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Biochemical and Biophysical Research Communications 358 (2007) 373–378
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Effective delivery with enhanced translational activity synergistically accelerates mRNA-based transfection

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Received 31 March 2007
Available online 1 May 2007

Abstract

mRNA instead of DNA provides a new and attractive approach for gene therapy and genetic vaccination. Current technologies for mRNA delivery are based on cationic lipids with DOTAP being the most efficient one. We previously reported on the synthesis of an inorganic–organic hybrid carrier by embedding inorganic nano-particles of carbonate apatite onto liposomal carrier DOTAP and demonstrated its high transfection potency of luciferase mRNA both in mitotic and non-mitotic cells. Here we show that in addition to the carrier design for effective endocytosis and release of mRNA to the cytoplasm, enhancement of mRNA translation efficiency is a prerequisite for maximum protein expression. We used the modified cap analog (ARCA) during in vitro transcription of luciferase DNA for proper cap orientation and demonstrated that transfection with ARCA–mRNA resulted in higher protein expression than the mRNA with usual cap structure for both DOTAP and DOTAP–apatite complex. Secondly, exogenous poly(A) was co-delivered with mRNA by the DOTAP–apatite, resulting in very significant expression compared to mRNA delivery only. Finally, when combined both of the effects of smart carrier and the modifications at mRNA translational level, a notable enhancement (100 times) was achieved as compared to the existing DOTAP-based liposome technology. Our findings, therefore, unveiled a novel approach that an effective delivery system can be developed by the improvement of the gene expression level in combination with the enhancement of the carrier potency.

Keywords: mRNA delivery; Liposome; Carbonate apatite; Inorganic–organic hybrid carrier; Anti reverse cap analog; Exogenous poly(A); Transgene expression

mRNA transfection is a very rare occurrence in the field of gene delivery or genetic vaccination. Considering the therapeutic application strategy in vivo where cells (hepatocytes, vascular endothelia, muscle cell, and brain) are quiescent or slowly proliferating, mRNA has been an attractive therapeutic genetic material to treat those cells. This is because the nuclear membrane provides a significant barrier for DNA-based non-viral gene therapy systems in non-dividing cells where cell cycle-dependent breakdown of the nuclear envelop is absent [1,2]. mRNA-based expression of certain protein sustains for a limited time but such transient expression is desirable in some clinical disorders specially in neurological diseases [3,4]. mRNA transfection strategy is also applicable in the field of cancer vaccination or immunotherapy [5,6]. Thus, an efficient mRNA delivery system is highly expected. Several synthetic vectors have been evaluated for their ability to mediated efficient mRNA transfection [7]. After the pioneering work by Malone et al. [8], so far most published reports are based on cationic lipids and DOTAP shows the highest activity [9–11]. We previously reported the potential effects of an inorganic nano-apatite on DOTAP-mediated luciferase mRNA expression into both dividing and non-dividing cells [12]. As a high level of protein expression following mRNA transfection should depend on both an efficient transfection reagent and an

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doi:10.1016/j.bbrc.2007.04.059

optimal mRNA transcript, we analyzed whether some structural modifications of mRNA to increase the translation efficiency could be beneficial for subsequently enhanced expression using our developed carrier. Since the incorrect incorporation of typical cap analog into mRNA during in vitro transcription of DNA decreases translation efficiency [13] and the use of anti reverse cap analog (ARCA) enhances translation efficiency by enabling the right orientation of cap [14], we investigated whether DOTAP–apatite-based mRNA transfection activity could be influenced by this mRNA modification strategy. Additionally, because of the major role of poly(A) tail in regulation of the translation efficiency and stability in association with poly(A)-binding protein (PABP) [15,16], we analyzed the effect of free poly(A) which, according to the previous reports [17,18], may have effects on PABP so that mRNA translation can be increased or degradation can be protected for further enhanced expression. While modification of mRNA using ARCA prior to mRNA delivery or co-delivery of free poly(A) with mRNA using DOTAP–apatite contributed individually to a significant increment of mRNA expression, the concerted effect of these two factors more dramatically enhanced transgene expression with an efficiency 100-fold higher than that achieved with the conventional DOTAP. Considering the expression as obtained by DOTAP, the so far reported most efficient non-viral carrier for mRNA transfection, this enhancement created a new concept that synergistically carrier efficacy and gene modification can facilitate to develop a very potent delivery system.

