mRNA Delivery

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A Single Methylene Group in Oligoalkylamine-Based Cationic Polymers and Lipids Promotes Enhanced mRNA Delivery

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Dedicated to the memory of Braydon (Brad) Guild

Abstract: *The development of chemically modified mRNA holds great promise as a new class of biologic therapeutics. However, the intracellular delivery and endosomal escape of mRNA encapsulated in nanoparticles has not been systematically investigated. Here, we synthesized a diverse set of cationic polymers and lipids from a series of oligoalkylamines and subsequently characterized their mRNA delivery capability. Notably, a structure with an alternating alkyl chain length between amines showed the highest transfection efficiency, which was linked to a high buffering capacity in a narrow range of pH 6.2 to 6.5. Variation in only one methylene group resulted in enhanced mRNA delivery to both the murine liver as well as porcine lungs after systemic or aerosol administration, respectively. These findings reveal a novel fundamental structure–activity relationship for the delivery of mRNA that is independent of the class of mRNA carrier and define a promising new path of exploration in the field of mRNA therapeutics.*

Recently the field of transcript therapy by treating diseases with messenger RNA (mRNA) has gained momentum and raised the interest in both academia and industry for widespread application in a variety of therapeutic areas.[1,2] In contrast to gene therapy, mRNA-based drugs do not bear the inherent risk of insertional mutagenesis. Moreover, they provide a transient protein production platform for in situ generation of the therapeutic protein due to the relatively short half-life of mRNA.[1] Additionally, chemical modifications of RNA nucleotides have been shown to decrease immune responses and improve the nucleic acid stability.[1] However, the direct delivery of mRNA to cells is hampered by its highly anionic character that prevents a passage across cell membrane unaided and the ubiquitous presence of RNases. Therefore, a delivery system that is designed for the efficient encapsulation of mRNA using carriers that possess effective cell penetrating properties is required. Although a variety of carriers for siRNA and pDNA have been investigated,[3–5] a tailored approach is required for mRNA due to its low stability, different size, and single-stranded nature. Thus, the de novo development of a delivery system is needed that can be adjusted to this nucleic acid.[2]

The aim of this study was to develop efficient mRNA carriers that can be used in vivo for both aerosol and intravenous application to face the challenge of diseases associated with the airways as well as metabolic disorders of the liver, respectively. Cationic polymers have proven to efficiently transport pDNA to the lung tissue after aerosol application,[6] while lipid nanoparticles are extremely capable in pDNA/siRNA delivery to hepatocytes via systemic administration. Since no carriers for mRNA-based therapies in vivo exist on the market so far, we chose these two distinct delivery systems and application routes as a starting point.

A key parameter that determines the transfection efficiency of any nucleic acid delivery agent is an acid dissociation constant (pK_a) in a pH range of 6.2 to 6.5, which is hypothesized to enhance endosomal escape of the carrier complex into the cytoplasm.[2,7,8] We expected this step to be critical for successful mRNA delivery as well. Since oligoalkylamine-based carriers are known effective delivery systems for various nucleic acids,[9–14] we chose a small diverse set of tri- (2-2, 3-3) and tetramines bearing ethylene (2-2-2) and/or propylene spacers (2-3-2, 3-3-3, Figure 1a) and determined the buffering capacity for each structure by potentiometric titration to predict their potency in mRNA delivery (Figure 1b).

To enable an accurate comparison in a non-polymeric system, the oligoalkylamines were succinylated (Supporting Information, Scheme S1), which imitates the occupation of one primary amine after chemical coupling to the polymer scaffold by formation of amide bonds. The modified oligoalkylamines were titrated with NaOH, revealing that diethylenetriamine (2-2) and the (2-3-2) tetramine bearing an alternating alkylamine chain length exhibited the highest buffering capacity in the endosomal pH range (Figure 1b). Hence, both were expected to transfect cells efficiently when incorporated in a polymeric delivery system.

