stability based on the PEG shell.

The carrier stability under cell culture conditions is a prerequisite for efficient cellular uptake of siRNA. In our previous study, ACO micelles were confirmed to stably entrap siRNA in 10% fetal bovine serum (FBS)-containing Dulbecco's modified Eagle's medium (DMEM) for a relatively short incubation time of 4 h.[17] Nevertheless, the stable entrapment of siRNA in the micelles for longer incubation time (e.g., 24 h) is crucial from the standpoint of systemic delivery. Thus, the stability of hybrid micelles in the DMEM containing 10% FBS was investigated over 24 h at 37 °C by fluorescence correlation spectroscopy.[17,23] By assuming the spherical shape of hybrid micelles,[17,18] the obtained diffusion coefficients of hybrid micelles incorporating Alexa647-siLuc were converted to the corresponding hydrodynamic diameters based on the Stokes-Einstein equation (Figure S5, Supporting Information), and then, normalized to the value of naked siRNA. Both hybrid micelles maintained their initial size during 24 h incubation, indicating the stable encapsulation of siRNA within the hybrid micelles in the serum-containing medium. These results strongly suggest that PEG–PAsp(DET–PMM) and PEG–PAsp(DET–ACO) should be stably bound to CaP core without hydrolysis of maleic acid amides in the serum-containing medium at pH 7.4.

Next, the cellular internalization behavior of hybrid micelles was examined by a flow cytometer. Luciferase-expressing human ovarian cancer (SKOV3-Luc) cells were incubated with Cy5-siLuc-loaded hybrid micelles for 6 and 24 h, followed by the flow cytometric analysis (Figure 3A and Figure S6, Supporting Information). The significant cellular uptake of Cy5-siLuc was confirmed for all three hybrid micelles, i.e., PMM micelles, ACO micelles,

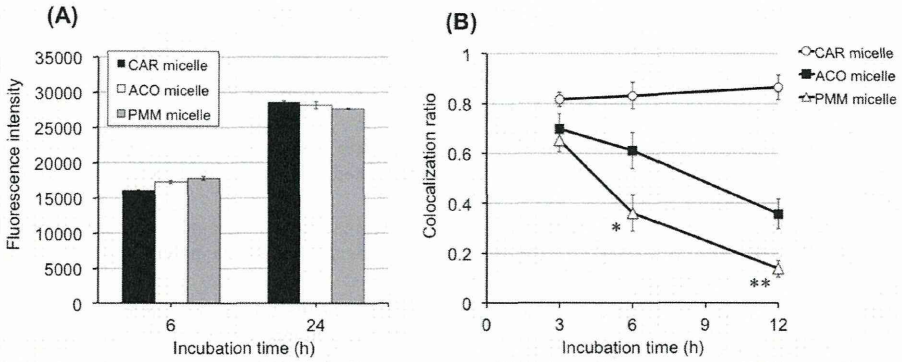


Figure 3. A) Cellular uptake efficiency of Cy5-siLuc delivered by hybrid micelles in SKOV3-Luc cells after 6 h incubation and 24 h incubation (100×10^{-9} M siRNA). Results are expressed as mean ± SEM ($n = 3$). (B) Time-dependent change in colocalization ratio of Alexa647-siLuc (red) with CellLight Late Endosomes-GFP (green) in SKOV3-Luc cells (200×10^{-9} M siRNA). The data are represented as mean ± SEM obtained from 14 cells ($^{*}p < 0.05$, $^{**}p < 0.01$).

and the micelles prepared with PEG–PAsp(DET–CAR) as a non-charge-conversion control polymer (nonCCP), in 6 h incubation. The uptake was further increased by prolonging the incubation time to 24 h. Importantly, almost the same cellular internalization profiles were observed among the three micelles, presumably because they have similar PEGylated shell structures. Next, the intracellular trafficking of hybrid micelles was investigated as a critical step following the cellular internalization. In particular, the colocalization of hybrid micelles (or the siRNA payload) with the late endosomes was focused to verify the endosome-escaping functionality of PEG–PAsp(DET–PMM) in comparison with PEG–PAsp(DET–ACO) and PEG–PAsp(DET–CAR). SKOV3-Luc cells were incubated with each hybrid micelle prepared with Alexa647-siLuc,