Materials and methods

Cell culture. HeLa cells were cultured in 25-cm^2 flasks in DMEM supplemented with 10% fetal bovine serum (FBS), 50 μg penicillin/ml, 50 μg streptomycin/ml, and 100 g neomycin/ml at 37 °C in a humidified 5% CO_2-containing atmosphere.

Synthesis of mRNA transcripts. mRNA encoding luciferase was prepared by in vitro transcription of the luciferase SP6 control DNA having a poly(A) tail (A30) (Promega) using an SP6 RiboMAX™ kit as described by the manufacturer (Promega) with m7G(5′)pppG(5′) cap analog (Ambion) or anti-reverse cap analog, ARCA (Ambion) at a ratio of 5:1 cap/GTP. When the cap analog was omitted, the GTP concentration was raised accordingly to 5 mM. In vitro transcribed mRNAs (with or without cap) were characterized by gel electrophoresis and were all ~1800 bp in length.

Preparation of liposomes. For the preparation of liposome, solid DOTAP {*N*-[1-(2,3-dioleoloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride; Sigma} was initially dried from chloroform, subsequently dispersed in dH_2O and shaken at a temperature above the gel-to-liquid-crystalline transition temperature of the lipid (~0 °C) for 10 min. The milky solution was sonicated for 10 min under temperature control using a Branson sonifier 250 (Branson Sci., USA) at minimum ultrasound power to avoid extensive bubble formation.

Complex formation. Cells from the exponential growth phase were seeded on the day before transfection at 100,000 cells per well into 24-well plates to attain 80% confluency. For mRNA transfection, 2 types of complexes were prepared mRNA–DOTAP and mRNA–DOTAP–apatite. For mRNA–DOTAP complex formation, 3 μg of heat-denatured mRNA (10 min at 65 °C) and 6–10 μg of DOTAP were diluted separately in 100 μl DMEM (pH 7.5). Then mRNA was mixed with or without poly adenylic acid {poly(A)} [Sigma] at a concentration of 0–6 μg/ml. After 15 min, DOTAP was added to the RNA solution containing poly(A) solution, followed by incubation for 40 min at room temperature as recommended by the manufacturer. Later, final volume was adjusted to 1 ml by DMEM (pH 7.5).

For preparation of mRNA–DOTAP–apatite complex, at first mRNA–DOTAP solution with or without free poly(A) was prepared like before and incubated for 15 min at room temperature. By adjusting the final volume to 1 ml with the same DMEM, 4 mM $CaCl_2$ was added prior to incubation for 30 min at 37 °C. The solution containing each type of transfection complexes was added with 10% FBS to the rinsed cells. Treated cells were incubated at 37 °C in a 5% CO_2 humidified environment for 2–4 h. The transfection mix was discarded and 1 ml of fresh serum medium was added to the cells. Cells were cultured for additional 6–10 h before analysis of reporter gene expression. The transfected cells were lysed by a lysis buffer (Promega) and the luciferase activity was measured using the luciferase assay system by a luminometer (TD-20/20 Luminometer, USA). Transfection efficiency was measured as mean relative light units per milligram of cell protein.

Labeling of mRNA. mRNA was labeled during in vitro transcription of luciferase control DNA using fluorescein RNA labeling mix (Roche, Germany) which contains fluorescein-12-UTP. For the labeled mRNA, all the complexes were formed using this mRNA instead of unlabeled capped mRNA according to the same procedure described above.

Flow cytometry. Fluorescein labeled mRNA-containing complexes were added with 10% FBS to 80% confluent HeLa cells seeded in a six-well plate and incubated for 3 h. Cells were treated with trypsin–EDTA and after detachment of the adherent cells centrifugation have been performed. Discarding supernatant the cell pallet was resuspended in 1 ml serum free DMEM and used for flow cytometry. The intensity of fluorescein labeled mRNA in HeLa cell was determined by gating cells at an excitation wavelength of 488 nm using an argon ion laser. The presence of the labeled mRNA was detected by emission at a wavelength of 525 nm.