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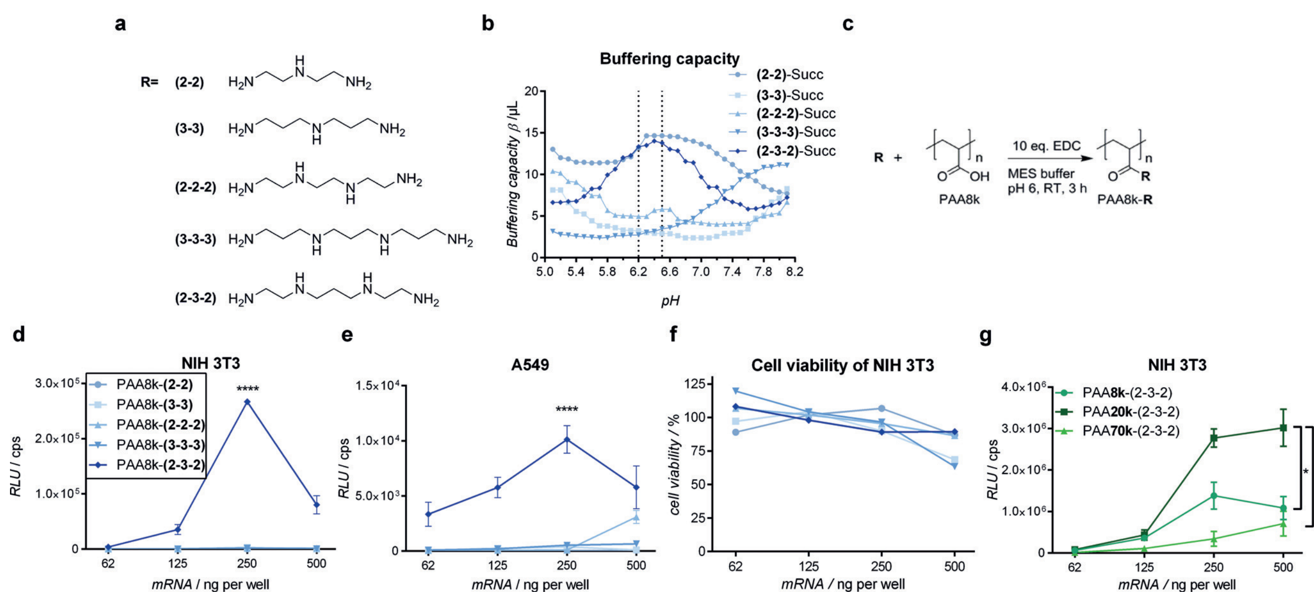

Figure 1. a) Structures of oligoalkylamines. b) Plots of buffering capacities of succinylated oligoalkylamines. The area in between the dotted lines indicates the ideal buffering range for nucleic acid carriers, enhancing endosomal escape. Buffering capacity β was defined as volume of 50 mM NaOH added to the solution containing 72 μmoles protonable amines resulting in a pH change of 0.1. c) Synthesis of oligoalkylamine-modified poly(acrylic acid) carriers. MES = 2-(*N*-morpholino)ethanesulfonic acid, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. d), e) Transfection efficiency of polyplexes carrying mRNA-FLuc on d) murine fibroblasts (NIH 3T3) and e) target tissue-derived human alveolar type II-like cells (A549). The reporter protein (firefly luciferase) level was determined 24 h post transfection by measuring bioluminescence expressed in counts per second (cps). f) Influence of polyplexes on cell viability of NIH 3T3 cells 24 h after transfection was evaluated using an MTT assay. g) Influence of poly(acrylic acid) molecular weight on transfection efficiency of (2-3-2)-based carriers. PAA20k-(2-3-2) exhibits superior transfection efficiency over both PAA70k-(2-3-2) and PAA8k-(2-3-2). The reporter protein (firefly luciferase) level was determined 24 h post transfection by measuring bioluminescence expressed in counts per second (cps). $^*p < 0.05$ and $^{****}p < 0.0001$.

