Targeting of Polyelectrolyte RNA Complexes to Cell Surface Integrins as an Efficient, Cytoplasmic Transfection Mechanism

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ABSTRACT: This is the first demonstration of receptor-mediated delivery of mRNA and establishes a new approach to gene therapy. Messenger RNA (mRNA) provides a promising alternative to plasmid DNA as a genetic material for delivery in non-viral gene therapy strategies. Since it does not require access to the nucleus and is less dependent on the cell cycle for expression, mRNA delivered using cationic lipids or short cationic polymers can be effectively translated within target cells. In this study, mRNA formed discrete nanoparticles following self assembly with a range of cationic polymers. Based on transfection activities, the low molecular weight polycations were more efficient than high molecular weight, while protamine and poly(ethylenimine) were far more efficient than poly(L-lysine). Receptor-mediated delivery of mRNA was demonstrated using the synthetic polyamino acid $(K)_{16}$GACDCRGDCFCA designed to promote cell entry following interaction with cell surface α_v integrins. RGD-bearing mRNA complexes showed very high levels of expression, reaching over 60% transduction of B16F10 cells.

KEY WORDS: RNA, transfection, RGD, integrin, gene therapy, gene delivery.

INTRODUCTION

Plasmid DNA is widely used for the development of non-viral gene therapy in a range of sophisticated gene delivery systems [1,2].

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However, difficulties of cellular targeting and inappropriate cellular processing restrict its expression and the overall efficiency of plasmid-based gene delivery is not very high. One important cellular factor is the requirement for the plasmid to translocate from the cytoplasm through the relatively impermeant nuclear membrane in order to gain access to cellular RNA polymerases [3,4], a process estimated to be less than 1% efficient [5].

Our recent studies have shown that messenger RNA (mRNA) can be used as an alternative transfection agent; undergoing translation in the cytoplasm and avoiding the requirement for entry of therapeutic nucleic acids into the nucleus [6]. However, the efficient use of mRNA demands certain properties of the delivery system. One of the most important requirements is that the RNA is presented to the ribosome either free or only loosely complexed to cationic delivery agents; presumably, because ribosomal components cannot recognise and translate the mRNA that is contained within the stable polyelectrolyte complexes.

RNA can be translated effectively when it is introduced into cells either using cationic lipids [7,8], which are thought to release some of the free nucleic acid in the cytoplasm [9], or using relatively low molecular weight polycations [6]. The latter are thought to form polyelectrolyte complexes with only limited stability due to the small number of positive charges on each polycation. They also provide the possibility to deliver RNA to specific cell surface receptors by condensing it with cationic polymers bearing appropriate targeting ligands.

The purpose of this study was to evaluate a range of cationic polymers for their ability to deliver mRNA effectively and to correlate the physicochemical and biological properties of the complexes formed. In addition, we examined the feasibility of delivering RNA complexes to cells using RGD sequences designed to bind to cell surface integrins and undergo internalization into endosomes. The encouraging results obtained suggest that cell-selective expression of RNA should be possible by targeting to specific cell surface receptors to yield high level cytoplasmic expression of therapeutic agents.

MATERIALS AND METHODS

Synthesis of mRNA Transcripts

The green fluorescent protein (GFP)-encoding plasmid pGEM4Z/GFP/A64 was a kind gift from Dr D. Boczkowski (Duke University Medical Center, USA). The plasmid was linearised using *Spe*1 and the

mRNA encoding GFP was generated using the T7 RiboMAX^{TM} kit (Promega) with $m^7G(5')pppG(5')$ cap analog (Ambion, Oxon, UK) at a ratio of 5 : 1 cap analog/GTP; this 894 bp mRNA contained a polyA tail (A_{64}). The product size was checked by electrophoresis.

Purification of the mRNA was performed by RQ1 DNase I digestion, followed by extraction with phenol: chloroform: isoamyl alcohol and chloroform and precipitation by the addition of sodium acetate (10% v/v, 3 M, pH 5.5) and ethanol (2.5 volume, 100%). The precipitated RNA was washed with 70% ethanol, dissolved in water, quantified spectrophotometrically at 260 nm, and examined by agarose gel electrophoresis after denaturation at 65°C for 15 min.

