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### Single-cell mRNA transfection studies: Delivery, kinetics and statistics by numbers

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#### Abstract

In artificial gene delivery, messenger RNA (mRNA) is an attractive alternative to plasmid DNA (pDNA) since it does not require transfer into the cell nucleus. Here we show that, unlike for pDNA transfection, the delivery statistics and dynamics of mRNA-mediated expression are generic and predictable in terms of mathematical modeling. We measured the single-cell expression time-courses and levels of enhanced green fluorescent protein (eGFP) using time-lapse microscopy and flow cytometry (FC). The single-cell analysis provides direct access to the distribution of onset times, life times and expression rates of mRNA and eGFP. We introduce a two-step stochastic delivery model that reproduces the number distribution of successfully delivered and translated mRNA molecules and thereby the dose–response relation. Our results establish a statistical framework for mRNA transfection and as such should advance the development of RNA carriers and small interfering/micro RNA-based drugs. © 2013 The Authors. Published by Elsevier Inc. All rights reserved.

Key words: mRNA transfection; Non-viral gene delivery; Expression kinetics; Single-cell studies; Pharmacokinetics

#### Introduction

Nucleic acid transfer is widely used in basic research as well as biomedical applications. In recent years, novel stabilized mRNA constructs have become more prevalent in therapeutic applications showing superior properties compared to plasmid DNA.<sup>1–3</sup> This progress is mostly due to the discovery of 5' mRNA anti-reverse cap analogues (ARCA), to the insertion of additional untranslated regions, and to poly(A) tails that significantly promote and prolong efficient translation of foreign mRNA inside cells.<sup>4–10</sup> In general, mRNA delivery has considerable advantages over pDNA delivery

in gene therapy applications. Firstly, mRNA does not require transfer into the nucleus and hence mRNA transfection is also effective in non-dividing cells, which is a major drawback of pDNA transfection.<sup>11–13</sup> This makes mRNA a particularly strong therapeutic agent in dendritic cells which are otherwise hard to transfect.<sup>14–16</sup> Secondly, immunogenic response to mRNA activated by Toll-like receptors (specifically TLR3) is less pronounced compared to unmethylated CpG motifs of DNA recognized by TLR9.<sup>2,9,17,18</sup> In addition, mRNA transfection remains transient, preventing the risk of permanently integrating into the genome. Hence, mRNA delivery is of increasing interest for future biomedical applications in particular with regards to strategies that aim to use mRNA as a programmable device for controlled intracellular mRNA targeting and *in situ* logic evaluation of disease-related conditions.<sup>19–24</sup>

The major hurdle to clinical trials remains the delivery of nucleic acid to eukaryotic cells. As a result, an ongoing search is still underway for non-viral delivery methods that are optimized for efficient and controlled delivery of mRNA. Since the first non-viral delivery of mRNA using cationic lipids by Malone, Felgner and Verma,<sup>25</sup> many synthetic delivery systems were found to be effective for mRNA delivery, with generally better efficiency found for liposomes than for polyplexes.<sup>26–31</sup> It is

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Figure 1. Comparison of mRNA and pDNA Vectors (both gene vectors encoding for the same eGFP protein) and their respective uptake pathways. (A) Linearized RNA (1192 bases) furnished with a stabilizing CAP sequence, an enhancing UTR sequence, and poly-(A) tail. (B) pDNA (4733 base pairs) under the control of the CMV promoter. The vector transfer under identical transfection protocols differs because mRNA is translated after endosomal escape, while plasmid DNA must be transferred into the nucleus for the initiation of transcription.

generally accepted that both mRNA as well as pDNA are translocated via endosomal uptake, cytosolic release and - in case of pDNA - nuclear entry. However, mechanistic insights are mostly limited to assessment of changes in the transfection efficiency as a function of biochemical or structural variations of the carrier. A full pharmacokinetic model, which in principle has been established using compartment models and rate equations,<sup>32–34</sup> lacks validation due to the multitude of kinetic rates. In comparative studies, it was shown that mRNA transfection compared to pDNA transfection is faster and yields a larger fraction of transfected cells.<sup>27,35</sup> However, a more detailed and quantitative understanding in particular of artificial mRNA delivery is of increasing importance for gaining a systems-level description of the kinetics of RNA-based devices.<sup>21,24,36</sup> The degree of predictive power describing synthetic RNA expression level and timing will nevertheless depend on the degree of accuracy with which the transfer efficiency and transfer kinetics can be described. Moreover, predictive modeling of mRNA transfection will be instrumental for the advancement of mRNA based therapies. Yet, any nonviral delivery is inherently stochastic and the expression level and timing of every single cell is different. Hence, measurements at the single-cell level and analysis of the corresponding distribution functions are necessary to acquire the true population response in transfection experiments. Using singlecell analysis, we recently showed that in the case of pDNA transfection, the distribution of gene expression levels can be reproduced using a stochastic model.<sup>37</sup> Similarly, a recent statistical analysis of nanoparticle dosing exhibited Poisson-type distribution in the number of nanoparticles being taken up.<sup>3</sup>