Results and discussion

Influence of the presence of ARCA on mRNA for enhanced gene expression

mRNA at the cytoplasm contains a cap structure [m7(5′)Gppp(5′) N, where N is any nucleotide] at its 5′-terminus. Previously, it was reported that the cap structure is required for efficient mRNA lipofection [8]. During in vitro-synthesis of mRNA, cap analog is incorporated into the RNA in both the forward [m7G(5′)pppG(pg)] and reverse orientation [G(5′)pppm7G(pN)] leading to the synthesis of two isomeric populations of RNA of approximately equal proportion [13]. RNA capped with reverse 5′ caps cannot be translated, resulting in a 50% reduction in expected yield of protein. Then it was shown that the use of 3′-*O*-methyl-m75′Gppp5′G, an anti-reverse cap analogue (ARCA), avoided the cap incorporation in the reverse orientation [14]. And, in vitro ARCA-capped transcripts (ARCA–mRNA) showed translation efficiency higher than that of typical capped transcripts (Cap–mRNA) in a rabbit reticulocyte lysate [14]. It was shown in dendritic cell that ARCA–mRNA is effective to enhance transfection potency [18]. Therefore, we expected that luciferase expression in HeLa cells transfected with ARCA–Luc-mRNA through our developed DOTAP–apatite carrier will also be more efficient. As shown in Fig. 1A,

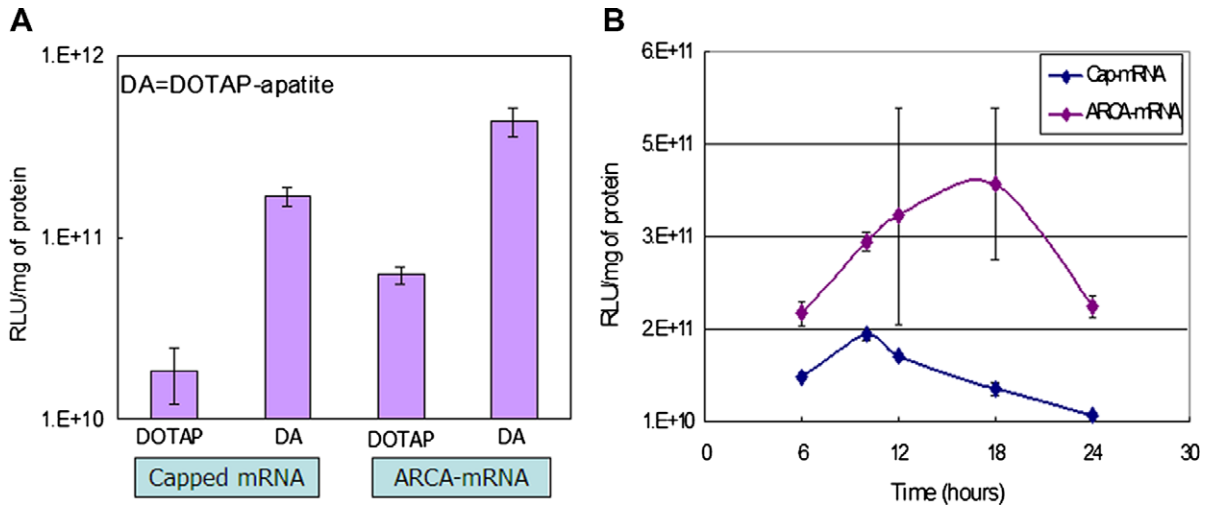

Fig. 1. Effect of the presence of ARCA on mRNA for enhanced gene expression. (A) In vitro transcribed ARCA-capped transcript (ARCA–mRNA) or capped-transcript (Cap–mRNA) was complexed with DOTAP and finally mRNA–DOTAP–apatite complex was formed as described in the materials and methods section. mRNA–DOTAP and mRNA–DOTAP–apatite complexes were transferred with 10% FBS to 80% confluent HeLa cells seeded in 24-well plate and Luc-mRNA expression was measured at 6 h post transfection. (B) For the time course of expression by DOTAP–apatite carrier, ARCA–mRNA/Cap–mRNA expression was measured at 6–24 h post transfection.

the luciferase activity was at least 2- to 9-fold higher with ARCA-capped Luc-mRNA than with Cap-Luc-mRNA.