Cell Culture and Transfection Studies

The mouse melanoma cell line B16-F10 was grown in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax (1 mM), glucose (1 g/L, GibcoBRL) and 10% foetal calf serum (FCS).

A range of commercially available cationic polymers was used for mRNA transfection, including branched polyethylenimine (PEI, 2 kDa and 25 kDa, Aldrich), poly(L-lysine) (PLL, 4.0 kDa and 211 kDa, Sigma) and protamine (Sigma). The synthetic poly(amino acid) $(K)_{16}$GACDCRGDCFCA was produced using solid phase chemistry by Severn Biotech Ltd (Kidderminster, UK). The transfection activities achieved were compared with those of DOTAP (*N*-(1-(2,3-dioleoyloxy) propyl)-*N*,*N*,*N*-trimethylammonium salts, Sigma).

For complexation of mRNA, the desired amount of polycation (9 μL of 10 mM aqueous amine nitrogen stock solution for N/P = 10), or lipoplex (1.2 μL of a 13 mM aqueous amine nitrogen stock solution of DOTAP for N/P = 1.8, equivalent to a w/w ratio DOTAP/nucleic acid of 4 as recommended by the manufacturer) was added to the appropriate amount of heat-denatured RNA (10 min at 65°C) in 10 mM HEPES pH 7.4. The mixture was gently vortexed and after 15 min the resulting lipoplexes or polyplexes were added directly to a 48-well plate (30 μL/well) containing 2×10^4 cells per well (original density; cells were plated at least 24 h before transfection) in 200 μL of DMEM. Transfection studies with cationic polymers were performed in the presence of 100 μM chloroquine. After 4 h, the mixture containing the complexes was discarded and 500 μL of fresh medium containing 10% foetal calf serum was added to each well. The cells were cultured for 24 h prior to analysis for reporter gene expression.

Assay of Reporter Gene Activity

The analysis for GFP expression was carried out on a Coulter Epics XL flow cytometer. The cells were trypsinised at appropriate times after transfection, washed with PBS and then fixed in 2% paraformaldehyde. GFP was excited using the 488 nm line of an Argon laser; the emitted light was measured at 520 nm (green fluorescence) and 575 nm (red fluorescence) to enable correction for autofluorescence by diagonal gating. Background fluorescence and autofluorescence were determined using mock treated cells. Cellular debris showing reduced side- and forward-scatter was excluded from the analysis. The software programme, WinMDI, was used to analyse the data which was expressed as the percentage of GFP-positive cells.

Photon Correlation Spectroscopy

The hydrodynamic diameters of the polyelectrolyte mRNA complexes (containing 10 μg/mL, mRNA) were measured by photon correlation spectroscopy (PCS) using a ZETASIZER 1000 (Malvern Instruments, Malvern, UK) equipped with a 70 mW external argon laser. Complexes containing PLL, protamine or RGD-K_{16} were made at N : P = 4; those containing PEI were made at N : P = 10, and those based on DOTAP were made at N : P 1.8 (weight ratio DOTAP/DNA = 4). The measurements were performed at 25°C in HEPES buffer (10 mM, pH 7.4) in triplicate with sampling time set to automatic, and the data were interpreted using Contin software.

RESULTS

Characterisation of the Complexes Formed Between mRNA and Polycations

The products from the self assembly of mRNA with cationic polymers or DOTAP were evaluated using PCS. To achieve comparable charge ratios, the complexes were compared at N/P ratios (i.e., the ratio of total amine groups to RNA phosphates) of 4.0 for PLL, $(K)_{16}$GACDCRGDCFCA and protamine, 10.0 for PEI and 1.8 for DOTAP. In the case of DOTAP/mRNA, relatively large aggregates with an average hydrodynamic diameter of 250 nm (Table 1) were formed. Complexes formed between mRNA and cationic polymers were all smaller than this; ranging from the smallest size of 86 nm

Table 1. Diameter of polyelectrolyte complexes formed between mRNA and cationic agents determined by photon correlation spectroscopy. Protamine, poly(L-lysine) (211 kDa, 4 kDa), poly(ethylenimine) (25 kDa, 2 kDa), DOTAP. PLL/RNA and protamine/RNA complexes were formed at an N : P ratio of 4.0; DOTAP/RNA complexes N : P 1.8 (w/w = 4) and PEI/RNA complexes N : P ratio of 10. Complexes were formed in 10 mM HEPES, pH 7.4.