Here, we study gene expression after non-viral delivery of synthetic mRNA analyzing single-cell expression traces in terms of numbers of complexes delivered and numbers of proteins being expressed. Using single-cell fluorescence time-lapse imaging and FC, we monitored expression of a cap-stabilized mRNA vector encoding for eGFP. Single-cell fluorescence timecourses were fitted based on rate equations for translation and mRNA/eGFP degradation yielding the onset time distribution, mRNA/eGFP degradation rates, and the expression rate. The mRNA expression model applies to at least three different cell lines. We interpret the cell-to-cell variability in eGFP levels, i.e. the distribution of expression rates, in terms of number of successfully delivered and translated mRNA. The latter is estimated using a two-step stochastic delivery model. The model assumes delivery of mRNA in finite size complexes that are taken up stochastically by endosomes and randomly released from endosomes into the cytosol. The model quantitatively reproduces the dose-response relation and yields the correct shape of the distribution function. As such, this work represents an advance in predictive modeling of mRNA transfection for quantitative gene expression studies, which we believe will be particularly useful for research on siRNA and miRNA kinetics.

#### Methods

#### pDNA and mRNA-vectors

Two different vectors for pDNA and mRNA transfection were designed. The peGFP-N1-Vector (commercially available at BD Biosciences Clontech, Germany, 4733 base pairs) is the standard eGFP vector. As an mRNA reference construct for *in vitro* transcription, we designed a vector that is based on the pSTI-A120-vector (4746 base pairs, transcript 1192 bases), which has previously been described in literature.<sup>10</sup> The complete vector map is presented in Figure S1. Both vectors contain the same eGFP gene but differ in their promoter region: The peGFP-N1-Vector has a strong CMV-promoter for expression *in vitro*. The mRNA is generated with a commercial *in vitro* transcription kit from the pSTI-A120-vector under the control of the T7 promoter. The backbone of both vectors is

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Figure 2. Representative FC scatter plots for mRNA- and pDNA- mediated eGFP expression in three different cell lines (arbitrary units). (A-F) Two-dimensional scatterplots (sideward scatter vs. fluorescence intensity) for HeLa, A549 and MDCKII cells 25 h post-transfection with mRNA and pDNA. (G-I) Average fluorescence intensity per fluorescent cell (RNA data are shown in blue, DNA data are shown in red); (J-L) Percentage of fluorescent cells (mean  $\pm$  SD).

based on the pCMV-Script vector. pSTI-A120 has a 120-bp poly(A) tail and a 3' untranslated region (UTR) from human  $\beta$ -globin enabling *in vitro* transcription of polyadenylated RNA.

To generate *in vitro*-transcribed mRNA (IVT RNA), the plasmid is linearized downstream of the poly(A) tract by SapI digestion and purified by phenol/chloroform extraction and sodium acetate precipitation. One  $\mu$ g of the linearized vector is used as a template for the *in vitro* transcription reaction using the Biozym Kit (MessageMAX<sup>TM</sup> T7 ARCA-Capped Message Transcription Kit). Having an Anti-Reverse Cap Analog (ARCA) (m<sub>2</sub><sup>-7, 3'-O</sup>G[5']ppp[5']G) cap on the 5' end, ARCA cannot be incorporated in the reverse orientation. Thus, 100% of the caps in the produced IVT RNA are in the correct orientation, increasing the translation efficiency of the IVT RNA.<sup>10,39</sup>

#### Materials

FBS, Leibovitz's L-15 Medium (Gibco), Lipofectamine<sup>™</sup>2000, OptiMEM (Gibco) and Sybr Gold were purchased from Invitrogen, Germany. Syto RNAselect was purchased from Life Technologies, Germany. 6-well culture plates (Falcon) were purchased from VWR International GmbH, Germany. Sterile PBS was prepared in-house. Ham's F-12K, MEM, DMEM and Trypsin-EDTA were purchased from c.c.pro GmbH, Germany.

#### Cell culture

A human alveolar adenocarcinoma cell line (A549, ATCC CCL-185) was grown in Ham's F12K medium supplemented with 10% FBS. HeLa cells (ATCC CCL-2) were cultured using minimum essential medium (MEM) with Earle's salts and L-Glutamine supplemented with 10% fetal bovine serum (FBS). A Madin-Darby Canine Kidney epithelial cell line (MDCKII, ATCC CCL-34) was cultured in DMEM with 4,5 g/L glucose and 110 mg/L pyruvate, supplemented with 10% fetal bovine serum. All cell lines were grown in a humidified atmosphere at 5% CO<sub>2</sub> level.

#### Transfection

The cells were transfected with equimolar amounts of pDNA and mRNA for FC measurements and with equal weight amounts

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Figure 3. mRNA- and pDNA-mediated gene expression kinetics. (A, B) Exemplary images of an average transfection of A549 cells 25 h post-transfection (overlay of bright field and eGFP fluorescence image. Scale bars 100  $\mu$ m). (C, D) Representative fluorescence time-courses of eGFP gene expression after transfection with mRNA (C) and pDNA (D). To highlight the characteristic differences, we chose and color-labeled three exemplary time-courses each. mRNA expression shows early onset and continuous rise in the eGFP level, while pDNA expression exhibits delayed onsets and S-shape expression time-courses.

of pDNA and mRNA for single-cell measurements (see Supplementary). The same transfection reagent (Lipofectamine2000<sup>®</sup>) and the same standard transfection protocols were used for pDNA and mRNA delivery. For transfection with fluorescently labelled mRNA, we followed the standard protocols for labelling mRNA with Sybr Gold/Syto RNAselect and prepared lipoplexes with labelled mRNA.

