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European Journal of Pharmaceutics and Biopharmaceutics 60 (2005) 279–285
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Cryoconserved shielded and EGF receptor targeted DNA polyplexes: cellular mechanisms☆

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Received 23 April 2004; accepted in revised form 16 August 2004
Available online 24 March 2005

Abstract

Recently, cryoconservable polyethylene glycol (PEG)-shielded and epidermal growth factor receptor (EGFR)-targeted polyplexes (EGF^+ polyplexes) were engineered in our laboratory for tumor-directed transfer and expression of DNA. Here, we further analyzed specificity and kinetics of EGFR-mediated cellular uptake of these polyplexes. Similar to our previous results, EGF^+ polyplexes significantly enhanced the transfection efficiency as compared to polyplexes without EGF (EGF^- polyplexes) in HUH-7 hepatoma cells and Renca-EGFR renal carcinoma cells. EGF^+ polyplexes rapidly associated with the cells within 30 min of exposure, and binding of EGF^+ polyplexes to the cells after 4 h was significantly higher than that of EGF^- polyplexes. In the presence of free EGF, both cell association and transfection efficiency of EGF^+ polyplexes were markedly reduced indicating that these effects were primarily mediated via ligand receptor interaction. Fluorescence microscopy revealed that the cell-associated EGF^+ polyplexes aggregated to micrometer sized clusters, resembling typical clustering of receptors upon ligand binding. In conclusion, EGFR-targeting enhances transfection efficiency due to accelerated and increased cell association followed by aggregation of the bound EGF^+ polyplexes.

Keywords: DNA complexes; Non-viral gene transfer; Polyethylenimine; Polyethylene glycol; Transfection

1. Introduction

Polycationic polymers such as polylysine or polyethyleneimine (PEI) bind and condense DNA due to charge interactions, triggering the formation of particles with virus-like dimensions termed 'polyplexes' [1–4]. Recently, surface-shielded DNA polyplexes [1,5–10] with virus-like characteristics have been synthesized which after systemic administration can deliver genes into distant target tissues such as tumors. In order to obtain a higher degree of cellular specificity for such tissue-directed delivery, targeting ligands have to be incorporated. For example, tumor-directed DNA delivery has been achieved by incorporating the serum protein transferrin, antibodies or growth factors into the complexes by covalent attachment to the cationic polymer [1,8,9].

The epidermal growth factor receptor (EGFR) is highly overexpressed in different human tumors. For example, hepatocellular carcinoma cells express EGF receptor levels of approximately 3×10^5 receptors per cell [11]. The EGFR has high affinity for EGF, 10% of the receptors exhibit a dissociation constant (K_d) of 3×10^{-10} M whereas the rest have a K_d of 2×10^{-9} M [12]. Because of these favorable characteristics we and others used this receptor as a target for cell attachment of polyplexes; DNA has been complexed with polylysine conjugates of anti-EGFR antibodies [13,14], fusion proteins containing the EGFR ligand TGF alpha [15], and polycation conjugates of epidermal growth factor EGF [16–19] or EGF derived peptides [20–22]. On the cellular level, such EGF containing polyplexes showed significantly enhanced transfection efficiency as compared to polyplexes lacking EGF. However, until now the process of enhanced cellular uptake of EGF containing polyplex has not been characterized in detail.

☆ Presented at the 5th International Conference on cell culture and in vivo models for drug absorption and delivery, Saarbrucken, Germany. February 25–March 5, 2004.

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doi:10.1016/j.ejpb.2004.08.014

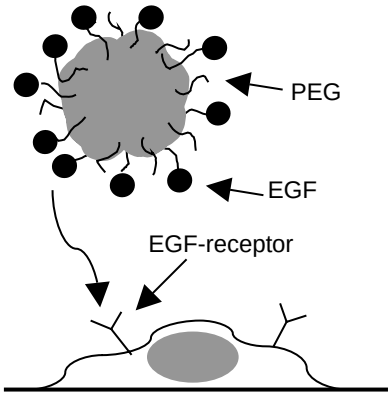

Fig. 1. Receptor mediated gene delivery via the EGF receptor. PEI polyplexes are shielded with covalently attached PEG. To obtain specific cellular binding to the EGF receptor, EGF is covalently attached to PEI via a PEG spacer.