Complex	Average Diameter/nm	Polydispersity
DOTAP/RNA	249.3 ± 6.6	0.24
Protamine/RNA	85.8 ± 1.5	0.25
pLL 211 kD_A/RNA	93.2 ± 2.5	0.42
PEI 2 kD_A/RNA	116.4 ± 4.1	0.34
PEI 25 kD_A/RNA	103.0 ± 7.7	0.58
pLL 4 kD_A/RNA	95.1 ± 4.6	0.57
RGD $(K)_{16}$/RNA	99.9 ± 4.7	0.56

diameter, for the protamine/mRNA complexes, up to 116 nm for the PEI (2 kDa)/mRNA complexes. Complexes of $(K)_{16}$GACDCRGDCFCA/mRNA showed an intermediate size of 100 nm. Polydispersities (defined as the peak width divided by the peak height) ranged from 0.24 (DOTAP/mRNA) up to 0.58 (for PEI 25 kDa/mRNA). The greater polydispersity of the PEI-based complexes may reflect the intrinsic polydispersity of the synthetic polymer, whereas DOTAP is a preparation of identical molecules.

Transfection Activity of Polyelectrolyte RNA Nanoparticles

Several cationic polymers were compared for their ability to mediate the expression of mRNA in B16F10 cells with DOTAP/mRNA as a positive control. Typically about 50% transduction frequency of B16F10 cells and a good efficiency of luciferase expression in the positive cells (over 500 r.f.u./cell; Figure 1) was observed. Polyelectrolyte complexes, formed using relatively high molecular weight PLL (211 kDa) to condense the mRNA, showed only limited transfection activity (approximately 10%), which is consistent with previous reports that cationic polymers for mRNA delivery preferably contain small numbers of positive charges, probably to enable release of mRNA within the cytoplasm. Of particular note is the low level of transgene expression achieved per cell using this high molecular weight polycation (19.7 r.f.u./cell). Similarly, PEI 25 kDa, an intermediate molecular weight polymer, showed a relatively high frequency of transfection (29.1%), but again the average transgene expression per cell was low (85.8 r.f.u./cell). In contrast, the low molecular weight PEI (2 kDa) achieved 42% frequency of transfection with a high

average transgene expression per cell (832 r.f.u./cell). However, the greatest transfection was by protamine/mRNA, showing over 50% transduction frequency with an average transgene expression of 830 r.f.u./cell. Generally the frequency of transfection is regarded to be important, since the level of transgene expression required depends on the therapeutic strategy employed; nevertheless, the low levels of