#### Data acquisition and quantitative image analysis

Live-cell imaging was performed on a motorized inverted microscope (Nikon, Eclipse Ti-E) equipped with an objective lens (CFI PlanFluor DL-10×, Phase1, N.A. 0.30; Nikon) and with a temperature-controlled mounting frame for the microscope stage. To acquire cell images, we used a cooled CCD camera (CLARA-E, Andor). A mercury light source (C-HGFIE Intensilight, Nikon) was used for illumination and a filter cube with the filter set 41024 (Chroma Technology Corp., BP450-490, FT510, LP510-565) was used for eGFP detection. An illumination shutter control was used to prevent bleaching. Images were taken at 10 fold magnification with a constant exposure time of 1300 ms at 10-minute intervals for at least 25 hours post-transfection. Fluorescence images were consolidated into single-image sequence files. Negative control images were taken to assess lamp threshold values and were subtracted from corresponding image sequence files to eliminate autofluorescence effects. Using SINGLECELLTRACKER, an in-housedevelopment software based on ImageJ,<sup>40</sup> fluorescence intensities were integrated over cell contours and corrected for background noise. The software calculates the cells' fluorescence over the entire sequence and connects corresponding intensities to time-courses of the fluorescence per cell.

#### eGFP quantification and calibration

To calculate numbers of eGFP molecules from grey values of the recorded time-lapse movies, a calibration-channel system was developed. Micro channels of known dimensions were filled with eGFP solutions of defined concentrations. Images of the channels were taken under the same experimental conditions as the monitored expression kinetics data, corrected for background and analysed to get calibration curves. For a detailed description of the calibration method, see Supplementary.

#### Flow cytometry

eGFP fluorescence intensity in cells was measured by FC (Partec, CyFlow space). Flow cytometer settings were adjusted to discriminate transfected and non-transfected cells. The Windows<sup>TM</sup> FloMax<sup>®</sup> software package was used for data analysis. See Supplementary for additional information.

#### Results

#### mRNA vs. pDNA transfection

In a first set of experiments, mRNA-mediated transfection was quantified using FC and compared to pDNA-mediated transfection as a reference. As schematically depicted in Figure 1, the design of the mRNA vector (Figure 1, A) was chosen for maximal analogy to the pDNA vector. The pDNA vector is a commercial eGFP plasmid equipped with a CMV promoter (Figure 1, B). The mRNA construct consists of polyadenylated RNAs enabling *in vitro* transcription under the control of the T7promoter and contains 2 sequential human  $\beta$ -globin 3'UTRs as well as the anti-reverse cap analog (ARCA) (see also Methods,

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Figure 4. Single-cell mRNA translation, analyzed by a kinetic rate model. (A) Time-courses of eGFP expression after mRNA transfection (gray lines). Blue lines are fits according to the rate equation model (shown schematically as insert in (B)). (B) Shows the same data as (A), normalized to their maximal value and shifted by their fitted onset times,  $t_0$ . (C) Distribution of the onset time  $t_0$  (mRNA data shown in blue, pDNA data shown in red). (D) Distribution of the expression rate  $k_{TL} \cdot m_0$ . (E) Distribution of the mRNA degradation rate. The black dashed line shows the Gaussian fit to the experimental data, whereas the red dashed line is the Gaussian fit to simulated data (see Supplementary) (F) Distribution of the eGFP decay rate. Dotted lines represent the Gaussian fit to experimental (black) and simulated (red) data.

Supplementary).<sup>10</sup> To collate the outcome of the transfection experiments, identical transfection protocols were followed for mRNA and pDNA transfection using the commercial cationic lipid agent Lipofectamine2000<sup>®</sup>.

The FC data shown in Figure 2 were taken 25 hours posttransfection. The scatterplots with the fluorescence intensity on

the x-axis and the sideward scattering signal on the y-axis show consistent bimodal populations. Both mRNA and pDNA mediated transfection exhibit eGFP-expressing cells and cells that do not express any eGFP. However, for three different cell types, the fluorescence level of eGFP expressing cells in case of pDNA mediated expression is more broadly distributed and shifted towards higher values than the eGFP distribution appearing in mRNA transfection. This effect is also seen in the integrated representation, where the distribution of the average number of eGFP molecules per eGFP expressing cell is shown (Figure 2, G-I). Here, pDNA transfection is shown in red and mRNA transfection in blue. Note that for pDNA transfection, 22% (HeLa), 7% (A549), and 28% (MDCKII) of the cells exhibit eGFP expression levels of 1000 (a.u.) and higher that are not shown for better clarity. In the last row (Figure 2, J-L), the percentage of transfected cells are depicted, which is a direct measure of the transfection efficiencies. We find slightly lower percentages of transfected cells for mRNA-transfected cells compared to pDNA-transfected cells except for MDCKII cells, which feature higher transfection for pDNA vectors.