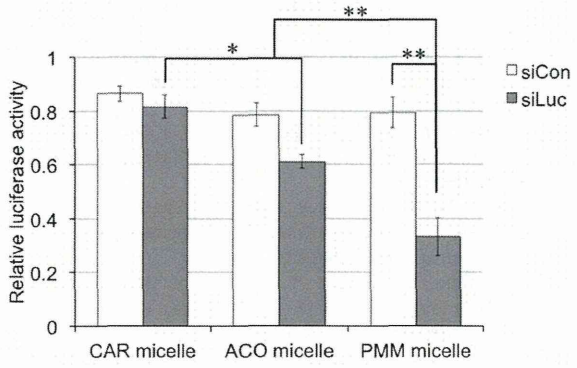


Figure 4. Luciferase gene silencing efficiency of CAR micelles, ACO micelles, and PMM micelles in SKOV3-Luc cells. The cells were incubated with each micelle incorporating siLuc (target sequence) or siCon (control sequence) at 50×10^{-9} M siRNA for 48 h, followed by a conventional luciferase assay. The obtained luminescence intensities from cell lysates were normalized to that from non-treated control cells. Results are shown as mean ± SEM ($n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$).

and then, the transfection medium containing the hybrid micelles was exchanged with the fresh one without micelle samples, followed by additional incubation before confocal laser scanning microscope (CLSM) imaging. The intracellular Alexa647-siLuc was shown red and also the endosomal membrane was stained with CellLight Late Endosomes-GFP (green), thereby their colocalization points within the cells should be shown yellow in the merged CLSM images (Figure S7, Supporting Information). Then, the colocalization ratio of Alexa647-siLuc with the stained late endosomes at the designated time points was calculated by pixel counting for each hybrid micelle (Figure 3B), as described in Supporting Information. The CCP-integrated micelles, i.e., PMM and ACO micelles, showed time-dependent decreases in the colocalization ratio from 3 to 12 h, whereas the colocalization ratio of the nonCCP-integrated, CAR micelles was not significantly altered. This result strongly suggests the facilitated endosomal escape of Alexa647-siLuc by the CCPs integrated into the hybrid micelles. Further, the colocalization ratio of PMM micelles was significantly lower than that of ACO micelles, demonstrating stronger endosome-escaping functionality of the CCP integrated with PAsp(DET–PMM). On the contrary, the apparently high colocalization ratios of CAR micelles indicate that the hybrid micelles without CCPs could not effectively induce the endosome disruption under the tested condition. These results are consistent with the polymer design concept that more rapid conversion of CCPs to the endosome-disrupting polycation PAsp(DET) at an endosomal acidic pH of 5.5 should enable more efficient endosomal escape of hybrid micelles.

The gene silencing efficiency of hybrid micelles was determined to elucidate the effect of endosome-escaping functionality of CCPs on the ultimate biological activity of siRNA. The hybrid micelles prepared with a target sequence of siRNA (siLuc) or a control sequence (siCon)

were incubated with SKOV3-Luc cells at 50×10^{-9} M siRNA for 48 h, followed by a luciferase assay of the cell lysates (Figure 4). The siLuc/CCP-loaded micelles (i.e., PMM and ACO micelles) achieved significantly lower luciferase activity compared to the siLuc/nonCCP-loaded micelles (i.e., CAR micelles), indicating the greater gene silencing efficiency of hybrid micelles equipped with CCPs. The PMM micelles revealed further improved efficiency in gene silencing compared to the ACO micelles. Considering the similar cellular internalization behaviors of the three hybrid micelles (Figure 3A), it is reasonable to conclude that the greater gene silencing efficiency of the PMM hybrid micelles is mainly due to their improved capability of translocating siRNA payloads from endosomal compartment to cytosol based on their prominent charge-conversion functionality (Figure 3B). It should be noted that the significant difference in relative luciferase activity was observed between siLuc-loaded and siCon-loaded micelles and also that the PMM micelles did not affect the viability of SKOV3-Luc cells under the same condition as the gene silencing assay (Figure S8, Supporting Information). These results confirm the sequence-specific gene silencing effect of siLuc-loaded hybrid micelles.

