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Development of Flexible Polycation-Based mRNA Delivery Systems for In Vivo Applications †

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Abstract: mRNA is a promising therapeutic nucleic acid though effective delivery systems are equired for its broad application. Polyion complex (PIC) micelles loading mRNA via polyion complexation with block catiomers are emerging as promising carriers for mRNA delivery while the PIC stability has been limited so far. Controlling the binding of polycations to mRNA could affect micelle stability. Nevertheless, the impact of binding affinity between polycations and mRNA on the function of mRNA-loaded PIC micelles (mRNA/m) remains unknown. Herein, we review our recent orthogonal approaches controlling the stiffness and the valency of polycations to improve the performance of mRNA/m toward enhancing stability and delivery efficiency. Thus, block catiomers with contrasting flexibility were developed to prepare mRNA/m. The flexible catiomer stabilized mRNA/m against enzymatic attack and polyanion exchange compared to the rigid catiomer, promoting protein translation in vitro and *in vivo*, and prolonged mRNA bioavailability in blood after systemic injection. Based on these observations, we also developed flexible catiomers with different valency. The guanidinated catiomer stabilized mRNA/m compared to the aminated catiomers, facilitating intracellular delivery and eventual gene expression. Our findings indicate the importance of controlling the polymer binding to mRNA for developing flexible polycation-based systems directed to in vivo applications.

Keywords: block copolymer; in vivo; mRNA delivery; polymeric micelles; RNA therapeutics

1. Introduction

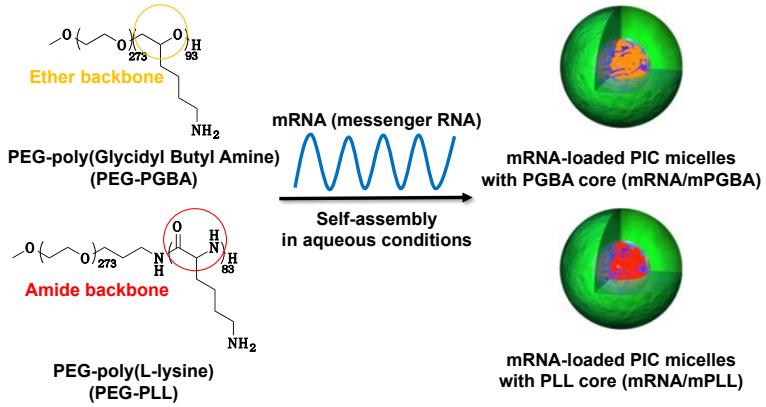
Messenger RNA (mRNA) is a promising therapeutic oligonucleotide though its broad application as a therapeutic agent is confronted by several challenges, including its rapid degradation by nucleases and limited cellular uptake [1]. Thus, the development of carrier systems capable of protecting mRNA is essential for mRNA-based therapies. Polyion complex (PIC) micelles comprising block copolymers with poly(ethylene glycol) (PEG) and polycation segments are promising nanocarriers for mRNA delivery [2]. These micelles can load mRNA in the core via electrostatic interaction with the polycation blocks, protecting it from the harsh biological environments by the PEG corona [3,4]. However, PIC micelles can be disassembled by biomolecules such as glycosaminoglycan existing as polyanions in kidney basement membrane [5]. Thus, several systems including the introduction of hydrophobic groups into the core of mRNA/m [6] and the crosslinking

of polymers with stimuli-responsive bonds [7] were developed to improve the stability of mRNA/m against polyelectrolytes though strategies enhancing the stability of mRNA/m by increasing the binding affinity between polycations and mRNA remain unexplored. Herein, we present our recent progress to develop PIC-based delivery systems by investigating the effect of the polycation flexibility and the valency on the stability and delivery efficiency of mRNA/m. Particularly, we have developed complemental block catiomers with different flexibility and valency to assemble mRNA/m. The physicochemical properties of these micelles and their delivery efficacies were investigated.

2. Results and Discussion

2.1. The Effect of Polymer Flexibility on the Stability and the Performance of mRNA/m

Recent observations by molecular dynamics simulation revealed that flexible polycations can increase the binding affinity to DNA and double stranded RNAs by promoting water release during the complexation [8]. Thus, we investigated the effect of polymer flexibility on the stability and the performance of mRNA/m by synthesizing PEG-poly(glycidyl butyl amine) (PEG-PGBA) with flexible polyether backbone and PEG-poly(L-Lysine) (PEG-PLL) with rigid polyamide backbone, and assembling mRNA/m to correlate with their performance in vitro and in vivo (Scheme 1) [9].