#### Single-cell mRNA expression kinetics

The most revealing difference between transfection with mRNA and pDNA is seen in the single-cell expression kinetics retrieved from time-lapse studies (Figure 3). Typically, beginning after 1.5 hours of incubation, fluorescence microscopy movies were taken over 25 hours using automated time-lapse microscopy. The total fluorescence intensity of each single cell was followed by image analysis<sup>40</sup> and converted into the number of eGFP molecules per cell (see Supplementary). Figure 3 shows two typical microscopy images of transfected cells 25 hours post-transfection (Figure 3, A and B). Bright field and fluorescence images were overlaid to illustrate the fraction of transfected cells. Figure 3, C and D show gene expression timecourses of single cells. To highlight the characteristic differences in the expression kinetics, we picked three representative traces each and show them in color. While mRNA-transfected cells show an early and steady rise to a maximum with a subsequent decrease, pDNA transfection results in sigmoidal intensity timecourses with a steady-state level of eGFP expression and random onset times. In contrast to the ubiquitous early onset of eGFP expression with mRNA that mainly occurs within 5 hours after transfection, the onset of eGFP expression after transfection with pDNA is spread over the range of 2 hours to 20 hours.

#### Modeling mRNA expression

Since mRNA transgene expression solely involves translation, quantitative modeling reduces to a simple biochemical reaction scheme defined by three kinetic rates as shown in Figure 4, *B*. The schematic shows a rate equation model for mRNA expression consisting of translation, mRNA, and eGFPdegradation. The model is described by the following set of equations for the changes in the number of eGFP molecules, G(t), and the number of mRNA molecules, m(t):

$$\frac{d}{dt}G = k_{TL} \cdot m - \beta \cdot G \tag{1}$$

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Figure 5. Dose–response relation. (A) Percentage of positively transfected A549 cells as a function of increasing amount of mRNA  $(0.05/0.1/0.5/1/2 \mu g)$ . Squares correspond to FC data. The dashed grey line is a single-Poisson fit, the black line is a double-Poisson fit according to our stochastic delivery model. (B) Corresponding fluorescence intensity distributions as measured by FC (bottom to top with increasing mRNA dose).

$$\frac{d}{dt}m = -\delta \cdot m \tag{2}$$

where  $k_{TL}$  denotes the translation rate and  $\delta$  and  $\beta$  the degradation rates of mRNA and eGFP, respectively. With  $t_0$  being the time of expression onset and the initial conditions  $G(t_0) = 0$  and  $m(t_0) = m_0$ , the following solution for the number of eGFP molecules is obtained:

$$G_{mRNA}(t) = \frac{k_{TL} \cdot m_0}{\delta - \beta} \cdot \left(1 - e^{-(\delta - \beta)(t - t_0)}\right) \cdot e^{-\beta(t - t_0)}$$
(3)

Applying Eq. 3 to the experimental time-courses, the data are indeed well fitted. The blue curves in Figure 4, A show exemplary best fits to single-cell time-courses (from a total of 281 time-courses). There are four free parameters: the onset time  $t_0$ , the product of translation ( $k_{TL}$ ) and initial number of effectively translated mRNA molecules ( $m_0$ ), as well as mRNA and protein degradation rates ( $\delta$  and  $\beta$ ). Eq. 3 entails a time-

course showing an exponential increase with rate  $\delta$ - $\beta$  and a longterm decay with decay rate  $\beta$  (see Supplementary). Each fit yields an individual set of parameters. Figure 4, *C*-*F* presents the corresponding distribution of the best-fit parameters, which will be discussed in the following.

#### Expression onset time distribution

In Figure 4, C, the onset time of mRNA (blue) is shown in comparison to the onset time for pDNA transfection (see Supplementary). The faster transfer of mRNA is clearly documented in this distribution. In the case of A549 cells shown here, the onset time distribution after transfection with mRNA peaks approximately 3 hours after transfection and hardly shows any delayed expression onset events after 5 hours, whereas the pDNA onset time distribution is spread over the interval between 2 and 20 hours post-transfection. The timedistribution is an indirect, yet quantitative measure for the transfer time of delivery. As known from microscopy studies, endosomal uptake already starts 10-30 minutes after transfection.<sup>30,41</sup> Therefore, the measured delay in case of mRNA transfer must be limited by endosomal escape rates. Remarkably, mRNA expression onset ceases after 10 hours, indicating that no more endosomes lyse or (more likely) that mRNA molecules are degraded in acidic late endosomes. The broadly distributed onset times for pDNA are associated with rare nuclear entry events, which are believed to occur predominately during mitosis.