4. Conclusions

In this study, PEG–PAsp(DET–PMM) was synthesized by utilizing PMM amide, which was more sensitive to acid hydrolysis compared to the previously developed ACO amide. The obtained PEG–PAsp(DET–PMM) successfully formed siRNA-loaded hybrid micelles having the size of ≈70 nm. The PMM micelles were stable in the 10% FBS-containing medium at least for 24 h, leading to the efficient cellular uptake of siRNA in cultured SKOV3-Luc cells, similar to ACO micelles. Ultimately, the PMM micelles achieved the greater gene silencing activity in the cells, compared to ACO micelles, presumably due to the more efficient endosomal escape of the siRNA payload by PEG–PAsp(DET-PMM). These results demonstrate that the fine-tuning of endosome-disrupting polymers improves the delivery efficacy of siRNA nanocarriers for enhanced gene silencing activity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements: This research was funded by JSPS through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)", JST through Center of Innovation (COI) and Adaptable and Seamless Technology Transfer Program through Target-driven R&D (A-STEP), National Institute of Biomedical Innovation (NIBIO), and the Japanese Ministry of Health, Labour and Welfare through Grants-in-Aid for Scientific Research. Part of this work was conducted in the Research Hub for Advanced Nano Characterization, The University of Tokyo, supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The authors are grateful to A. Miyoshi for their assistance with experiments.

Received: January 24, 2014; Revised: March 3, 2014; Published online: April 8, 2014; DOI: 10.1002/marc.201400049

Keywords: calcium phosphate; poly(ethylene glycol); polymeric micelles; pH-responsive polymer; siRNA

[1] J. C. Burnett, J. J. Rossi, *Chem. Biol.* **2012**, *19*, 60.
[2] J. Gruenberg, F. G. van der Goot, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 495.
[3] E. Wagner, *Acc. Chem. Res.* **2012**, *45*, 1005.
[4] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297.
[5] Z. U. Rehman, D. Hoekstra, I. S. Zuhorn, *ACS Nano* **2013**, *7*, 3767.
[6] T. Bieber, W. Meissner, S. Kostin, A. Niemann, H. P. Elsasser, *J. Controlled Release* **2002**, *82*, 441.
[7] C. W. Evans, M. Fitzgerald, T. D. Clemons, M. J. House, B. S. Padman, J. A. Shaw, M. Saunders, A. R. Harvey, B. Zdyrko, I. Luzinov, G. A. Silva, S. A. Dunlop, K. S. Iyer, *ACS Nano* **2011**, *5*, 8640.
[8] S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, A. Szewczyk, *Mol. Ther.* **2005**, *11*, 990.
[9] G. Grandinetti, N. P. Ingle, T. M. Reineke, *Mol. Pharm.* **2011**, *8*, 1709.
[10] N. Kanayama, S. Fukushima, N. Nishiyama, K. Itaka, W. D. Jang, K. Miyata, Y. Yamasaki, U. I. Chung, K. Kataoka, *ChemMedChem* **2006**, *1*, 439.
[11] K. Miyata, N. Nishiyama, K. Kataoka, *Chem. Soc. Rev.* **2012**, *41*, 2562.
[12] K. Masago, K. Itaka, N. Nishiyama, U.-I. Chung, K. Kataoka, *Biomaterials* **2007**, *28*, 5169.
[13] K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* **2008**, *130*, 16287.
[14] H. Uchida, K. Miyata, M. Oba, T. Ishii, T. Suma, K. Itaka, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* **2011**, *133*, 15524.
[15] Y. Lee, K. Miyata, M. Oba, T. Ishii, S. Fukushima, M. Han, H. Koyama, N. Nishiyama, K. Kataoka, *Angew. Chem. Int. Ed.* **2008**, *47*, 5163.
[16] F. Pittella, M. Zhang, Y. Lee, H. J. Kim, T. Tockary, K. Osada, T. Ishii, K. Miyata, N. Nishiyama, K. Kataoka, *Biomaterials* **2011**, *32*, 3106.
[17] F. Pittella, K. Miyata, Y. Maeda, T. Suma, S. Watanabe, Q. Chen, R. J. Christie, K. Osada, N. Nishiyama, K. Kataoka, *J. Controlled Release* **2012**, *161*, 868.
[18] F. Pittella, H. Cabral, Y. Maeda, P. Mi, S. Watanabe, H. Takemoto, H. J. Kim, N. Nishiyama, K. Miyata, K. Kataoka, *J. Controlled Release***2014**, *178*, 18.
[19] A. Tabaković, M. Kester, J. H. Adair, *WIREs Nanomed. Nanobiotechnol.* **2012**, *4*, 96.
[20] W. A. Blattler, B. S. Kuenzi, J. M. Lambert, P. D. Senter, *Biochemistry* **1985**, *24*, 1517.
[21] D. B. Rozema, K. Ekena, D. L. Lewis, A. G. Loomis, J. A. Wolff, *Bioconjugate Chem.* **2003**, *14*, 51.
[22] R. L. Juliano, X. Ming, O. Nakagawa, *Bioconjugate Chem.* **2012**, *23*, 147.
[23] K. Buyens, M. Meyer, E. Wagner, J. Demeester, S. C. De Smedt, N. N. Sanders, *J. Controlled Release* **2010**, *141*, 38.