We have used polyplexes modified with EGF (EGF^+ polyplexes) covalently attached to PEI via a PEG spacer. In addition the polyplexes contained the hydrophilic polymer polyethylene glyocol (PEG) in order to prevent undesired interactions with non-target cells [16] (see Fig. 1). Upon systemic application the PEG shield enables prolonged blood circulation of polyplexes and the EGF ligand enables targeting of distant subcutaneous EGFR-rich hepatoma tumors in SCID mice. This results in high and specific reporter gene expression in the tumor [18].

Another major challenge in the development of non-viral vectors for systemic delivery of therapeutic genes is the engineering of polyplexes that can be stored for a reasonable time period without changes in particle size or transfection efficiency. In previous work considerable aggregation of EGF^+ polyplexes was observed [18]. Our polyplexes were therefore generated in HEPES-buffered glucose to allow cryoconservation at −80 °C. Fluorescent dye-labeled DNA was incorporated in these particles to analyze whether EGF accelerated and increased binding of polyplexes to EGFR expressing cells. Moreover, we sought to find out whether such accelerated association and enhanced transfection efficiency of EGF^+ polyplexes was indeed due to specific ligand receptor interaction, and whether these effects were obtained after cryoconservation.

2. Materials and methods

2.1. Chemicals and reagents

Branched PEI with an average molecular weight of 25 kDa (B-PEI) was obtained from Sigma–Aldrich (Munich, Germany). Linear PEI with an average molecular weight of 22 kDa (L-PEI) is available from Euromedex (Exgen 500, Euromedex, Souffelweyersheim, France). PEI was used at a 1 mg/ml stock solution, neutralized with HCl. PEI22-PEG20 (PEI-SPA-3PEG 20 kDa) was synthesized and purified as previously described in Kursa et al. [8], and EGF-PEG-PEI25 was synthesized as described [18].

Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) is described in [4] and was produced endotoxin-free by Elim Biopharmaceuticals (San Francisco, CA, USA) or Aldevron (Fargo, ND, USA). A plasmid pEGFP-N1 (encoding Enhanced Green Fluorescent Protein (EGFP) under control of the CMV promoter) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA).

Cell culture media and fetal calf serum (FCS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). HUH-7 cells (JCRB 0403; Tokyo, Japan) were grown in DMEM: F12 (1:1) with Glutamax I medium supplemented with 10% FCS at 37 °C in 5% CO_2 humidified atmosphere. Renca-EGFR cells, these are mouse renal carcinoma Renca cells stably cotransfected with the plasmids pLTR-EGFR and pSV2neo (kindly provided by Winfried Wels, Georg-Speyer-Haus, Frankfurt am Main, Germany), were cultured in RPMI-1640 with Glutamax I medium supplemented with 10% FCS and 0.5 mg/ml geneticin under the standard conditions mentioned before.

2.2. Polyplex formation

DNA complexes containing EGF (EGF^+ polyplexes) were prepared by first diluting and mixing EGF-PEG-PEI and PEG-PEI conjugates with free PEI22 at a molar ratio of EGF:PEG:PEI of 13:22:100% in HBG (HEPES buffered glucose: 5% (w/v) glucose, 20 mM HEPES, pH 7.1). For the EGF^- polyplexes, the PEG-PEI conjugate was mixed with free PEI22 at a molar ratio of PEG: PEI of 22:100%. The PEI conjugate buffer solution was then mixed with plasmid DNA diluted in HBG at a molar ratio of PEI nitrogen to DNA phosphate (N/P) of 6 (i.e. 156 μg/ml) and a final DNA concentration of 200 μg/ml. Complexes were incubated for 30 min at room temperature, snap-frozen in liquid nitrogen, subsequently stored at −80 °C and, before use, allowed to stand for 30 min at room temperature after thawing. Median particle size of shielded EGF^+ polyplexes was determined by laser-light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) after dilution to 10 μg/ml prior to measurement as described in [23]. The presence of glucose in the formulation before freezing was important to avoid aggregation during the freezing/thawing process.

2.3. Reporter gene expression

Cells were seeded in 96 well plates at a density of 10^4 cells per well 24 h prior to transfection. pCMVLuc transfection complexes were added to the cells in 100 μl fresh culture medium. Culture medium was replaced 4 h after transfection and gene expression was measured after 24 h. Luciferase detection was carried out as described before [24]. Transfection efficiency was expressed as relative light units

(RLU) per 10,000 seeded cells (mean ± SD of triplicates). Two nanograms of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units.