Scheme 1. Preparation of mRNA/m after complexation with PEG-PGBA (mRNA/mPGBA) or PEG-PLL (mRNA/mPLL). Reproduced with permission from ref [9].

By isothermal titration calorimetry (ITC), mRNA/m from PEG-PGBA (mRNA/mPGBA) showed lower formation enthalpy and higher formation entropy compared to mRNA/m from PEG-PLL (mRNA/mPLL), indicating flexible polyether backbone increased the contact area between PGBA segments and mRNA, and promoted the water release during the ionic pair formation, resulting in lower free energy of mRNA/mPGBA and higher binding affinity of PEG-PGBA to mRNA (Table 1). This thermodynamically stable mRNA/mPGBA protected mRNA against polyanion exchanges and nuclease attacks, indicating mRNA/mPGBA is stable in the biological settings. This enhanced stability resulted in augmented cellular uptake in cultured cells (Figure 1a) and efficient gene expression in vitro and in vivo (Figure 1b). After intravenous injection, the mRNA/mPGBA showed longer circulation in blood compared to the mRNA/mPLL (Figure 1c).

Table 1. Thermodynamic parameters for mRNA/m formation. Reproduced with permission from ref 9.

Micelles	ΔH [kcal/mol] [a]	ΔS [cal/(mol·K)] [a]	ΔG [kcal/mol] [a]	K_A [M^{-1}] [a]
mRNA/mPGBA	6.07 ± 0.06	57.7 ± 0.5	57.7 ± 0.5	(1.40 ± 0.51) × 10^8
mRNA/mPLL	7.00 ± 0.19	52.8 ± 0.2	52.8 ± 0.2	(2.59 ± 0.54) × 10^6

[a] Determined by ITC.

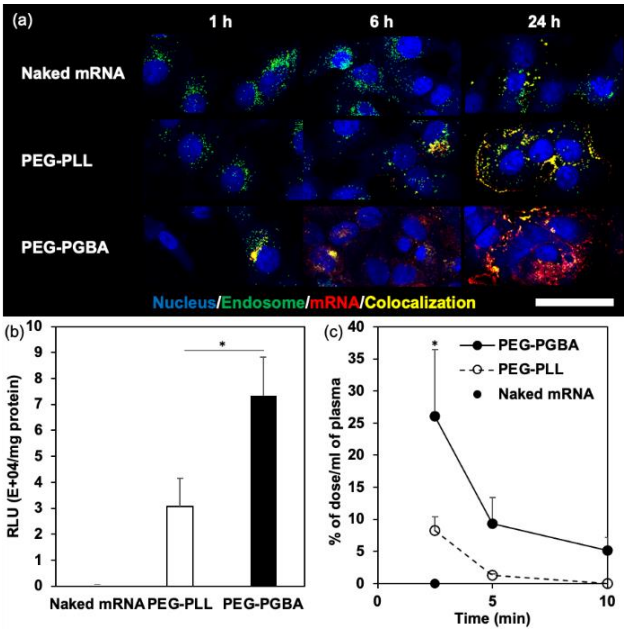
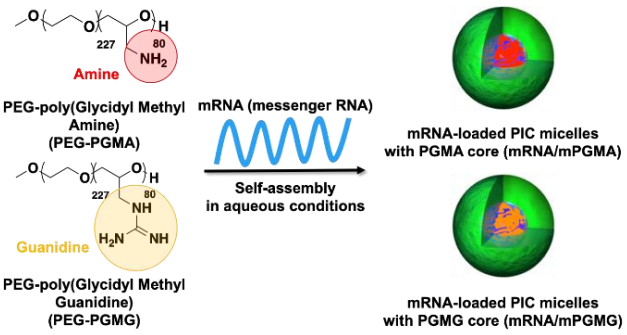


Figure 1. (**a**) Fluorescence in cultured HuH-7 cells with micelles loading Cy5-labeled mRNA, observed by confocal laser scanning microscopy (CLSM). Scale bar, 50 μm; (**c**) Bioluminescence in mouse lungs after pulmonary administration of micelles loading GLuc mRNA. Data shown as the mean ± S.D. (n = 7). (**d**) Plasma concentration of mRNA after intravenous injections of mRNA/m, determined by qRT-PCR. Data shown as the mean ± S.D. (n = 5). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ determined by Student's t-test. Reproduced with permission from ref. [9].

These results indicate that polymer flexibility affects the thermodynamical parameters of mRNA/m by facilitating the polyion complexation with flexible mRNA to enhance the stability of mRNA/m against polyanion and enzymes as biological barriers, resulting in high performance of mRNA/m in vitro and *in vivo*. Our observations highlight the importance of controlling the polymer structure and developed flexible polycations can be a platform for effective mRNA delivery.