Nutrition and Cancer, 67(1), 105–111

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2015.976312

Routledge
Taylor & Francis Group

Oral Nutritional Support Can Shorten the Duration of Parenteral Hydration in End-of-Life Cancer Patients: A Randomized Controlled Trial

Hiroto Ishiki
Department of Palliative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Satoru Iwase
Department of Palliative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; Data Center, Japanese Organisation for Research and Treatment of Cancer, Tokyo, Japan

Yasuaki Gyoda
Division of Home Palliative Care, Genki Healthcare Corporation, Tokyo, Japan

Yoshiaki Kanai
Department of Palliative Medicine, The University of Tokyo Hospital, Tokyo, Japan

Keisuke Ariyoshi
Division of Biostatistics, Tohoku University Graduate School of Medicine, Miyagi, Japan; Data Center, Japanese Organisation for Research and Treatment of Cancer, Tokyo, Japan

Tempei Miyaji
Department of Clinical Trial Data Management, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; Data Center, Japanese Organisation for Research and Treatment of Cancer, Tokyo, Japan

Yukiko Tahara
Data Center, Japanese Organisation for Research and Treatment of Cancer, Tokyo, Japan

Takashi Kawaguchi
Department of Practical Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

Mieko Chinzei
Department of Palliative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Takuhiro Yamaguchi
Division of Biostatistics, Tohoku University Graduate School of Medicine, Miyagi, Japan; Department of Clinical Trial Data Management, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and Data Center, Japanese Organisation for Research and Treatment of Cancer, Tokyo, Japan

Tube feeding or hydration is often considered for end-of-life cancer patients despite the negative effects on quality of life.

Submitted 4 February 2014; accepted in final form 24 August 2014.
Address correspondence to Tempei Miyaji, Department of Clinical Trial Data Management, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: +81-3-5800-9086. Fax: +81-3-5800-9087. E-mail: tmiyaji@m.u-tokyo.ac.jp
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/hnuc.

The efficacy of oral nutritional support in this setting is unknown. We conducted a randomized trial to compare the efficacies of an amino acid jelly, Inner Power® (IP), and a liquid enteral product, Ensure Liquid® (EL), in terminally ill cancer patients. We randomly assigned patients to 3 arms: EL, IP, and EL+IP. The primary endpoint was drip infusion in vein (DIV)-free survival, which was defined as the duration from nutritional support initiation to administration of parenteral hydration. Twenty-seven patients were enrolled in the study, of whom 21 were included in the intention-to-treat analysis. The median age of the subjects was 69 yr. There were significant differences between the arms with regard to the median DIV-free survival (0.5, 6.0, and 4.5 days in the EL, IP, and EL + IP arms,

respectively; $P = 0.05$). The median overall survival was 7, 9, and 8 days in the EL, IP, and EL + IP arms, respectively. IP may shorten the duration of parenteral hydration in terminally ill cancer patients and does not affect their survival.