To determine the percentage of transfected cells, pEGFP-N1 complexes were added to the cells seeded 24 h prior to transfection in 12 well plates at a density of 10^5 cells per well. The medium was replaced 4 h after the transfection. After 48 h the number of transfected cells was determined using a Cyan™ MLE flow cytometer (DaKoCytomation, Copenhagen, Denmark). GFP fluorescence was excited at 488 nm and emission was detected using a 530 ± 40 bandpass filter. To discriminate between viable and dead cells and to exclude doublets, cells were appropriately gated by forward/side scatter and pulse width, and 2×10^4 gated events per sample were collected.

2.4. Covalent labeling of plasmid DNA and flow cytometry

Plasmid pCMVLuc was covalently labeled with the fluorophores Cy3 or Cy5 using the Label IT kits (MIRUS, Madison, WI) according to the manufacturers instructions. On average, one Cy3 or Cy5 molecule was bound per 50 or 120 bp, respectively.

For analysis by flow cytometry, cells were seeded in 12 well plates at a density of 10^5 cells per well 24 h prior to transfection. Polyplexes containing Cy5-labeled DNA (20% Cy5-DNA) were added to the cells at a DNA concentration of 625 ng/well in 1 ml of fresh culture medium, and cells were harvested after 4 h incubation at 37 °C by treatment with trypsin/EDTA solution (Invitrogen GmbH, Karlsruhe, Germany). Cell association of polyplexes was assayed using a Cyan™ MLE flow cytometer (DaKoCytomation, Copenhagen, Denmark). The fluorophore Cy5 was excited at 635 nm and emission was detected at 665 ± 20 nm. Data were analyzed in logarithmic mode. To discriminate between viable and dead cells and to exclude doublets, cells were appropriately gated by forward/side scatter and pulse width, and 2×10^4 gated events per sample were collected. Median channel values ± SD of triplicates (representing the median intensity of cell-associated DNA) were determined and presented as relative fluorescence units (RFU), with the highest value defined as 100%.

2.5. Fluorescence microscopy

Cells were seeded on medco glass slides (10 well, Medco, Munich, Germany), coated with collagen, at a density of 3×10^3 cells per well 24 h prior to transfection. Cells were transfected with Cy3-labeled DNA complexes (4% Cy3-DNA) at a concentration of 250 ng DNA per well. After 2 h incubation at 37 °C the slides were washed twice with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and the nuclei were stained with DAPI. Afterwards the cells were mounted with vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and covered with a coverslip. Samples were viewed on an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam camera. Light was collected through a 63 × 1.4 NA oil immersion objective (Zeiss). Cy3 fluorescence was excited using a 546 ± 12 nm bandpass filter and emission was detected using a 575–640 nm bandpass filter. Digital image recording and image analysis were performed with the Axivision 3.1 (Zeiss) software.

3. Results and discussion

3.1. Formation of cryoconserved, EGF receptor targeted and shielded polyplexes

Strategies towards the development of non-viral vectors for tissue-targeted delivery of therapeutic genes requires the engineering of stable polyplexes that are storable without significant changes in size, charge or transfection efficiency. Here, we used a novel formulation of EGFR--targeted, PEG-shielded polyplexes that maintained their physical properties as well as transfection efficiency after storage at −80 °C. Polyplexes were generated following a 'pre-PEGylation' strategy. Defined conjugates of PEI, PEG, and EGF were diluted in buffer and then mixed with plasmid DNA in defined ratios to form stabilized DNA complexes. Without a cryoprotectant such as glucose, however, large aggregates are formed after the freezing/thawing procedure [10,18,25]. A 5% glucose buffer allowed freezing and storage of polyplexes at −80 °C. Since we found that particle size (124.2 ± 17 nm for EGF^- and 195.8 ± 26 nm for EGF^+ before freezing) remained small after thawing (248 ± 26 nm for the EGF^- control polyplexes without EGF, 266 ± 22 nm for EGF containing EGF^+ polyplexes), the polyplexes can also be applied systemically.