Time course of ARCA−mRNA expression

Very low protein expression over a certain time is the most concerned factor for mRNA-mediated gene expression. In almost all cell lines protein expression following mRNA delivery sustains up to 6–10 h [9,10], rapidly declines at 24 h and reaches an undetectable level after 48 h. Translation inefficiency of template mRNA, its quick degradation and short half life of the expressed protein are the prime reasons for this low expression. As modification at the mRNA transcript level through the addition of modified cap analog (ARCA) showed enhanced mRNA expression which could be effective over long time. Thus, the time course of the expression of ARCA–mRNA was analyzed. As already shown mRNA expression was maximum at 10 h and rapid decline led to only 6% of the maximum protein level at 24 h post transfection. Kinetic study in Fig. 1B shows a higher protein level in each time scale following transfection with ARCA–mRNA than Cap–mRNA. It was observed that 2-fold increase at 6 h, 3-fold at 12 h, 6-fold at 18 h and finally 9-fold at 24 h, the most significant enhancement due to ARCA incorporation into mRNA. As shown in Fig. 2, expression of Cap–mRNA at 24 h was only 13% of the maximum level, whereas at the same time ARCA–mRNA expression was almost 50% of the maximum level. It is noteworthy that ARCA–mRNA expression level at 24 h is even more than that observed at 10 h, the maximum expression achieved by Cap–mRNA. These results suggest that increased translation efficiency due to right cap orientation on mRNA led higher expression kinetics.

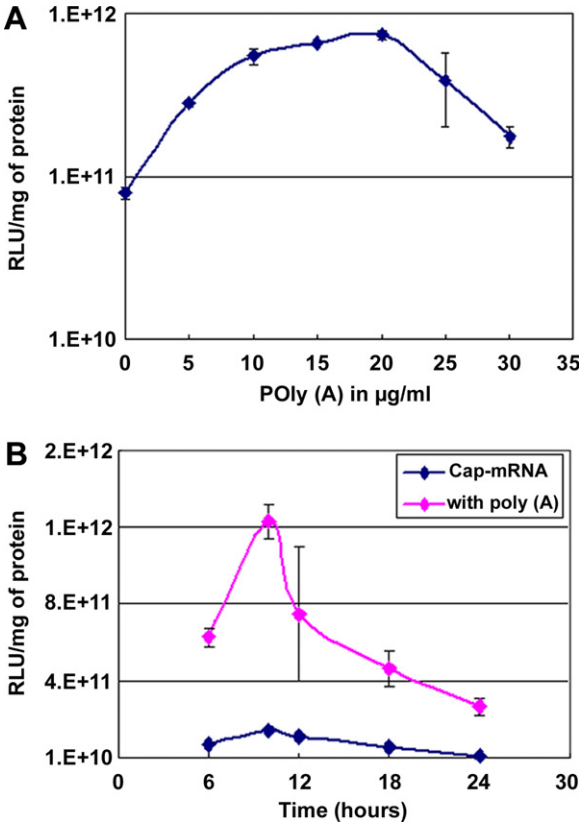

Fig. 2. Influence of the co-delivery of free poly(A) with mRNA for enhanced expression. (A) Luc-mRNA was mixed with poly(A) (0–30 µg/ml) and then the mRNA–DOTAP–apatite complex was formed as described in method. HeLa cells were incubated with the complex for 4 h and replaced with serum media. Luciferase expression was measured 6 h post transfection in RLU/mg of protein. (B) For the time course of expression by DOTAP–apatite carrier, ARCA–mRNA/Cap–mRNA expression was measured at 6–24 h post transfection.

Effect of exogenous poly(A) on the transfection efficiency of delivered mRNA

While the potential importance of mRNA stability in the regulation of gene expression has been recognized, the structures and mechanism involved in the determination of individual mRNA decay rate have just been begun to be elucidated. Current evidence suggests that mRNA turnover is a very specific process requiring *cis*-acting sequences, *trans*-acting factors and ongoing translation. According to the report [19], some mRNA-binding proteins, endo- and exoribonucleases, factors involved in poly(A) metabolism, antisense RNAs and ribosome are identified and characterized as *trans*-acting factors to regulate mRNA decay.

It was shown by Borman et al. [17] that the addition of exogenous poly(A) chains of 50–180 residues *in trans* in ribosome-depleted rabbit reticulocyte lysates stimulated 11-fold the translation of capped mRNA containing a 100 adenosine residue poly(A) tail. They showed that poly(A) can stimulate in vitro capped mRNA translation in *trans* without the need of mRNA circularization. Mockey et al. also performed transfection in dendritic cells using poly(A) and showed better transfection efficiency [18]. Therefore, we performed co-transfection of mRNA with exogenous poly(A) [0–30 μg/ml] through DOTAP–apatite carrier in HeLa cell (Fig. 2A). It was found that 2–9 times enhancement of mRNA expression could be achieved due to co-delivery of exogenous poly(A) with mRNA compared to the delivery of only mRNA using the same carrier. Twenty micrograms per milliliter poly(A) showed maximum efficiency in this respect.