#### mRNA degradation rates

Figure 4, E shows the distribution of the mRNA degradation rate retrieved from fitting single-cell time-courses with the described model. The average mRNA degradation rate of 0.062/h (corresponding to an mRNA life time of  $t_{1/2} \approx 11$  hours) is in rough agreement with the literature value of 0.028/h.<sup>10</sup> The value is clearly smaller than the degradation rate of endogenous mRNA  $(\delta < 0.14/h^{42})$ , which is consistent with the reportedly higher stability of ARCA capped mRNA vectors. The distribution of mRNA degradation is well described by a Gaussian with halfwidth 0.024/h. This variability in the degradation rate is on the order of the so-called "extrinsic noise" in eukaryotes.43 The values for the degradation of eGFP (with a mean of 0.056/h) are higher than values that have been reported previously.<sup>42,44</sup> In general, it is noteworthy that the single-cell analysis yields estimates for  $\delta$  and  $\beta$  with high accuracy. The Gaussian fit yields mean values with less than 6% relative error. Knowing the degradation rates is of great value for the improvement of novel vectors and capping sequences. Furthermore, the degradation times are a key to predicting the time-course of expression. In fact, analysis of Eq. (3) predicts that the maximum of expression is reached approximately at  $t_{max} = 17$  h. The time point of half maximum expression value in the declining late phase of expression is  $t_{1/2} = 45$  h. The latter is important because it is a measure for the duration of the transient mRNA expression. Note that Eq. 3 also holds for the case  $\delta < \beta$  (see Supplementary). Moreover, the expression rate  $k_{TL} \cdot m_0$  and the difference in the degradation rates  $(\delta - \beta)$  both determining the amplitude and hence the maximal expression levels, are uncorrelated (see

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Supplementary, Figure S3C). In Figure 4, *E* and *F*, Gaussian fits to simulated data are additionally shown. For simulation, we used the experimentally measured mean degradation rates (see Supplementary). These fits should represent intrinsic noise only, which accounts for about 30% of the total noise. The additional width of the experimental data can be attributed to extrinsic sources of noise involved in the gene transfer process. The kinetics of mRNA proves to be generic because different cell types show the same mRNA expression curves (see Supplementary).

#### A stochastic delivery model by Numbers

It is generally understood that mRNA as well as pDNA delivery via artificial, non-viral vectors is stochastic and dominated by rare processes. In the case of mRNA transfection, the limiting steps are endosomal uptake, endosomal lysis, and mRNA release from lipoplexes. Here, we ask the question whether the measured distribution of expression levels can be reproduced in a stochastic rate model, where each step is assumed to be described by a random process with defined transition probability. The fact that a large fraction of cells does not express eGFP at all indicates that there is a finite probability that no nucleic acid is successfully transferred. Figure 5, A shows the dose-response curve in terms of the percentage of transfected cells versus the concentration of mRNA in µg RNA per ml transfection medium. The corresponding distribution of eGFP expression levels can be seen in Figure 5, B. Data were taken 25 h after transfection using FC. The number of transfected cells monotonically increases with mRNA dosage. It is instructive to describe the transfection process in terms of number of lipoplexes: Lipoplexes form when cationic lipid liposomes are complexed with nucleic acid. Each lipoplex contains a large average number of mRNA molecules (as discussed below). Hence, the delivery of a single lipoplex results in a burst of eGFP expression. If lipoplexes were delivered by overcoming a single barrier, the dose-response function would be described by a Poisson-like process as represented by the dashed line in Figure 5, A (see Supplementary). In this case, the average number of effectively delivered lipoplexes would be  $\langle C \rangle_{\rm SP} = 0.5$ . However, as shown in Figure 5, A, the fraction of transfected cells can be more closely described by a chain of two successive Poisson processes. In this case, the response does not rise up to 100% at large mRNA concentration, which is due to the fact that the two Poisson processes are sequential. A physical interpretation of such a chain of events is shown in Figure 6. A: The scheme shows endosomal uptake of lipoplexes, endosomal lysis, and mRNA release from lipoplexes. It is assumed that Nendosomes are stochastically loaded with a small number of lipoplexes,  $L_{eff}$  and that subsequently a small fraction of endosomes,  $N_{eff}$ , undergoes lysis. These two stochastic steps are modeled as Poisson processes and determine the number of delivered lipoplexes, C. If we assume the lipoplex load  $L_{eff}$  to be proportional to the mRNA concentration, i.e.  $L_{eff} = \lambda \cdot c_{mRNA}$ , we obtain a two-parameter expression for the dose-response function (see Figure 5, A and Supplementary). The best fit yields  $N_{eff} = 0.9$  and  $\lambda = 1.1 \ \mu g^{-1}$ , meaning that at the highest dose of 2  $\ \mu g$ , an effective number of  $L_{eff} = 2.2$  lipoplexes are contained per endosome and



Figure 6. Two-step stochastic mRNA delivery model. (A) Schematic drawing of the stochastic uptake of lipoplexes by endosomes, lysis of the endosomes, and release of the mRNA load by lipoplexes. The model reproduces the dose-response relation shown in Figure 6, A. (B) Fluorescence autocorrelation function of lipoplexes showing an average hydrodynamic radius of  $R_{hydr.} = 60$  nm. (C) Fluorescence image of fluorescently labeled mRNA lipoplexes adsorbed to a petri dish at the concentration that was used for timelapse transfection experiments (dose: 1 µg/ml mRNA). Image analysis led to a typical lipoplex density of order 4000/mm<sup>2</sup> corresponding to about 4-8 lipoplexes per cell (intensity scale inverted for clarity, scale bar 25  $\mu$ m). (**D**) Typical A549 cell five hours after transfection with fluorescently labeled mRNA-lipoplexes (shown in red, scale bar 25 µm). (E) Predicted distribution of delivered lipoplexes derived from the dose-response relation. (F) Predicted distribution of delivered mRNA molecules, based on an average of 350 mRNA molecules per lipoplex. (G) Experimental probability distribution of expression rates ( $k_{TL} \cdot m_0$ , black bars) derived from singlecell data. Blue line indicates best fit of mRNA distribution to the expression distribution, yielding an approximate translation rate of  $k_{TL} = 170/h$ .

