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Synthetic Polyamines to Regulate mRNA Translation through the Preservative Binding of Eukaryotic Initiation Factor 4E to the Cap Structure

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Supporting Information

ABSTRACT: Polyion complexes (PICs) of mRNA with synthetic polyamines are receiving increasing attention as mRNA delivery vehicles, and the search for polyamine structure maximizing the translational efficiency of complexed mRNA becomes a critical research topic. Herein, we discovered that fine-tuning of the protonation status of synthetic polyamines can regulate mRNA translation through the preservative binding of eukaryotic initiation factor 4E to m^{7}GpppN (cap structure) on the 5′ end of mRNA. A series of polyamines with varied numbers of aminoethylene repeats in their side chains were prepared by an aminolysis reaction of poly(β-benzyl-L-aspartate) and paired with mRNA to form PICs. PICs formed from polyamines with higher numbers of aminoethylene repeats preserved the original translational efficiency to naked mRNA, whereas the efficiency significantly dropped by decreasing the number of aminoethylene repeats in the polyamines. Immunoprecipitation assays using anti-eIF4E antibodies revealed that the binding affinity of eIF4E to the cap structure of mRNA in the PIC was sensitive to the number of charged aminoethylene repeats in the polyamine side chain and was strongly correlated with their translational efficiency. These results indicate that the fine-tuning of the polyamine structure plays a critical role in maximizing the translational efficiency of mRNA in the PICs having potential utility as mRNA delivery vehicles.

Regulation of mRNA translation is a fundamental cellular process. Translational initiation, which involves recruitment of the ribosome to the 5′ end of mRNA, is recognized as a rate-liming step in mRNA translation. Binding of eukaryotic initiation factor 4E (eIF4E) to m^{7}GpppN (cap structure) on the 5′ end of mRNA is critical for the regulation of translational initiation.[1,2] Here, we report a discovery that fine-tuning of the chemical structure of synthetic polyamines can regulate the translation process through polyion complex (PIC) formation with mRNA to allow the smooth binding of eIF4E to the cap structure.

Inspired by the structure of natural polyamines, including spermine and spermidine, we prepared cationic N-substituted polyaspartamides with varying numbers of aminoethylene repeats in the side chain by an aminolysis reaction of poly(β-benzyl-L-aspartate) (PBLA; degree of polymerization [DP] = 102, M_w/M_n = 1.06). Ethylenediamine (EDA), diethylenetriamine (DET), triethylenetetramine (TET), and tetraethylenepentamine (TEP) were used as reactants to obtain poly[*N*-(2-aminoethyl)aspartamide] (PAsp(EDA)), poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PAsp(DET)), poly(*N*″-{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}aspartamide) (PAsp(TET)), and poly[*N*‴-(*N*″-{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}-2-aminoethyl)aspartamide] (PAsp(TEP)), respectively (Figure 1). Note that these cationic N-substituted polyaspartamides were prepared from the same platform polymer, PBLA, and therefore had the similar degree of polymerization and molecular weight distribution. In addition, poly-L-lysine (PLL) with a similar DP (= 98) was synthesized as a control polycation.

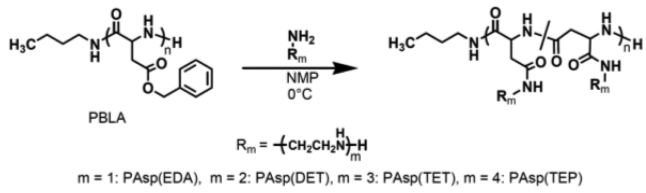

Figure 1. Synthesis scheme of N-substituted polyaspartamides.

Buffered solutions of mRNA encoding Gaussia luciferase (GLuc; 50 ng/μL) and the synthetic polyamines described above were mixed at a residual molar ratio of protonated amino groups in the polymer to phosphate groups in mRNA (N^+/P ratio) = 1.5 to prepare PICs (mRNA polyplexes). The protonation degrees

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of the synthetic polyamines at pH 7.2 (cytoplasmic pH) were determined from titration experiments (PAsp(EDA): 95%, PAsp(DET): 53%, PAsp(TET): 58%, and PAsp(TEP): 52%) as reported elsewhere.[3,4] The residual amino groups of PLL should be fully protonated because of its high pK_a = 10.44.[5] The N^+/P ratio of each polyplex was calculated from the determined protonation degree. All of these polyplexes had comparable size of ~60 nm diameter (SI Table 1) with unimodal size distribution (SI Figure 1) as determined by dynamic light scattering.