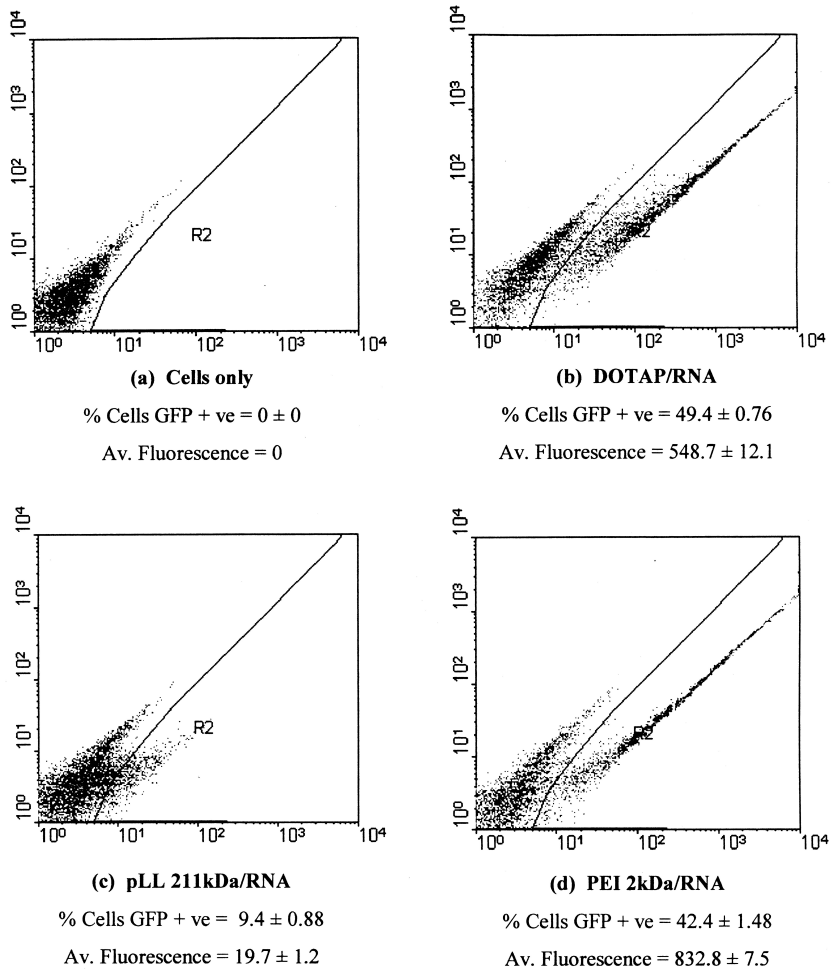

Figure 1. Transfection activity of mRNA complexes measured in B16F10 melanoma cells in the presence of chloroquine (100 μM). Complexes were formed at the same charge ratios as in Table 1. Complexes were transfected in the absence of serum for 4 h (37°C, 5% CO_2) and then reincubated in the presence of serum for a further 20 h before analysis of expression of green fluorescence protein.

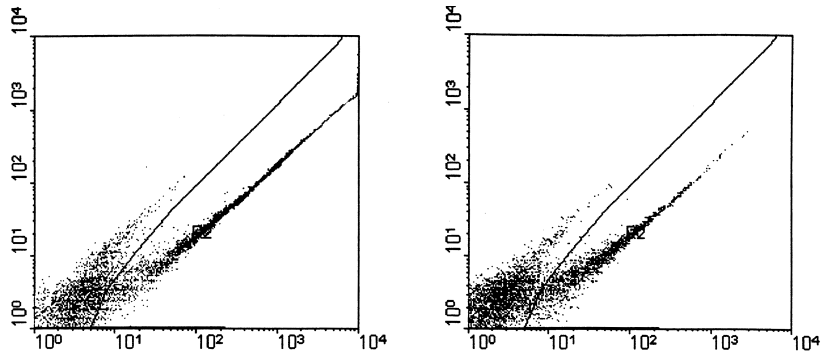

Figure 1. Continued.

expression achieved, using high molecular weight cationic polymers, is consistent with poor processing of these relatively stable complexes by cytoplasmic ribosomes.

Targeted Delivery of mRNA Using Integrin-Binding Peptides

To examine the possibility of receptor-mediated delivery of mRNA, complexes were formed using the synthetic peptide $(K)_{16}$GACDCRGDCFCA, the transfection activity was compared with that of complexes formed using PLL 4 kDa, which has an average of 19 lysines per molecule. Whereas the simple PLL 4kDa/mRNA complexes showed no transfection activity at all in B16F10 cells, the mRNA complexes, formed using $(K)_{16}$GACDCRGDCFCA, showed over 60% frequency of transduction, with an average cellular expression of 170 r.f.u./cell. The profound difference in activity by these two agents is thought to represent increased cell entry following peptide-mediated binding of $(K)_{16}$GACDCRGDCFCA/mRNA complexes to cell surface integrins; although it is also possible that the two types of complexes follow different intracellular routings. It is interesting that, although integrin-targeting gave dramatic increases in the frequency of transduction, the average fluorescence per cell was not particularly high. This may be a property of the $(K)_{16}$ component, and might be improved on by optimising the mRNA-binding component of the conjugate (Figure 2).