that an average of  $\langle C \rangle = N_{eff} \cdot L_{eff} = 2$  successfully delivered complexes is obtained. To demonstrate that such a surprisingly small number of effectively delivered lipoplexes is realistic, we assessed the average number of lipoplexes resting on a single cell in an experiment. At a dose of 1 µg mRNA and after one hour

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incubation time, we found a lipoplex surface density of about  $4000/\text{mm}^2$ , corresponding to an average of 4–8 lipoplexes per cell (Figure 6, *C*). This number is strongly dependent on incubation time due to the diffusion limited transport of the lipoplexes. After five hours of incubation, the number of lipoplexes doubles as seen in Figure 6, *D*. We can safely assume that almost all lipoplexes that hit the cell surface will be taken up by endocytosis over time as reported by others.<sup>29,30</sup> However, not every endosome releases its lipoplex cargo into the cytosol. We find that a lysis rate of about 25–50% leads to accordance of the experimental dose–response relation with the above theoretical estimate.

A single lipoplex contains an average of  $\langle m \rangle = 350$  mRNA molecules. This number is derived knowing the size and packing density of lipoplexes (see Supplementary). The mRNA lipoplexes used here exhibit an average hydrodynamic radius of 60 nm as measured by fluorescence correlation spectroscopy (FCS) (Figure 6, B). The structure and packing density have been measured previously using small angle X-ray scattering (SAXS).  $^{45,46}$  Figure 6, E shows the theoretical distribution of delivered lipoplexes based on the double-Poisson model and the mRNA dose that was used for these experiments (1 µg). If this distribution is multiplied with the number of mRNA molecules per lipoplex, we obtain the theoretical distribution of mRNA per cell as shown in Figure 6, F. It is noteworthy that the theoretical distribution (Figure 6, G, blue curve) is in very satisfying agreement with the shape of the experimental distribution (Figure 6, G, black bars) of expression rates. Comparing the theoretical mRNA distribution with the actually measured distribution of expression rates,  $k_{TL} \cdot m_0$ , we find  $k_{TL} = 170/h$ . This translation rate, which emerges from the analysis of singlecell expression rates, is in the range of independently published values of translation rates.42

#### Discussion

We studied the expression kinetics of eGFP following transfection mediated by mRNA and pDNA. While pDNA complexes have to enter the nucleus, mRNA molecules released from mRNA lipoplexes can be translated immediately after endosomal escape. Consequently, mRNA-induced expression is profoundly earlier and more homogeneously timed than pDNAinduced expression. This behavior is generic and similar onset time distributions are observed e.g. for HeLa and MDCKII cells (data not shown). The high transfection efficiencies for pDNA transfected cells as compared to mRNA transfected cells might be a result of size-dependent lipoplex uptake that has been reported previously.<sup>47</sup> We determined the pDNA-lipoplexes to be about 230 nm in diameter (data not shown), as opposed to 120 nm for mRNA-lipoplexes. The narrow timing of mRNA expression onset at approximately 3 hours post-transfection is in agreement with the observed timing found for endosomal uptake and release in single-particle tracking studies.<sup>30,41</sup> Therefore, the mRNA expression onset distribution might serve as a valuable indicator for the endosomal release time distribution and could be useful for the advancement of artificial endosomolytic agents. Furthermore, our data imply that mRNA expression modeling

can predict the transient course of therapeutic efficacy of mRNA therapeutics in preclinical studies. For example, the development of improved capping sequences of mRNA vectors can be carried out using destabilized eGFP variants. In this case, the protein level decreases substantially faster and long observation times causing experimental difficulties can be circumvented (see Figure S7, Supplementary). Based on kinetic rates obtained in such studies, the time-course of arbitrary gene products with longer half-life times can be inferred. In this context, it should be noted that the half-life of about 12 hours for eGFP determined from single-cell tracks is shorter than previously reported in ensemble measurements, which necessarily average over the somewhat heterogeneous timing of whole populations.<sup>42,44</sup> We also showed that the cell-to-cell variability in the expression levels is well described by a two-step Poisson process. The twostep stochastic model is capable of reproducing the measured dose-response curve consistently with the statistical distribution of expression rates. However, it is limited to transfection in vitro and provides only an approximate description of the underlying delivery cascade. The most important element provided by our model is the account of quantal delivery of mRNA in form of lipoplexes, which is in quantitative agreement with the measured distribution functions. The small number of successfully delivered lipoplexes per cell is the key to understanding the stochastic outcome of transfection experiments that inherently allow a finite number of non-transfected cells. More refined modeling has to be done to picture the dynamics of transfection and to reproduce the onset time distribution. Here, computational representation of size-dependent uptake rates, the nature of endosome lysis, and intracellular diffusion need to be solved. Furthermore, computational modeling of extracellular delivery, mimicking in vivo situations, needs to be advanced to gain impact on translational medicine.