The translational behaviors of the mRNA polyplexes were evaluated using a rabbit reticulocyte lysate system (Promega, Madison, WI, USA). This cell-free assay permitted evaluation of the pure translational efficiency of mRNA polyplexes without interference from endogenous genomic DNA and mRNA, which may exchange with mRNA in the polyplexes. GLuc expression was measured by luciferase assays after 90 min of incubation (Figure 2a). As shown in Figure 2a, mRNA polyplexes from N-substituted polyaspartamides possessing three and four aminoethylene repeats in their side chains (PAsp(TET) and PAsp(TEP)) exhibited GLuc expression comparable to naked mRNA, even in the complex form. This was in a sharp contrast with the significant reduction in expression observed for polyplexes from those with one or two aminoethylene repeats (PAsp(EDA) and PAsp(DET)). A similar reduction was also observed for the polyplex from PLL. These results demonstrate that the structure of polyamines indeed influenced the translational efficacy of mRNA. It is worth noting that PAsp(TET) and PAsp(TEP) did not interfere with the translation scheme of mRNA in the polyplexes.

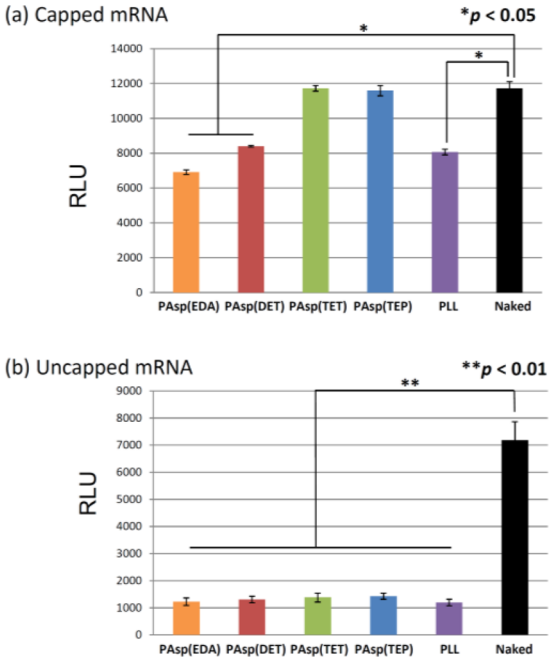

Figure 2. Translational efficiencies of GLuc mRNA polyplexes prepared from N-substituted polyaspartamides and PLL. (a) Capped and (b) uncapped mRNA. * and ** indicate significantly lower expression compared with capped (a) or uncapped (b) naked mRNA evaluated by Student's *t*-test.

In order to get insight into the mechanisms involved in mRNA translation in the polyplexes, we focused on the initiation process of translation, which is considered a rate-limiting step for mRNA translation.[1,2] On the 5′ end of mRNA, 7-methylguanosine is linked by a 5′-5′-triphosphate bridge to the first nucleotide, called the cap structure. At the first step of mRNA translation, eIF4E recognizes the cap structure. Subsequently, eIF4E binds with eIF4A and eIF4G, resulting in the construction of eIF4F complex. This complex recruits the ribosome to mRNA. Herein, we synthesized uncapped mRNA from the same GLuc template to investigate the role of the cap structure on the translation of mRNA polyplexes and measured the translational efficiency of uncapped mRNA polyplexes in the cell-free system. Naked uncapped mRNA showed about half of the expression observed for naked capped mRNA, consistent with a previous report.[6] Interestingly, all of the uncapped mRNA polyplexes showed significantly lower expression than uncapped naked mRNA ($p < 0.01$; Figure 2b), indicating that the capped mRNA polyplexes were mainly translated through the cap-dependent pathway. Translation of uncapped mRNA requires the direct binding of ribosomes to mRNA without cap-dependent initiation.[6] Therefore, the poor expression of uncapped mRNA polyplexes suggests that all of the polyamines strongly bind to uncapped mRNA to inhibit direct binding of the ribosome with mRNA, excluding the possibility that the differences in translational efficiency shown in Figure 2(a) may derive from differential disassembly of the polyplexes during the cell-free assay.