For this purpose, oligoalkylamines were grafted to an 8000 Da poly(acrylic acid) (PAA8k) scaffold (Figure 1 c) resulting in polymers that maintained the previously observed pattern in buffering capacity (Supporting Information, Figure S4). When complexed with chemically modified mRNA at nitrogen to phosphate (N/P) ratio 20 in water (Aqua ad iniectabilia, B. Braun), all resulting polyplexes formed decent monodisperse particles with hydrodynamic diameters of 65 to 236 nm and overall positive surface charge (Supporting Information, Table ST1). Next, the polymeric carriers were screened on murine fibroblasts (NIH 3T3) for their ability to transfect cells using chemically modified mRNA encoding for firefly luciferase (mRNA-FLuc) as a reporter (Figure 1 d). Suprisingly, the activity of PAA8k-(2-2) was at the background level, whereas PAA8k-(2-3-2) transfected cells up to 85-fold more efficiently (at 250 ng mRNA per well) compared to the second best PAA8k-(3-3) at similar cell viability (ca. 90 %). Since the polymeric carrier was intended for aerosol application,[1] human alveolar type II like cells (A549) were transfected with the polymeric particles, resulting in highest levels of the reporter enzyme in PAA8k-(2-3-2)-treated cells as observed during the screening on NIH 3T3 cells (Figure 1 e). Interestingly, irrespective of the applied N/P ratio (Supporting Information, Figure S1), the alternating structure (2-3-2) provided superior mRNA transfection compared to all other compounds including (2-2), which exhibited similar buffering capacity.

Since sufficient particle uptake is a prerequesite for high transfection efficiency, we evaluated the amounts of reporter mRNA in NIH 3T3 cells treated with polyplexes (ca. 3 pg mRNA per cell) via qPCR. Briefly, cells were incubated 0.5, 1, 2, or 4 h with polyplexes and the amounts of the reporter sequence were quantified after each time point. The lowest uptake was detected for PAA8k-(2-2), which led to even 4-fold lower mRNA levels compared to PAA8k-(2-3-2) (0.04–0.09 pg per cell; Supporting Information, Figure S2 a). This explains the finding that PAA8k-(2-2)-based carriers did not result in efficient transfection although they showed similar buffering capacity in the critical pH range. In contrast, all other polyplexes provided higher cellular uptake of reporter mRNA (PAA8k-(3-3) being most efficient resulting in 0.17–0.25 pg per cell). These findings support the notion that the increased uptake of complexes does not correlate with higher transfection levels,[15] although for mRNA delivery a certain threshold needs to be exceeded.

Next, MTT assays were performed to exclude the effect of cell viability on polyplex efficacy (Figure 1 f). The highest toxicity was observed in the case of PAA8k-(3-3-3), which led to only 63 % viable cells 24 h after transfection with 500 ng of mRNA. In contrast, the same dose of mRNA in complex with tetramines PAA8k-(2-3-2) or PAA8k-(2-2-2) resulted in a significantly higher amount of viable cells (89 or 86 %, respectively), whereas only the alternating (2-3-2) scaffold led to reasonable reporter enzyme activity. Therefore, cell viability after incubation with polyplexes is independent of transfection efficiency, implying that after sufficient particle uptake, buffering capacity is a driving force for augmented mRNA translation.