In our experiments, the single-cell time-courses of mRNAmediated transfection showed excellent agreement with the standard biochemical rate model of translation. Hence, singlecell analysis enables direct determination of expression rates as well as decay rates for both mRNA and eGFP with great accuracy and provides a quantitative foundation for kinetic studies on mRNA translational regulation as for example RNA interference. The fact that mRNA transfection exhibits a narrow time window of delivery is beneficial for kinetic studies. This advantage should be of practical importance for future timeresolved studies on siRNA knockdown and RNA constructs for programmed gene regulatory operations.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2013.11.008.

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#### References

- Tavernier G, Andries O, Demeester J, Sanders NN, De Smedt SC, Rejman J. mRNA as gene therapeutic: how to control protein expression. *J Control Release* 2011;150(3):238-47.
- Yamamoto A, Kormann M, Rosenecker J, Rudolph C. Current prospects for mRNA gene delivery. *Eur J Pharm Biopharm* 2009; 71(3):484-9.
- Andries O, De Filette M, Rejman J, De Smedt SC, Demeester J, Van Poucke M, et al. Comparison of the gene transfer efficiency of mRNA/ GL67 and pDNA/GL67 complexes in respiratory cells. *Mol Pharm* 2012;9(8):2136-45.
- Stepinski J, Waddell C, Stolarski R, Darzynkiewicz E, Rhoads RE. Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl (3'-deoxy) GpppG. RNA (New York, NY) 2001;7(10):1486-95.
- Jemielity J, Stepinski J, Jaremko M, Haber D, Stolarski R, Rhoads RE, et al. Synthesis of novel mRNA 5¢ cap-analogues: dinucleoside P1, P3-Tri-, P1, P4-Tetra-, and P1, P5-Pentaphosphates. *Nucleosides Nucleotides Nucleic Acids* 2003;22(5–8):691-4.
- Zohra FT, Chowdhury EH, Tada S, Hoshiba T, Akaike T. Effective delivery with enhanced translational activity synergistically accelerates mRNA-based transfection. *Biochem Biophys Res Commun* 2007;**358**(1): 373-8.
- Kuhn AN, Diken M, Kreiter S, Selmi A, Kowalska J, Jemielity J, et al. Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. *Gene Ther* 2010;17(8):961-71.
- Pesole G, Grillo G, Larizza A, Liuni S. The untranslated regions of eukaryotic mRNAs: structure, function, evolution and bioinformatic tools for their analysis. *Brief Bioinform* 2000;1(3):236-49.
- Kormann MSD, Hasenpusch G, Aneja MK, Nica G, Flemmer AW, Herber-Jonat S, et al. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat Biotech* 2011;29(2):154-7.
- Holtkamp S, Kreiter S, Selmi A, Simon P, Koslowski M, Huber C, et al. Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. *Blood* 2006;108(13):4009-17.
- Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995;270(32):18997-9007.
- Wilke M, Fortunati E, vandenBroek M, Hoogeveen AT, Scholte BJ. Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Ther* 1996;3(12):1133-42.
- Brunner S, Sauer T, Carotta S, Cotten M, Saltik M, Wagner E. Cell cycle dependence of gene transfer by lipoplex polyplex and recombinant adenovirus. *Gene Ther* 2000;7(5):401-7.
- Mockey M, Goncalves C, Dupuy FP, Lemoine FM, Pichon C, Midoux P. mRNA transfection of dendritic cells: synergistic effect of ARCA mRNA capping with Poly(A) chains in cis and in trans for a high protein expression level. *Biochem Biophys Res Commun* 2006;**340**(4):1062-8.
- Kuhn AN, Diken M, Kreiter S, Vallazza B, Türeci Ö, Sahin U. Determinants of intracellular RNA pharmacokinetics: implications for RNA-based immunotherapeutics. *RNA Biol* 2011;8(1):35-43.
- De Haes W, Van Mol G, Merlin C, De Smedt SC, Vanham G, Rejman J. Internalization of mRNA lipoplexes by dendritic cells. *Mol Pharm* 2012;9(10):2942-9.
- Arthur JF, Butterfield LH, Roth MD, Bui LA, Kiertscher SM, Lau R, et al. A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther* 1997;4(1):17-25.
- Strobel I, Berchtold S, Gotze A, Schulze U, Schuler G, Steinkasserer A. Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. *Gene Ther* 2000;7(23):2028-35.
- Seelig G, Soloveichik D, Zhang DY, Winfree E. Enzyme-free nucleic acid logic circuits. *Science* 2006;314(5805):1585-8.