3.2. Kinetics of receptor-mediated association with cultured cells

To determine whether the cryoconserved EGFR-targeted polyplexes showed EGF receptor specificity, we performed cell association studies using human HUH-7 hepatoma cells (Fig. 2A and B) and Renca-EGFR renal carcinoma cells (Fig. 2C and D), both expressing high levels of EGFR. PEG-shielded PEI polyplexes with or without EGF ligand (EGF^+, EGF^-) were generated with Cy5-labeled DNA to enable the measurement of cell-associated polyplexes by flow cytometry analysis. Incubating cells for 0.5–4 h in standard medium with EGF^+ polyplexes resulted in strong cellular association, which was two- to three-fold higher as compared to EGF^- polyplexes (Fig. 2A and C). The difference in cellular association was already pronounced after only 30 min of incubation indicating a fast interaction of ligands attached to the polyplex with receptors located on the cell surface. Cell binding of EGF^+ polyplexes was reduced to levels of

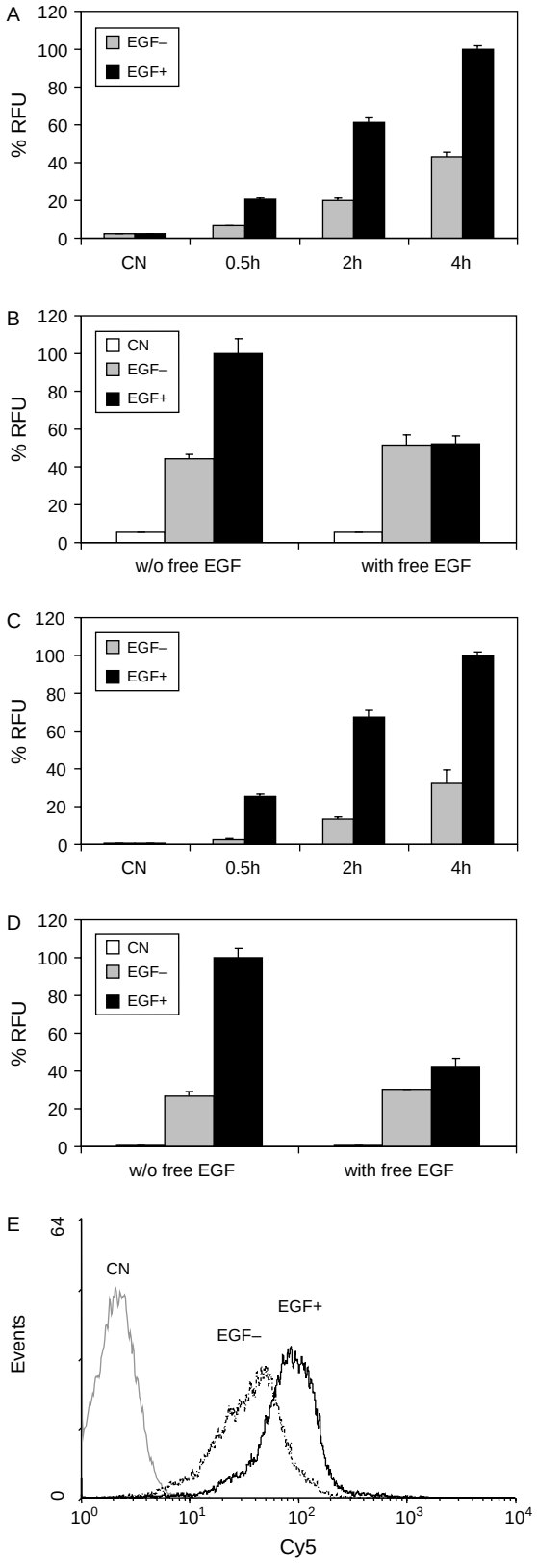

Fig. 2. Association of plasmid DNA with HUH-7 (A, B) or Renca-EGFR (C, D) cells. Time course (A and C) of cell associated Cy5-labeled DNA of PEG20 kDa-shielded polyplexes with or without EGF (EGF^+, EGF^-) as measured by flow cytometry. Competitive inhibition of cell association (B and D) by incubation with murine EGF (as free competing ligand) or BSA (as irrelevant control). Cells were harvested and analyzed after a total of 2 h incubation. CN, Untransfected control cells. Flow cytometry experiments were performed as described in Section 2, and median intensity of cell associated DNA is presented in relative fluorescence units (RFU). (E) Flow cytometry histogram for HUH-7 cell association. CN, grey line; EGF^-, dotted line; EGF^+, dark line.