INTRODUCTION

The oral intake of terminally ill cancer patients generally decreases gradually owing to symptoms arising from their overall clinical condition (e.g., fatigue, cachexia), gastrointestinal dysfunction (e.g., anorexia, nausea, vomiting, constipation, dysphagia), and psychological status (e.g., coma, narcosis, delirium). Artificial nutrition and hydration (ANH) is often used in hospitals. However, the procedures often have negative effects on the quality of life (QoL) of patients. Despite this, 53–88% of patients receive either artificial nutrition or hydration during the terminal phase of cancer (1). There is no doubt that tube placement is painful and ANH requires a restriction of body movements during the procedures. Controversy exists about whether ANH is necessary for cancer patients. Although ANH is said to have advantages, including prolonged survival and improvement of symptoms caused by dehydration (2,3), it requires tube placement and can aggravate fluid retention symptoms (4). Furthermore, there is an opinion that a decreased oral intake is a natural process of dying and that we should perhaps not take a medical approach toward such as decreased intake (5).

According to guidelines for the care of incurable cancer patients, ANH should be started when patients cannot eat orally and may die because of malnourishment, rather than because of their cancer (6). Before ANH is started, a modified diet (e.g., form, volume, taste) should be considered. However, there is no best practice consensus regarding form, ingredients, or volume (6,7). As a consequence, we are uncertain about what to serve patients when their oral intake is decreasing. In Japan, liquid enteral nutrition is often provided to such patients in general hospitals (8,9). Although these liquids are useful in terms of health preservation, care management, and cost (because they are covered by Japanese social insurance), patient adherence to these products is not always good. Terminally ill cancer patients are frail and can easily aspirate liquid feeds, which frequently leads to fatal pneumonia (10), and they may also suffer from diarrhea if they consume high-calorie, high-fat liquids. For frail patients at risk of aspiration, thickened liquids and pudding-like forms are a safer diet than normal food and liquids (11).

Inner Power® (IP), an amino acid jelly, does not contain fat and its semisolid form is suitable for preventing aspiration. We thought that a prolonged duration of oral food intake by patients would lead to an improvement in QoL, especially by reducing the use of ANH and lessening its impact on activities of daily living. We hypothesized that 1) IP would prolong the duration of oral intake; 2) IP would shorten the duration of artificial hydration (AH); and 3) IP would not affect survival rates. This exploratory study aimed to investigate the efficacy of this amino acid jelly in the terminal phase of cancer patients with decreased oral intake.

METHODS

Study Design

This study was an open-label, multicenter, randomized controlled study with 3 treatment arms. Randomization was performed at the central data center using an allocation table. Patients were recruited at local palliative care units at 2 medical institutions in and around Tokyo, Japan.

Eligibility Criteria

Eligibility criteria were as follows: 1) provision of written informed consent; 2) age >20 yr; 3) cancer diagnosis; 4) incurable disease by any treatment, including surgery, radiotherapy, or chemotherapy; 5) ability to eat orally when enrolled in the study; 6) life expectancy >2 wk; 7) Eastern Cooperative Oncology Group performance status between 0 and 3; and 8) inpatient status, or being eligible for hospital admission.

Patients were regarded as ineligible if they met 1 of the following criteria: 1) presence of dysphagia; 2) gastrointestinal obstruction or stricture; 3) cognitive problems; and 4) allergy to milk or soybean.

Treatment

For the oral nutritional support protocol, IP and a liquid enteral nutrition product, Ensure Liquid® (EL), were used. The compositions of these products are shown in Table 1. The product information for IP and EL is available on the companies' websites (12,13).

Patients were randomly allocated to 1 of 3 arms: EL, IP, or EL + IP. Observations were started soon after registration. When a patient's oral intake decreased to 10% or less of their normal daily intake, we started the oral nutritional support allocated to each arm. For this, patients in the EL arm received EL and patients in the IP arm received IP. Patients in the EL + IP arm received EL initially, and when the amount of EL intake became insufficient (i.e., less than 125 ml/day), they

TABLE 1
Compositions of the products

	Ensure Liquid®	Inner Power®
Volume	250 ml	125 g
Protein	8.8 g	1.7 g
Fat	8.8 g	0 g
Carbohydrate	34.3 g	33 g
Calorie	250 kcal	139 kcal

received IP. If the total amount of daily nutritional support decreased to less than 125 ml, AH at 500–1000 ml/day was started and continued until the patient's death.