- Rinaudo K, Bleris L, Maddamsetti R, Subramanian S, Weiss R, Benenson Y. A universal RNAi-based logic evaluator that operates in mammalian cells. *Nat Biotechnol* 2007;**25**(7):795-801.
- Win MN, Smolke CD. Higher-order cellular information processing with synthetic RNA devices. *Science* 2008;**322**(5900):456-60.
- Leisner M, Bleris L, Lohmueller J, Xie Z, Benenson Y. Rationally designed logic integration of regulatory signals in mammalian cells. *Nat Nanotechnol* 2010;5(9):666-70.
- Xie Z, Wroblewska L, Prochazka L, Weiss R, Benenson Y. Multi-input RNAi-based logic circuit for identification of specific cancer cells. *Science* 2011;333(6047):1307-11.
- Carothers JM, Goler JA, Juminaga D, Keasling JD. Model-driven engineering of RNA devices to quantitatively program gene expression. *Science* 2011;334(6063):1716-9.
- Malone RW, Felgner PL, Verma IM. Cationic liposome-mediated RNA transfection. *Proc Natl Acad Sci U S A* 1989;86(16):6077-81.
- Bettinger T, Carlisle RC, Read ML, Ogris M, Seymour LW. Peptidemediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. *Nucleic Acids Res* 2001;29(18):3882-91.
- Rejman J, Tavernier G, Bavarsad N, Demeester J, De Smedt SC. mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *J Control Release* 2010;147(3):385-91.
- Debus H, Baumhof P, Probst J, Kissel T. Delivery of messenger RNA using poly(ethylene imine)-poly(ethylene glycol)-copolymer blends for polyplex formation: biophysical characterization and in vitro transfection properties. *J Control Release* 2010;**148**(3):334-43.
- Lin AJ, Slack NL, Ahmad A, George CX, Samuel CE, Safinya CR. Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes. *Biophys J* 2003;84(5):3307-16.
- Chan C-L, Majzoub RN, Shirazi RS, Ewert KK, Chen Y-J, Liang KS, et al. Endosomal escape and transfection efficiency of PEGylated cationic liposome–DNA complexes prepared with an acid-labile PEG-lipid. *Biomaterials* 2012;33(19):4928-35.
- Safinya CR, Ewert K, Ahmad A, Evans HM, Raviv U, Needleman DJ, et al. Cationic liposome–DNA complexes: from liquid crystal science to gene delivery applications. *Philos Transact R Soc A, Math Phys Eng Sci* 2006;**364**(1847):2573-96.
- Varga CM, Hong K, Lauffenburger DA. Quantitative analysis of synthetic gene delivery vector design properties. *Mol Ther* 2001;4(5):438-46.
- Dinh AT, Pangarkar C, Theofanous T, Mitragotri S. Understanding intracellular transport processes pertinent to synthetic gene delivery via stochastic simulations and sensitivity analyses. *Biophys J* 2007;92(3):831-46.
- Kamiya H, Akita H, Harashima H. Pharmacokinetic and pharmacodynamic considerations in gene therapy. *Drug Discov Today* 2003;8(21): 990-6.
- Zou S, Scarfo K, Nantz MH, Hecker JG. Lipid-mediated delivery of RNA is more efficient than delivery of DNA in non-dividing cells. *Int J Pharm* 2010;**389**(1–2):232-43.
- Guo P. The emerging field of RNA nanotechnology. *Nat Nanotechnol* 2010;5(12):833-42.
- Schwake G, Youssef S, Kuhr JT, Gude S, David MP, Mendoza E, et al. Predictive modeling of non-viral gene transfer. *Biotechnol Bioeng* 2009;105(4):805-13.
- Summers HD, Rees P, Holton MD, Rowan Brown M, Chappell SC, Smith PJ, et al. Statistical analysis of nanoparticle dosing in a dynamic cellular system. *Nat Nano* 2011;6(3):170-4.
- Grudzien E, Stepinski J, Jankowska-Anyszka M, Stolarski R, Darzynkiewicz E, Rhoads RE. Novel cap analogs for in vitro synthesis of mRNAs with high translational efficiency. RNA (New York, NY. 2004;10(9):1479–87.
- Youssef S, Gude S, R\u00e4dler JO. Automated tracking in live-cell timelapse movies. *Integr Biol* 2011;3(11):1095-101.
- de Bruin K, Ruthardt N, von Gersdorff K, Bausinger R, Wagner E, Ogris M, et al. Cellular dynamics of EGF receptor-targeted synthetic viruses. *Mol Ther* 2007;15(7):1297-305.

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- Sacchetti A, El Sewedy T, Nasr AF, Alberti S. Efficient GFP mutations profoundly affect mRNA transcription and translation rates. *FEBS Lett* 2001;492(1-2):151-5.
- Blake WJ, Kaern M, Cantor CR, Collins JJ. Noise in eukaryotic gene expression. *Nature* 2003;422(6932):633-7.
- 44. Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, et al. Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 1998;273(52):34970-5.
- Rädler JO, Koltover I, Salditt T, Safinya CR. Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science* 1997;275(5301):810-4.
- Rädler JO, Koltover I, Jamieson A, Salditt T, Safinya CR. Structure and interfacial aspects of self-assembled cationic lipid–DNA gene carrier complexes§. *Langmuir* 1998;14(15):4272-83.
- Ross PC, Hui SW. Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther* 1999;6(4):651-9.