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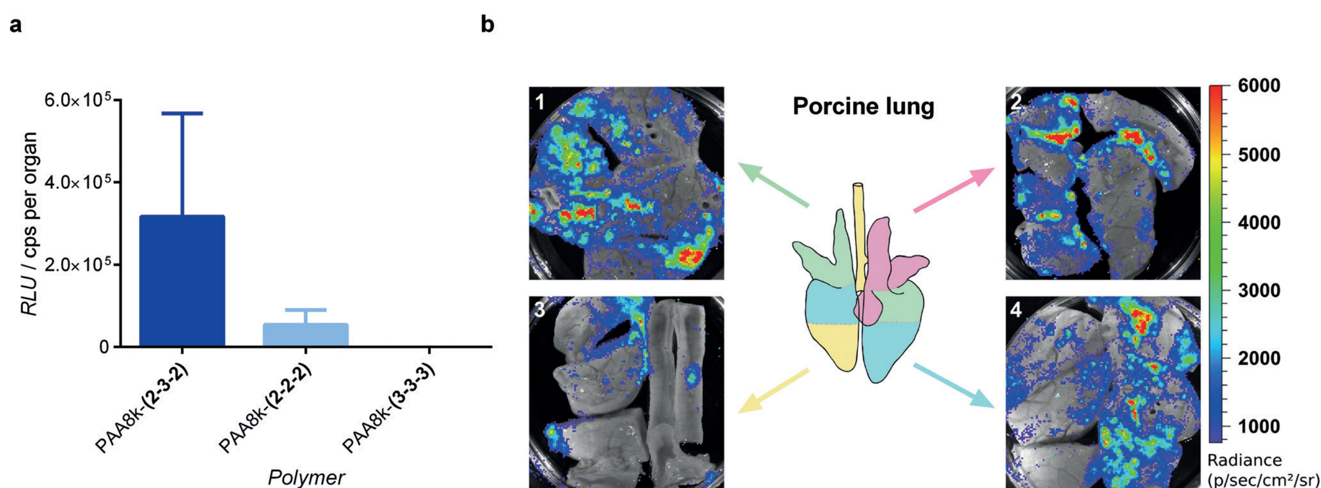

Figure 2. a) Ex vivo enzyme activity of firefly luciferase in murine lungs after treatment with 12.5 μg mRNA-FLuc complexed with PAA8k-(2-3-2), PAA8k-(2-2-2) or PAA8k-(3-3-3); $n=3$. b) Bioluminescence imaging of porcine lung tissue after aerosol application of PAA20k-(2-3-2) mRNA-FLuc polyplexes. Applied dose: 2 mg in 8 mL of total volume; Picture 1: *L. cranialis sinister, L. caudalis dexter*; Picture 2: *L. accessorius, L. cranialis dexter, L. medius*; Picture 3: *L. caudalis sinister, Trachea*; Picture 4: *L. caudalis dexter, L. caudalis sinister*; *L. Lobus*.

To further confirm the in vitro findings of best performing tetramine transfection agents PAA8k-(2-3-2), -(2-2-2), and -(3-3-3) as mRNA carriers in vivo, we tested mRNA-FLuc polyplexes under different conditions in both mice and a pig. After pulmonary application in mice, the order of carrier efficacy (2-3-2) > (2-2-2) > (3-3-3) observed in A549 cells was confirmed as a trend in vivo (Figure 2a).

Next, we extended our investigation to a pig model, which is a more relevant human disease animal model as the nanoparticles can be applied via aerosol into a ventilated body as performed in clinical application. For this purpose, the PAA molecular weight was optimized regarding transfection efficiency in vitro, showing that 20 kDa is noticeably more effective than 8 kDa and 70 kDa (Figure 1g). Thus, mRNA-FLuc complexed with (2-3-2)-modified PAA20k was nebulized into the lungs of a pig, which resulted in firefly luciferase enzyme activity in well-ventilated cranial parts of the lung (Figure 2b; images of a water-treated control in the Supporting Information, Figure S4).

Encouraged by our findings with the polymer-based delivery system, we investigated whether the correlation between buffering capacity and transfection efficiency remains valid in lipid-derivatized alkylamine carriers. Thus, a small set of oligoalkylamine cationic lipid structures were generated by applying the method reported by Love et al. (Figure 3a).[9] Briefly, a ring-opening of $CH_3(CH_2)_9$-epoxide by oligoalkylamines resulted in a library of C12-decorated carrier scaffolds (Supporting Information, Scheme S2). As in the case of the polymer library, the lipidic compounds were analysed for their buffering capacity in a presumed optimal pH range (6.2 to 6.5) for endosomal release. While the lipid-derivatized triamines did not possess increased buffering capability in the tested range, the tetramines exhibited clear peaks (Figure 3b). However, for C12-(3-3-3) and -(2-2-2), these were above and/or below pH 6.2 and 6.5. Only the C12-(2-3-2) displayed a peak buffering capacity in the desired range.