Endpoints

The primary endpoint was drip infusion in vein (DIV)-free survival. This was defined as the duration between the initiation of nutritional support and the start of AH. Secondary endpoints included overall survival, duration of parenteral hydration, and duration of nutritional support. The relationships among these endpoints are depicted in Fig. 1. Other secondary endpoints included adverse events and QoL measures. Adverse events were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events v4.0 (NCI-CTCAE v4.0) (14). QoL data were obtained using the Japanese version of the EORTC QLQ-C15-PAL questionnaire, and scored according to its scoring manuals (15,16). The EORTC QLQ-C15-PAL consists of 15 items and represents an abbreviated version of the EORTC QLQ-C30 (version 3.0) developed for patients with advanced, incurable, and symptomatic cancer (median life expectancy of less than a few months). Each of the scales and single-item measures ranged from 0 to 100. A high scale score represents a higher response level, meaning that a high score for the functional scale and global health status represents a healthy level of functioning and a high QoL. Meanwhile, a high score for a symptom scale or item represents a high level of symptomatology or problems (16). The validity of the Japanese version of EORTC QLQ-C15-PAL has been confirmed (17). In addition, for EORTC QLQ-C15-PAL, a questionnaire about diarrhea was added for QoL data collection, and its wording was created by reference to the diarrhea item of EORTC QLQ-PAL-C30. The data for adverse events and QoL were collected on a weekly basis after patient registration.

Statistical Analysis

For each variable, descriptive statistics were presented and compared between groups. Analyses were performed with JMP Pro 9 and SAS version 9.3. (SAS Institute Inc., Cary, NC).

Ethical Considerations

This study was performed in accordance with the Helsinki Declaration and the Japanese ethical guidelines for clinical research (18,19). The study protocol was reviewed and approved by the ethical review committee of the not-for-profit organization TACTICS. All patients provided written informed consent before entry into the study. This trial was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) under UMIN000006936 (20).

RESULTS

Patient Characteristics

Between August 2011 and December 2012, 27 patients were enrolled and 9 patients were allocated to each arm. Six patients dropped out because of ineligibility ($n = 2$), or because they received a nonprotocol treatment ($n = 1$), withdrew consent ($n = 1$), relocated to a hospice ($n = 1$), or died before commencement of a protocol treatment ($n = 1$). Thus, 21 patients were included in an intention-to-treat analysis (Fig. 2), and all of these patients were followed until death. The patient characteristics are shown in Table 2. The median age and age range of the patients in the EL, EL + IP, and IP arms were 69 (66–81), 73 (59–88), and 77 (54–89) years, respectively. Most of the patients (95.2%) had a poor performance status (PS 3). The primary cancer sites were the lung

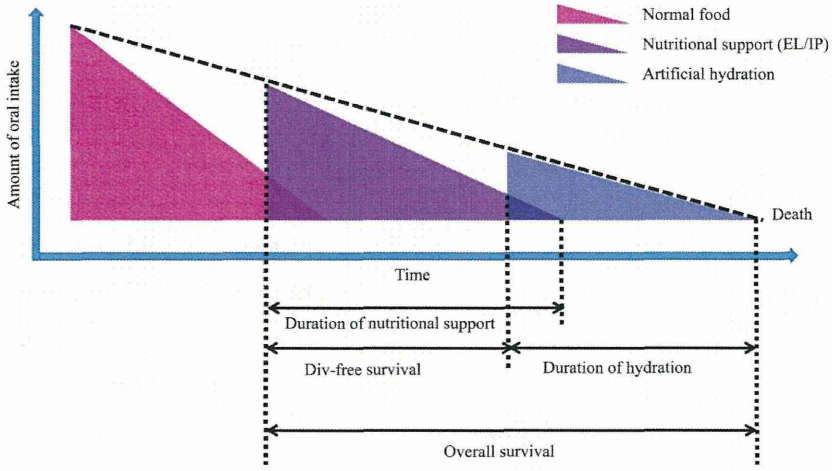


FIG. 1. Relationships among primary and secondary endpoints EL/IP = Ensure Liquid®/Inner Power®.