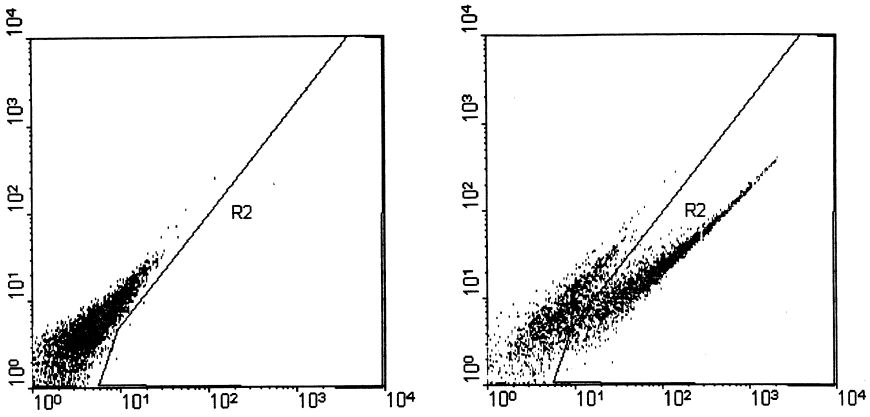

Figure 2. RGD-targeted mRNA transfection. Transgene expression was measured in B16F10 cells following delivery of mRNA using (a) 4 kDa PLL, or (b) $(K)_{16}$GACDCRGDCF-CA. Complexes were formed at an N : P ratio of 4.0 and incubated with B16F10 cells in the presence of chloroquine (100 μM) for 4 h in the absence of serum, before being reincubated for a further 20 h in the presence of serum.

DISCUSSION

Therapeutic strategies based on DNA delivery have been widely explored, while mRNA has received little attention and factors regulating its delivery have not been elucidated. Apart from recent studies using mRNA to transduce dendritic cells *ex vivo* [10–12], few therapeutic strategies have been developed. This is surprising given the breadth of opportunities presented by the use of mRNA and its advantages over DNA. A chief advantage of mRNA is that the cytoplasmic site of action enables it to function in post mitotic or non-cycling cells [13], where the nuclear membrane remains intact and delivery of DNA is usually difficult [5,6]. mRNA expression can achieve a reasonable duration, largely controlled by the presence of an appropriate poly(A) tail, while avoiding the possibility of genetic integration [6]. A disadvantage of mRNA for therapy is the difficulty of gaining cell-specific expression. Whereas the regulation of DNA transcription can be controlled using appropriate tissue-selective promoters, factors regulating cell-selective RNA translation are poorly understood. Hence it is likely in the forseeable future that mRNA will be employed mainly

for short-to-medium duration expression of locally-acting proteins and that its site of expression will be dependent on targeted delivery of the mRNA vector.

This report presents the first example of targeted delivery of mRNA to cell surface receptors. The use of integrin-binding peptides was selected because this approach is known to function effectively for DNA delivery [14,15], although the widespread expression of α_v integrins means that this system is not selective for specific target cells. Nevertheless, the high frequency of mRNA expression achieved using the integrin-targeting system demonstrate that this approach can be successful and paves the way for the development of more cell-specific mRNA targeting systems to achieve higher levels of transgene expression.

A comparison of the condensation of mRNA with a range of cationic polymers showed the formation of nanoparticles, approximately 100 nm diameter, in every case. These results are similar to those reported for polycation-mediated condensation of plasmid DNA [16], and indicate that the single chain structure and relatively small size of mRNA, compared to DNA plasmids, has no dramatic effect on the condensation process. Despite their similar sizes, the non-targeted complexes showed significant variations in transfection activity. As expected, complexes based on large polycations showed only low levels of transduction, consistent with limitations on cytoplasmic unpacking for translation [6]. In comparing the activity of RNA complexes formed using small polycations, however, it was interesting that PLL 4 kDa/RNA complexes were virtually inactive, while protamine/mRNA and PEI 2 kDa/mRNA complexes were highly active. While the pH-responsive nature of PEI is often invoked to explain the good DNA transfection activity of PEI [17,18], such a mechanism is unlikely to apply to protamine. Hence the precise reasons underlying these differential activities are not yet known, although it is tempting to wonder if the high arginine content of protamine could promote membrane translocation as documented for the HIV tat protein [19].

Taken together these data show that certain cationic polymers are more effective than others at delivering mRNA, while integrin-targeting appears to be a very promising way forward. It would be very interesting to combine these approaches and evaluate mRNA delivery using protamine-RGD or PEI-RGD conjugates. Such agents might show high efficiency of mRNA expression with very little toxicity and could find a range of applications *in vitro*, *in vivo* and *ex vivo*.

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