In a sharp contrast with the poor expression of uncapped mRNA polyplexes, capped mRNA polyplexes from PAsp(TET) and PAsp(TEP) exhibited the same level of expression as naked capped mRNA, indicating that these polyamines did not inhibit cap-dependent initiation. Cap-dependent initiation requires the binding of eIF4E to the cap structure.[1,2] Hence, the binding affinity of eIF4E with the cap structure in the cell-free system was evaluated by co-immunoprecipitation using anti-eIF4E antibodies. Notably, the co-immunoprecipitation results (Figure 3) were consistent with the GLuc assay results shown in Figure 2a. Capped mRNA in the PAsp(TET) and PAsp(TEP) polyplexes, which showed similar levels of expression to naked capped mRNA, possessed a binding affinity similar to that of naked capped mRNA. Alternatively, decreased affinity was obvious for PAsp(EDA), PAsp(DET), and PLL polyplexes (Figure 3). The consistent results of the GLuc assays with the co-immunoprecipitation indicate that the association affinity between the cap structure and eIF4E is critical for the translation of mRNA in the

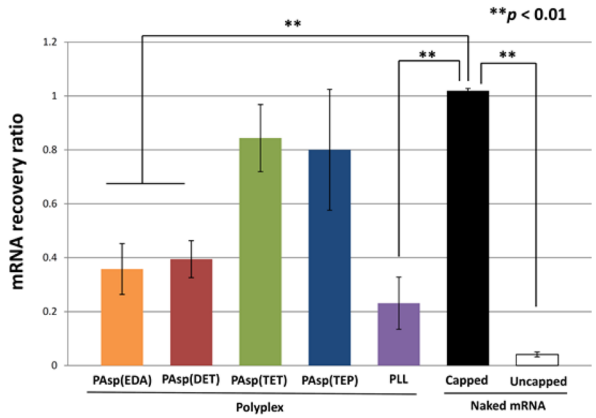

Figure 3. Relative fold differences in mRNA bound to eIF4E. mRNAs and polyplexes were co-immunoprecipitated from the cell-free system using anti-eIF4E antibodies. mRNA recovery ratios were normalized to that of naked capped mRNA (black bar). ** indicates significant lower mRNA recovery ratio than naked capped mRNA ($p < 0.01$).

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polyplexes. Worth noting is that the polyamines with particular structures, i.e., PAsp(TET) and PAsp(TEP), did not inhibit the binding of eIF4E to the cap structure of mRNA in the polyplexes. Thus, it is reasonable to assume that, in PAsp(TET) and PAsp(TEP) polyplexes, the cap structure of mRNA can still take a conformation available for eIF4E binding and the following process of the eIF4F complex formation with eIF4A and eIF4G, recruiting the ribosome for initiation of protein synthesis. In addition, the co-immunoprecipitation assay using uncapped mRNA polyplexes was performed in the same condition. All uncapped mRNA polyplexes showed poor mRNA recovery ratios, suggesting that the nonspecific binding of eIF4E to polyplexes could be negligible in this experimental condition (SI Figure 2).

The poor expression of uncapped mRNA polyplexes, regardless of polyamine structure shown in Figure 2b, indicates that all of the examined polyamines, including PAsp(TET) and PAsp(TEP), interact with mRNA strongly enough to inhibit direct ribosome binding with mRNA strands. Thus, the dependency of polyamine structure observed in translational efficiency of capped mRNA (Figure 2a) as well as in eIF4E binding to the cap structure (Figure 3) cannot simply be explained by a difference in the ensemble electrostatic affinity between the polyamine and mRNA strands, but may involve distinctive interaction localized in the region of the cap structure, keeping it available for eIF4E binding. To get insight into the mechanisms involved in the distinctive interaction of the cap structure with polyamines, we focused on the protonation status of the aminoethylene repeats in the side chain of polyamines. This status can be estimated from the titration results of polyamines, as reported previously.[3,4] The estimated protonated structures of each polyamine at pH 7.2 (cytoplasmic pH) are summarized in Figure 4. PAsp(TET) and PAsp(TEP),

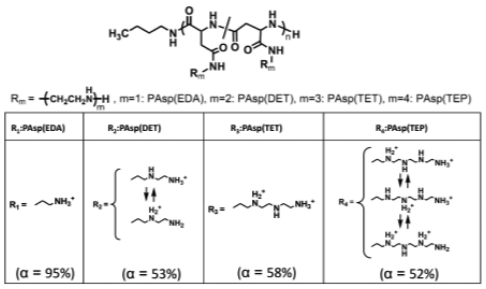

Figure 4. Estimated protonation structures of amino groups in N-substituted polyaspartamide side chains at pH 7.2. α indicates the percentage of protonated amino groups at this condition.

composing polyplexes with preservative translational efficiency and eIF4E binding ability, are characterized by the presence of two positive charges (two protonated amino groups) per side chain. In contrast, PAsp(EDA) and PAsp(DET), composing polyplexes with significantly lowered translational efficiency and poor binding ability to eIF4E, possess only a single positive charge (single protonated amino group) per side chain. Although, PLL, which is known as a potent nucleic acid binder and used here as a control polycation, has no amide linkage in the side chain unlike other polyamines derived from PBLA, it shares the similarity with PAsp(EDA) in terms of having a single protonated amino group in the side chain of every residual units and is also categorized into the group with the poor translational efficiency and binding ability to eIF4E. Hence, it may be reasonable to assume that the number of the positive charges per side chain may play a key role in the binding of eIF4E to the cap structure.