The transfection efficiency of all the lipids was tested by complexation of mRNA-FLuc, as described in the Supporting Information. Lipid-based nanoparticles were found to comprise uniform spherical particles with hydrodynamic diameter between 54 and 61 nm and a neutral or slightly positive surface charge (Figure 3c; Supporting Information, Table ST2). The transfection of NIH 3T3 cells with lipoplexes comprised of carriers with low buffering capacity in the endosomal pH range exhibited only low levels of reporter enzyme activity (Figure 3d). In contrast, C12-(2-3-2)-based lipoplexes demonstrated high transfection efficiency with up to a nearly 7-fold increase compared to the second best C12-(2-2-2) at a comparable cell viability of about 100%.

Next, lipoplexes were investigated with the aim of protein production in hepatocytes, targeting the liver via systemic administration as described for lipidic systems for siRNA applied intravenously.[7,9] Transfection of human liver carcinoma cells (HepG2, Figure 3e) with the lipidic particles confirmed the trend observed during the previous screening on NIH 3T3 cells, namely, the alternating (2-3-2)-structure exerts the highest transfection capabilities compared to other oligoalkylamines. We found that this trend was maintained when extended to studies using other cell lines (Supporting Information, Figure S3). Furthermore, transfection efficiency of lipoplexes proved to be independent of intracellular reporter mRNA levels or cell viability as determined by qPCR and MTT assays, respectively (Supporting Information, Figure S2b; Figure 3f).

As a final proof of concept, the potency of lipoplexes consisting of tetramine-based lipid to deliver mRNA-FLuc was investigated in female Balb/c mice. Six hours post intravenous injection, D-luciferin was applied intraperitoneally as a substrate. After 10 min of incubation, bioluminescence in animals treated with cationic lipids C12-(2-2-2) and C12-(3-3-3) was at the background level, whereas a strong signal in the liver of C12-(2-3-2)-treated mice was observed (Figure 3g–i).

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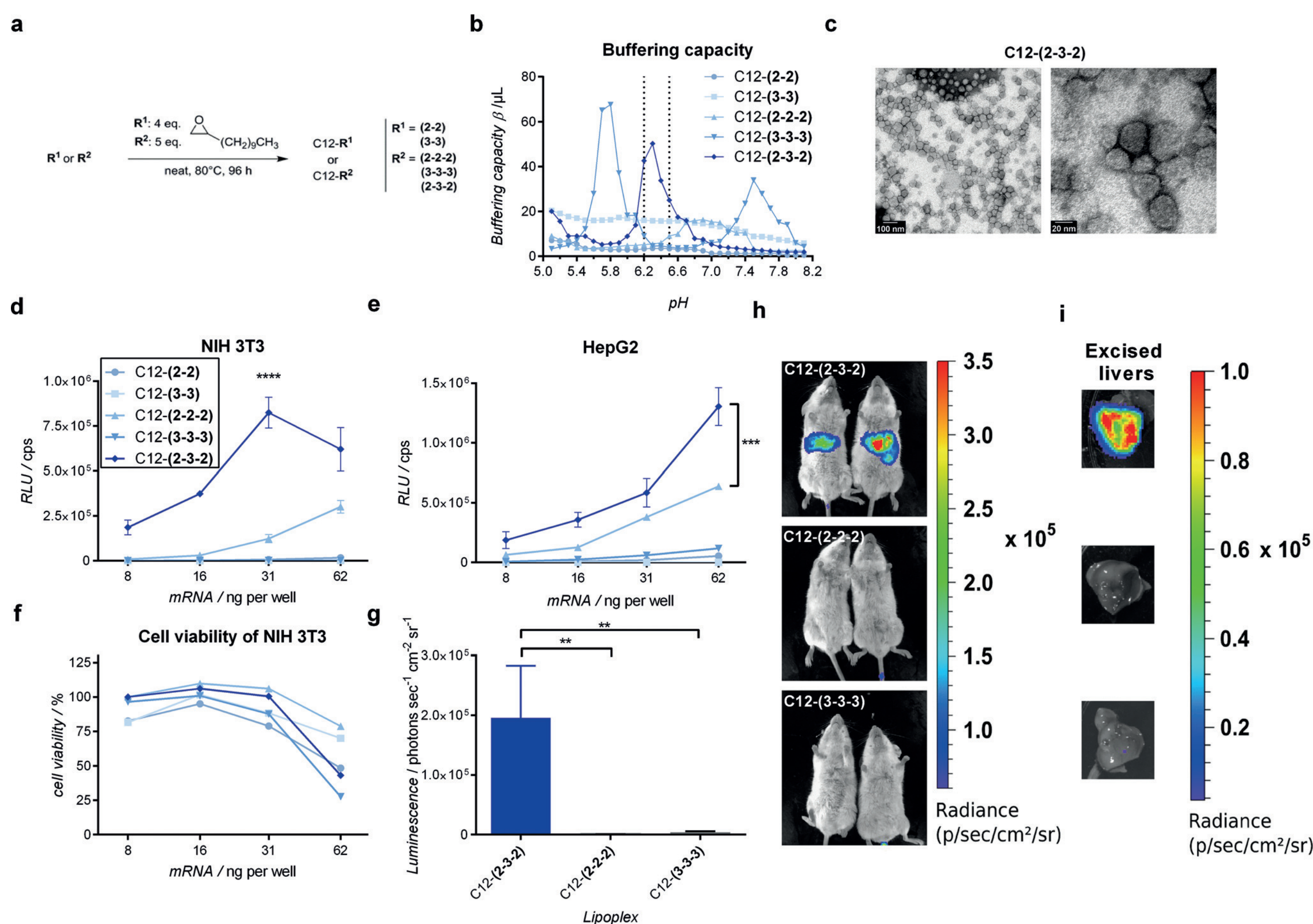