Time course of mRNA expression co-delivered with free poly(A)

We, next, examined the time profile of mRNA expression with or without free poly(A). As we can see in Fig. 2B, similarly like ARCA effect, time course was very interesting in the aspect that at each time mRNA expression with poly(A) was quite higher than the expression without poly(A). Maximum expression was at 10 h in both of the cases and declined afterwards. Expression was increased at least 6–10 times for poly(A) co-transfected mRNA as compared to only mRNA. Moreover, mRNA expression level with poly(A) at 24 h is even more than that observed at 10 h by mRNA in absence of poly(A). The impressive time course data (Figs. 1B and 2B) indicates the use of modified cap analog or exogenous free poly(A) could be very effective when expression over a longer time is a prior requirement of any therapeutically important mRNA for clinical application.

Cellular uptake analysis of fluorescein-labeled Luc-mRNA

To speculate the reason of poly(A) effect, the pattern of uptake of fluorescein-labeled mRNA delivered with or without poly(A) was examined in HeLa cell. In Fig. 3, it was shown that there is almost no change in the uptake level of mRNA as evaluated quantitatively by flow cytometry and also qualitatively by fluorescence microscope. Thus, it is reasonable to assume that the effect of free poly(A) is totally intracellular and it is likely to be related specifically to mRNA translation. It is known that poly(A) tail of cytoplasmic mRNA is important for both transla-

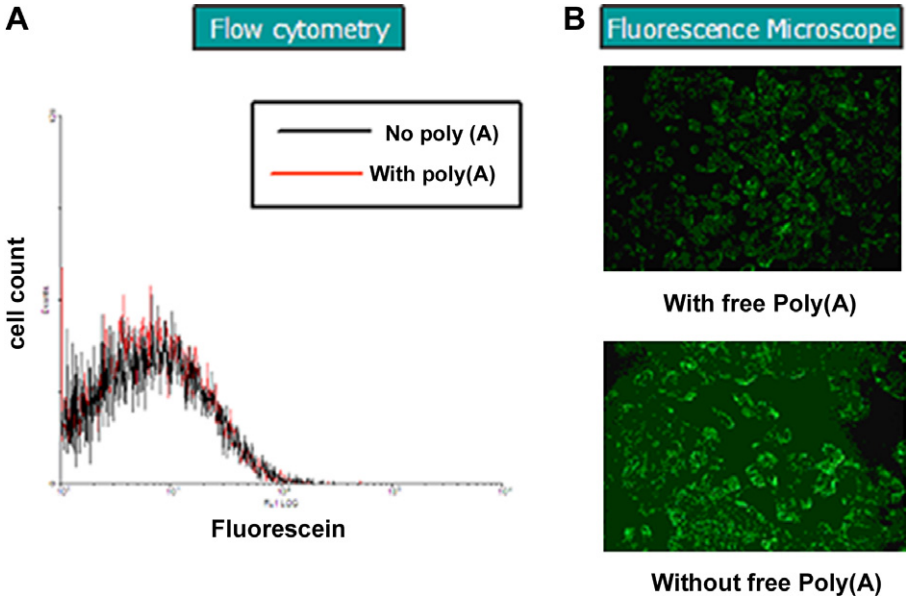

Fig. 3. Cellular uptake analysis of fluorescein labeled Luc-mRNA into HeLa cells. Fluorescein labeled mRNA–DOTAP–apatite complexes were added with 10% FBS to 80% confluent HeLa cells seeded in a six-well plate and incubated for 3 h. Cells were treated with trypsin–EDTA and after detachment of the adherent cells, centrifugation has been performed. Discarding supernatant the cell pallet was resuspended in 1 ml serum free DMEM and used for flow cytometry (A) and for analysis under fluorescence microscope, extracellular particles were removed by EDTA and observed after 3 h of incubation (B).

tion and stability. poly(A) tail binds to PABP which interacts with the N-terminal part of eIF4G leading to mRNA circularization via the cap-eF4E-eF4G-PABP-poly(A) complex [15]. This synergy between the poly(A) tail and the 5′cap is supposed to increase the expression at the level of translation. According to the previous report [17,18], it could be assumed that the binding of PABP molecules on free poly(A) chains increases the binding of multimerized PABP to eIF4G and the affinity of eIF4E for the 5′ cap and increases translation efficiency.