In order to confirm the importance of multiprotonated side chain on the translation of mRNA polyplexes, we synthesized a poly{*N*′-[*N*-(3-aminopropyl)-3-aminopropyl]aspartamide} (PAsp(DPT)) (Figure 5) and used it to prepare mRNA

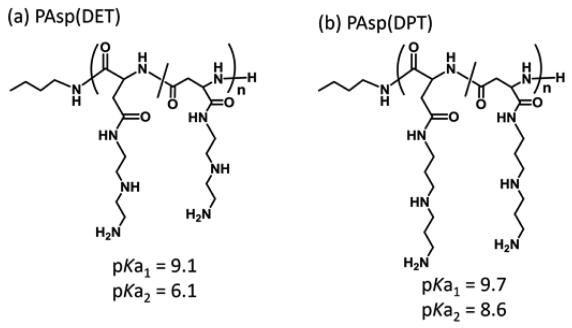

Figure 5. Chemical structures and pK_a values of PAsp(DET) and PAsp(DPT).

polyplexes. Similarly to PAsp(DET), PAsp(DPT) possesses two amino groups in the side chain. Nevertheless, even at pH 7.2, both of these amino groups of PAsp(DPT) are in double-protonated form because of their high pK_a values (pK_{a1} = 9.7 and pK_{a2} = 8.6), which is in a sharp contrast with PAsp(DET) characterized by the monoprotonated form of diaminoethane unit in the side chain due to the lowered pK_{a2} value (pK_{a1} = 9.1 and pK_{a2} = 6.1).[7,8] The differences in pK_a arise because the propyl spacer between the two amino groups in PAsp(DPT) relieves the electrostatic repulsion to allow diaminopropane unit to take a double-protonated structure at neutral pH. Accordingly, comparison between PAsp(DET) and PAsp(DPT), both having two amino groups in their side chain with a difference in protonation status, further reveals an indispensable role of multiprotonated side chain structure in the translation process of mRNA polyplexes. Interestingly, the PAsp(DPT) polyplex achieved translational efficiency (Figure 6a) and eIF4E binding ability (Figure 6b) comparable to naked mRNA and significantly

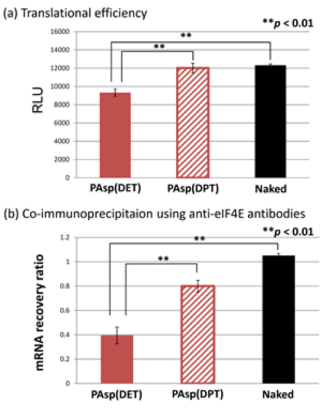

Figure 6. (a) Translational efficiencies of GLuc capped mRNA polyplexes prepared from PAsp(DET) and PAsp(DPT). (b) Relative fold differences in mRNA bound to eIF4E. PAsp(DET) and PAsp(DPT) polyplexes were co-immunoprecipitated from the cell-free system using anti-eIF4E antibodies. mRNA recovery ratios were normalized to that of naked capped mRNA (black bar). ** indicates significantly higher expression or mRNA recovery ratio compared with PAsp(DET) polyplexes ($p < 0.01$).

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higher than PAsp(DET) polyplex. These results are consistent with our hypothesis that the presence of positively charged repeats in the side chain may be a critical factor for efficient translation through the preservative binding of eIF4E to the cap structure of mRNA in the polyplexes.

Although detailed insights into the molecular mechanisms have yet to be clarified, it may be worth discussing the possible interaction of the cap structure with the positively charged repeats in the side chain of polyamines. We here notice that the m^7G group in the cap structure is protonated at cytoplasmic pH and is the only positively charged group in an mRNA strand.[9−11] Then, it may be reasonable to assume that this positive charge would generate electrostatic repulsion against the positively charged repeat in the side chains of N-substituted polyaspartamides. The cap structure locates at the 5′ end of mRNA and presumably has flexibility high enough to expose itself to the exterior, so as to minimize electrostatic repulsion against protonated amino groups in complexed polyamines. Alternatively, triphosphate group, which bridges the m^7G group to the first nucleotide unit at the 5′ end of mRNA, possesses three negative charges. The side chain with positively charged repeat could have more opportunity to strongly bind with this negatively charged repeat of triphosphate group than single-protonated side chain and eventually may anchor the cap structure on the exterior of polyplexes to allow eIF4E binding even in the form of polyplexes.