2.2. The Effect of Valency between Polycations and mRNA on the Stability and the Performance of mRNA/m

Based on our flexible polycations as a platform technology, we attempted to develop a new polymeric system for efficient mRNA delivery. Recent approaches performed that guanidinated polycations stabilized DNA-loaded PIC micelles [10] or PICsomes [11] by the multivalent interactions between guanidine groups and phosphate groups. Thus, we examined the effect of valency between polycations and mRNA on the stability and the performance of mRNA/m by developing PEG-poly(glycidyl methyl guanidine) (PEG-PGMG) with guanidine pendants and PEG-poly(glycidyl methyl amine) (PEG-PGMA) with amine pendants, and preparing mRNA/m to investigate the relationship with their performance in vitro (Scheme 2) [12].



Scheme 2. Preparation of mRNA/m after complexation with PEG-PGMG (mRNA/mPGMG) or PEG-PGMA (mRNA/mPGMA). Reproduced with permission from ref. [12].

mRNA/m from PEG-PGMG (mRNA/mPGMG) encapsulated mRNA at higher concentration of heparin as polyanions compared to mRNA/m from PEG-PGMA (mRNA/mPGMA), indicating the multivalent interactions between guanidinium in PGMG segments and phosphate in mRNA stabilized the core of mRNA/m. This stable mRNA/mPGMG protected mRNA payload against urea attacks and enzymatic degradation, indicating mRNA/mPGMG is stable in biological environments. This augmented stability resulted in facilitated intracellular delivery (Figure 2a,b) and efficient protein translation in cultured cells (Figure 2c).

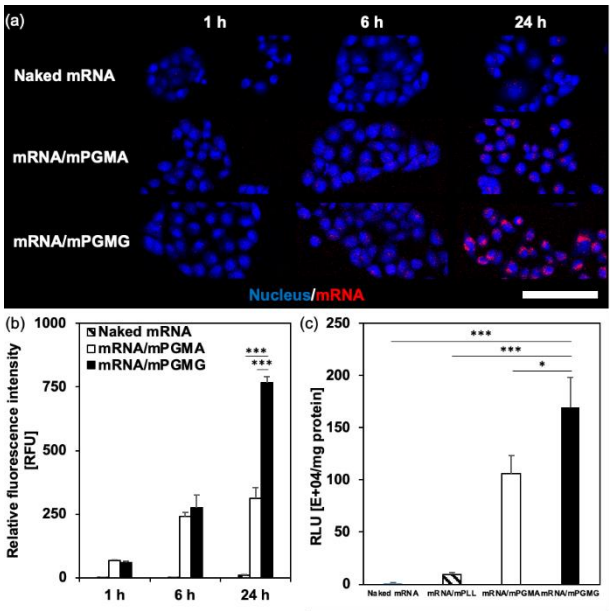


Figure 2. (**a**) Fluorescence in cultured HuH-7 cells with micelles loading Cy5-labeled mRNA, observed by confocal laser scanning microscopy (CLSM). Scale bar, 50 μm; (**b**) Quantification of Cy5 fluorescence in HuH-7 cells based on the CLSM images, including those shown in a. Data shown as the mean ± S.D. (n = 4). (**c**) mRNA introduction efficiency in cultured cells. HuH-7 cells were incubated with naked *GLuc* mRNA or mRNA/m loading *GLuc* mRNA. Data shown as the mean ± S.D. (n = 6).* $p < 0.05$, *** $p < 0.001$ determined by Student's t-test. Reproduced with permission from ref. [12].

These observations indicate that the multivalent interactions between polycations and mRNA stabilize mRNA/m against polyanion, urea and nucleases, enhancing their in vitro transfection. Our findings emphasize the importance of controlling the interactions between polymers and mRNA for the development of PIC-based systems for RNA therapeutics.

3. Conclusions

Our efforts successfully solved several challenges in mRNA delivery, such as rapid enzymatic degradation and limited intracellular delivery. By merging orthogonal approaches including flexible polymers and guanidination, guanidinated polycations with flexible backbone were developed to improve the binding affinity with mRNA, resulting in enhanced stability against polyanions and nucleases. Moreover, charge neutralization between such polycations and mRNA boosted the cellular uptake of intact mRNA, promoting eventual protein translation in vitro and in vivo. Systemically administrated mRNA/m exhibited long circulation in blood, which could allow broad applications of mRNA/m combined with targeting systems. Through further development of our flexible polycation-based mRNA delivery systems, we can overcome various obstacles which are difficult to solve with conventional approaches, promoting preclinical and clinical studies for clinical translation.