On the other hand, free poly(A) may have effects on protecting mRNA degradation. Physiologically degradation of mRNA having poly(A) tail occurs generally in two pathways. In both cases, the degradation is initiated by the removal of the 3′ poly(A) tail and is followed by either cleavage of the 5′ cap structure (decapping) and 5′–3′ exonucleotidic digestion or 3′–5′ degradation. Decapping occurs mainly in cytoplasmic foci called as P-bodies having many enzymes involved in mRNA degradation [21]. PABP bound to free poly(A) could also act as an inhibitor of mRNA decapping via the 5′-terminus by preventing its entry in P-bodies [20,21]. In other words, it could be simply the effect of intracellular RNases which would degrade co-delivered poly(A) protecting degradation of the real mRNA in a certain dose.

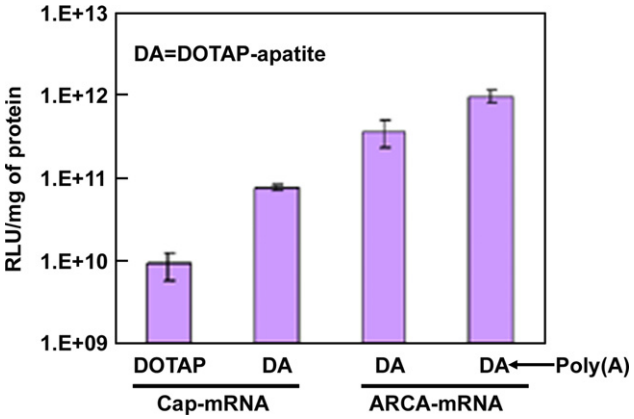

Fig. 4. Effect of the presence of ARCA on mRNA co-delivered with free poly(A). During in vitro transcription mRNA was prepared in the presence of cap analog or anti-reverse cap analog (ARCA). Cap–mRNA/ARCA–mRNA was allowed to form mRNA–DOTAP–apatite complex. ARCA–mRNA was mixed with poly(A) [20 μg/ml] and then the mRNA–DOTAP–apatite complex was formed as described in method. As a control, cap–mRNA–DOTAP complex was also generated. All the complexes were transferred with 10% FBS to 80% confluent HeLa cells seeded in 24-well plate and Luc-mRNA expression was measured at 6 h post transfection.

Synergistic effect of ARCA and free poly(A) on DOTAP–apatite-mediated mRNA transfection

So far, we have demonstrated mRNA expression enhancement through an efficient carrier as well as increasing translation efficiency of mRNA separately. We then examined the final mRNA expression of the modified ARCA–mRNA in presence of free poly(A). Co-transfection of ARCA–mRNA with free poly(A) was performed through the DOTAP–apatite carrier. Surprisingly, we could achieve more than 100 times enhancement of mRNA expression when we applied advanced strategies both at the carrier and mRNA level to increase expression. In Fig. 4, it was shown that ARCA–mRNA co-transfected with free poly(A) by DOTAP–apatite carrier led to more than 100 times expression than Cap–mRNA transfected by only DOTAP. This is an excellent combination effect of carrier efficacy and gene modification what we are reporting first time. Indeed after gene packaging with the carrier, the gene–carrier complex pass through a common route i.e. binding onto the cell membrane, internalization of the complex through membrane fusion or endocytosis and releasing the gene from the complex into the cytoplasm after the endosomal membrane destabilization. The need of carrier efficacy in any or all of the steps finally results in enhanced gene expression. On the other hand, after the release of gene in the cytoplasm, the role of carrier efficacy is completed, but at that time modified gene or enhancement of transcription rate (in case of DNA-based gene delivery) or translation efficiency (mRNA or DNA-based gene delivery) could play a major role to enhance further gene expression.

Taken together, our findings reveal an attractive and new strategy that in combination with the increased efficacy of a carrier, if we can perform modifications in the gene level, or if we can add some factors at an appropriate time which can improve transcription or translation intracellularly, it will lead to a remarkable gene delivery system for basic research and clinical medicine.

Acknowledgment

This work was supported in parts by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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