Finally, it should be noted that mRNA has attracted increased attention in recent years as a novel therapeutic agent to treat intractable diseases, such as enzyme deficiencies.[12−14] Nevertheless, there are still issues in stability, immunogenicity, and translational efficiency *in vivo*, and accordingly, delivery systems of mRNA to compromise these issues have strongly been demanded. The results of the present study clarify the optimized chemical structure of polyamines to preserve the function of the cap structure in mRNA, thereby providing a novel principle for the chemical design of polycations feasible as polyplex carriers for mRNA delivery. Indeed, we recently confirmed that polyplex micelles composed of PAsp(TET), possessing multiprotonated side chain structure, can deliver mRNA encoding a cartilage-anabolic transcription factor to chondrocytes *in vivo*, successfully treating the osteoarthritis in a mouse model.[15]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b11726.

Materials and methods, Table 1, and Figures 1 and 2 (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sonenberg, N.; Hinnebusch, A. G. *Cell* **2009**, *136*, 731−745.
(2) Jackson, R. J.; Hellen, C. U.; Pestova, T. V. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 113−127.
(3) Uchida, H.; Miyata, K.; Oba, M.; Ishii, T.; Suma, T.; Itaka, K.; Nishiyama, N.; Kataoka, K. *J. Am. Chem. Soc.* **2011**, *133*, 15524−15532.
(4) Uchida, H.; Itaka, K.; Nomoto, T.; Ishii, T.; Suma, T.; Ikegami, M.; Miyata, K.; Oba, M.; Nishiyama, N.; Kataoka, K. *J. Am. Chem. Soc.* **2014**, *136*, 12396−12405.
(5) Pereira, F. C.; Fogg, A. G.; Zanoni, M. V. *Talanta* **2003**, *60*, 1023−1032.
(6) Svitkin, Y. V.; Ovchinnikov, L. P.; Dreyfuss, G.; Sonenberg, N. *EMBO J.* **1996**, *15*, 7147−7155.
(7) Miyata, K.; Oba, M.; Nakanishi, M.; Fukushima, S.; Yamasaki, Y.; Koyama, H.; Nishiyama, N.; Kataoka, K. *J. Am. Chem. Soc.* **2008**, *130*, 16287−16294.
(8) Suma, T.; Miyata, K.; Ishii, T.; Uchida, S.; Uchida, H.; Itaka, K.; Nishiyama, N.; Kataoka, K. *Biomaterials* **2012**, *33*, 2770−2779.
(9) Marcotrigiano, J.; Gingras, A. C.; Sonenberg, N.; Burley, S. K. *Cell* **1997**, *89*, 951−961.
(10) Niedzwiecka, A.; Stepinski, J.; Darzynkiewicz, E.; Sonenberg, N.; Stolarski, R. *Biochemistry* **2002**, *41*, 12140−12148.
(11) Casanova, E.; Priego, E. M.; Jimeno, M. L.; Aguado, L.; Negri, A.; Gago, F.; Camarasa, M. J.; Pérez-Pérez, M. J. *J. Org. Chem.* **2010**, *75*, 1974−1981.
(12) Kormann, M. S.; Hasenpusch, G.; Aneja, M. K.; Nica, G.; Flemmer, A. W.; Herber-Jonat, S.; Huppmann, M.; Mays, L. E.; Illenyi, M.; Schams, A.; Griese, M.; Bittmann, I.; Handgretinger, R.; Hartl, D.; Rosenecker, J.; Rudolph, C. *Nat. Biotechnol.* **2011**, *29*, 154−157.
(13) Zangi, L.; Lui, K. O.; von Gise, A.; Ma, Q.; Ebina, W.; Ptaszek, L. M.; Später, D.; Xu, H.; Tabebordbar, M.; Gorbatov, R.; Sena, B.; Nahrendorf, M.; Briscoe, D. M.; Li, R. A.; Wagers, A. J.; Rossi, D. J.; Pu, W. T.; Chien, K. R. *Nat. Biotechnol.* **2013**, *31*, 898−907.
(14) Sahin, U.; Karikó, K.; Türeci, O. *Nat. Rev. Drug Discovery* **2014**, *13*, 759−780.
(15) Aini, H.; Itaka, K.; Fujisawa, A.; Uchida, H.; Uchida, S.; Fukushima, S.; Kataoka, K.; Saito, T.; Chung, U. I.; Ohba, S. *Sci. Rep.* **2016**, *6*, 18743.

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