4. Materials and Methods

Details of materials and methods are available in our previous publications [9,12]. Briefly, The PEG-PGBA, PEG-PLL, PEG-PGMG, PEG-PGMA were synthesized by ring-opening polymerization.

The mRNA-loaded micelles were prepared from polymers and mRNA in water and characterized by dynamic light scattering (DLS). The thermodynamic parameters were measured by isothermal titration calorimetry (ITC). The resistance of the micelles against polyanion exchange was studied by fluorescence correlation spectroscopy (FCS). The ability of the micelles to protect the loaded mRNA in serum containing nucleases was measured by qRT-PCR. The translation ability of the micelles was assessed by using mRNA encoding luciferase and luminometer. The pharmacokinetics were examined in mice by qRT-PCR following intravenous administration.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sahin, U.; Karikó, K.; Türeci, Ö. mRNA-based therapeutics—Developing a new class of drugs. *Nat. Rev. Drug Discov.* **2014**, *13*, 759–780, doi:10.1038/nrd4278.
2. Cabral, H.; Miyata, K.; Osada, K.; Kataoka, K. Block Copolymer Micelles in Nanomedicine Applications. *Chem. Rev.* **2018**, *118*, 6844–6892, doi:10.1021/acs.chemrev.8b00199.
3. Uchida, S.; Kataoka, K. Design concepts of polyplex micelles for in vivo therapeutic delivery of plasmid DNA and messenger RNA. *J. Biomed. Mater. Res. Part A* **2019**, *107*, 978–990, doi:10.1002/jbm.a.36614.
4. Uchida, S.; Perche, F.; Pichon, C.; Cabral, H. Nanomedicine-Based Approaches for mRNA Delivery. *Mol. Pharm.* **2020**, *17*, 3654–3684, doi:10.1021/acs.molpharmaceut.0c00618.
5. 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. USA* **2012**, *109*, 3137–3142, doi:10.1073/pnas.1200718109.
6. Uchida, S.; Kinoh, H.; Ishii, T.; Matsui, A.; Tockary, T.A.; Takeda, K.M.; Uchida, H.; Osada, K.; Itaka, K.; Kataoka, K. Systemic delivery of messenger RNA for the treatment of pancreatic cancer using polyplex nanomicelles with a cholesterol moiety. *Biomaterials* **2016**, *82*, 221–228, doi:10.1016/j.biomaterials.2015.12.031.
7. Dirisala, A.; Uchida, S.; Tockary, T.A.; Yoshinaga, N.; Li, J.; Osawa, S.; Gorantla, L.; Fukushima, S.; Osada, K.; Kataoka, K. Precise tuning of disulphide crosslinking in mRNA polyplex micelles for optimising extracellular and intracellular nuclease tolerability. *J. Drug Target.* **2019**, *27*, 670–680, doi:10.1080/1061186X.2018.1550646.
8. Grasso, G.; Deriu, M.A.; Patrulea, V.; Borchard, G.; Möller, M.; Danani, A. Free energy landscape of siRNA-polycation complexation: Elucidating the effect of molecular geometry, polymer flexibility, and charge neutralization. *PLoS ONE* **2017**, *12*, e0186816, doi:10.1371/journal.pone.0186816.
9. Miyazaki, T.; Uchida, S.; Nagatoishi, S.; Koji, K.; Hong, T.; Fukushima, S.; Tsumoto, K.; Ishihara, K.; Kataoka, K.; Cabral, H. Polymeric Nanocarriers with Controlled Chain Flexibility Boost mRNA Delivery In Vivo through Enhanced Structural Fastening. *Adv. Healthc. Mater.* **2020**, *9*, 2000538, doi:10.1002/adhm.202000538.
10. Carlson, P.M.; Schellinger, J.G.; Pahang, J.A.; Johnson, R.N.; Pun, S.H. Comparative study of guanidine-based and lysine-based brush copolymers for plasmid delivery. *Biomater. Sci.* **2013**, *1*, 736–744, doi:10.1039/C3BM60079C.
11. Hori, M.; Cabral, H.; Toh, K.; Kishimura, A.; Kataoka, K. Robust Polyion Complex Vesicles (PICsomes) under Physiological Conditions Reinforced by Multiple Hydrogen Bond Formation Derived by Guanidinium Groups. *Biomacromolecules* **2018**, *19*, 4113–4121, doi:10.1021/acs.biomac.8b01097.
12. Miyazaki, T.; Uchida, S.; Hatano, H.; Miyahara, Y.; Matsumoto, A.; Cabral, H. Guanidine-phosphate interactions stabilize polyion complex micelles based on flexible catiomers to improve mRNA delivery. *Eur. Polym. J.* **2020**, *140*, 110028, doi:10.1016/j.eurpolymj.2020.110028.

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