Figure 3. a) Synthesis of oligoalkylamine-based lipids. b) Plots of buffering capacities of C12-lipids with oligoalkylamine backbones. The area in between the dotted lines indicates the ideal buffering range for nucleic acid carriers, enhancing endosomal escape. Buffering capacity β was defined as volume of 50 mM NaOH added to the solution containing 10 µmoles lipid resulting in a pH change of 0.1. c) Transmission electron microscopic (TEM) images of C12-(2-3-2) lipoplexes. d), e) Transfection efficiency of lipoplexes carrying mRNA-FLuc on d) murine fibroblasts (NIH 3T3) and e) target tissue-derived human liver carcinoma cells (HepG2). The protein level was determined 24 h post transfection by measuring bioluminescence expressed in counts per second (cps). f) Influence of lipoplexes on cell viability of NIH 3T3 cells 24 h after transfection was evaluated using an MTT assay. g) Quantification of bioluminescence intensity in treated mice (observed in (h)). $n=3$. $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$. h) Exemplary bioluminescence images of mice treated intraveneously with 18 µg mRNA-FLuc complexed with C12-(2-3-2), C12-(2-2-2), and C12-(3-3-3). i) Exemplary bioluminescence images of excised livers.

In conclusion, by screening a set of oligoalkylamines for efficient delivery of mRNA in nanoparticles, we found that a tetramine with alternating ethyl–propyl–ethyl spacers exhibited a high ability to mediate robust levels of protein translation in vitro, although the oligoalkylamines within the tested carriers hardly varied. A trend towards higher efficiency of the (2-3-2)-based polymer/lipid was further confirmed in animal models. The observation of enhanced transfection properties of mRNA nanoparticles in two distinct delivery systems indicates a universal mechanism of action. We hypothesize that the scaffold with an alternating alkyl chain length between amines combines good buffering capacity with sufficient complex stability. As a consequence, endosomal release of (2-3-2) tetramine is facilitated and the intact complex is able to enter the cytosol. These surprising findings reveal that even subtle changes in the structure of carriers hold high potential for carrier improvements. However, a detailed investigation regarding the underlying mechanism of this structure–activity relationship has to be the topic of further studies.

Since the (2-3-2)-motif bridges the gap between in vitro and in vivo efficiency, this work provides a foundation to develop mRNA delivery systems that may have future clinical applications.

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