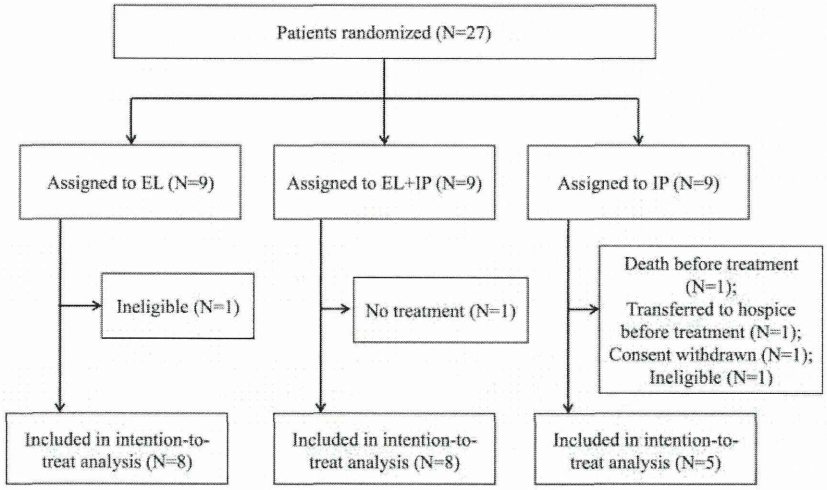


FIG. 2. Consort diagram. EL = Ensure Liquid®; IP = Inner Power®.

TABLE 2
Patient characteristics

	EL ($n = 8$)	EL + IP ($n = 8$)	IP ($n = 5$)
Gender (male/female)	4/4	2/6	4/1
Median age (range)	69 (66-81)	73 (59-88)	77 (54-89)
Performance status (1/3)	0/8	0/8	2/4
Primary site			
Lung	2	2	2
Hepatobiliary	2	2	1
Breast	2	1	1
Colorectal	1	1	1
Head and neck	1	1	0
Cervix	0	1	0

EL = Ensure Liquid®; IP = Inner Power®.

($n = 6$), hepatobiliary ($n = 5$), breast ($n = 4$), colorectal ($n = 3$), head and neck ($n = 2$), and cervix ($n = 1$).

Efficacy

For the primary endpoint, the median DIV-free survival was 0.5 days in the EL arm, 4.5 days in the EL + IP arm, and 6.0 days in the IP arm. For the primary endpoint analysis of DIV-free survival, we used a median test to compare whether the 3 groups differed with respect to their median values. There were significant differences between the 3 arms ($P = 0.050$) (Fig. 3).

The median overall survival was 7, 8, and 9 days (Fig. 4) and the median duration of parenteral hydration was 3.5, 4, and 3 days in the EL, EL + IP, and IP arms, respectively (Fig. 5).

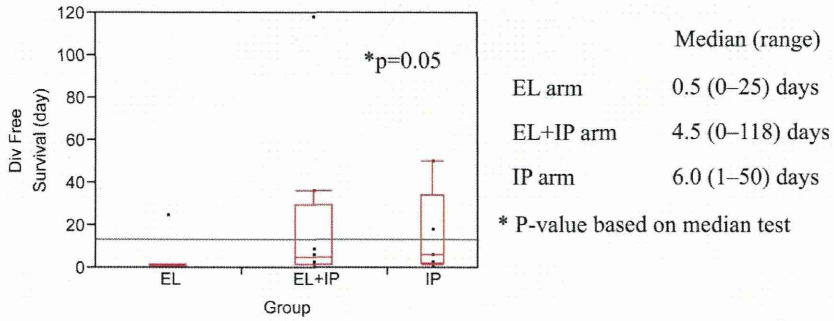


FIG. 3. Drip infusion in vein (DIV)-free survival. EL = Ensure Liquid®; IP = Inner Power®.