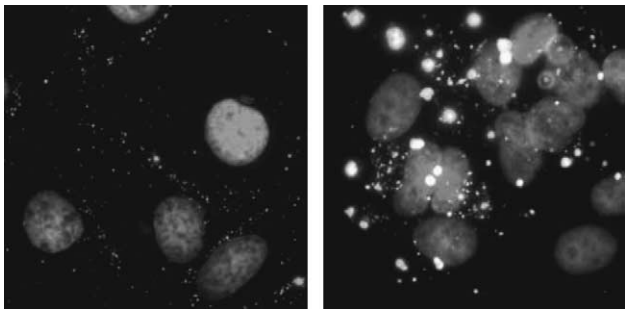

Fig. 3. Epifluorescence microscopy of HUH-7 cells transfected with polyplexes. HUH-7 cells were incubated with Cy3-labeled polyplexes with or without EGF (EGF^+, EGF^-). After 2 h, cells were washed, fixed and counterstained with DAPI to visualize cell nuclei. Large, grey areas: DAPI stained nuclei. Bright dots: Cy3-labeled polyplexes.

EGF^- polyplexes in the presence of a 1000-fold molar excess of free EGF over polyplex bound EGF (Fig. 2B and D) pointing out the specificity of interaction. Addition of albumin as control did not influence cell association (Fig. 2B).

3.3. Clustering of EGF^+ particles upon cell binding

Fluorescence microscopy of HUH-7 cells transfected with Cy3-labeled polyplexes revealed that the increased cell binding was also associated with clustering of EGF^+ polyplexes (Fig. 3). EGF^- polyplexes shielded with PEG appeared as a faint punctuate pattern on the cells (left picture), whereas EGF^+ polyplexes were observed as large (micrometer) aggregates associated with the cells. Both, EGF^+ and EGF^- polyplexes had a similar particle size (approximately 260 nm) prior to transfection, opening the hypothesis that the clustering observed is due to the interaction of polyplex bound EGF with the EGFR. Clustering of EGFR after binding of EGF^+ polyplexes could account for this phenomenon of polyplex aggregation since such clustering (in clathrin structures) has been described for this receptor family upon ligand binding. In addition to the accelerated binding of EGF^+ polyplexes the observed aggregation may be an important mechanism to enhance transfection efficiency. Such particle aggregation was only observed after binding of EGF^+ polyplexes. This indicated that polyplex aggregation required specific ligand receptor interaction and is another feature contributing to the enhanced transfection efficiency of EGF^+ polyplexes. Consistent with this hypothesis we observed that upon incubation of EGF^+ polyplexes with (EGFR-positive) Renca-EGFR cells or (EGFR-negative) Renca-LacZ cells,

only the Renca-EGFR cells mediated clustering of the polyplexes (data not shown).

3.4. Pivotal role of ligand receptor interaction for enhanced transfection efficiency

Transfection studies using a luciferase marker gene construct showed that EGF^+ polyplexes mediated an at least 10-fold and up to 50-fold higher level of reporter gene expression as compared to polyplexes lacking EGF on both tested cell lines (Fig. 4). This is in good agreement with previous studies using similar polyplexes and other cell lines [16,18]. The percentage of successfully transfected cells was determined using a plasmid encoding Green Fluorescent Protein (GFP) for transfection. As the transfection rate of Renca-EGFR cells was very low with the small complexes prepared in HBG, bigger particles, generated in HBS (HEPES buffered saline: 150 mM NaCl, 20 mM HEPES, pH 7.1), were used for the analysis of this cell line. With EGF^- polyplexes, the percentage of transfected cells was very low (0.5%) on HUH-7 and moderate (up to 11%) on Renca-EGFR cells (Fig. 5). With EGF^+ polyplexes, however, 13 and 20% of HUH-7 and Renca-EGFR cells expressed GFP, respectively (29-fold and two-fold increase as compared to non-targeted EGF^- polyplexes). These results demonstrate that EGF bound to a PEI polyplex enhances transgene delivery to EGFR-positive cells due to increased and accelerated cellular binding followed by aggregation to micrometer particles. The biological activity of EGF in terms of receptor binding and subsequent clustering does not seem to be impaired,

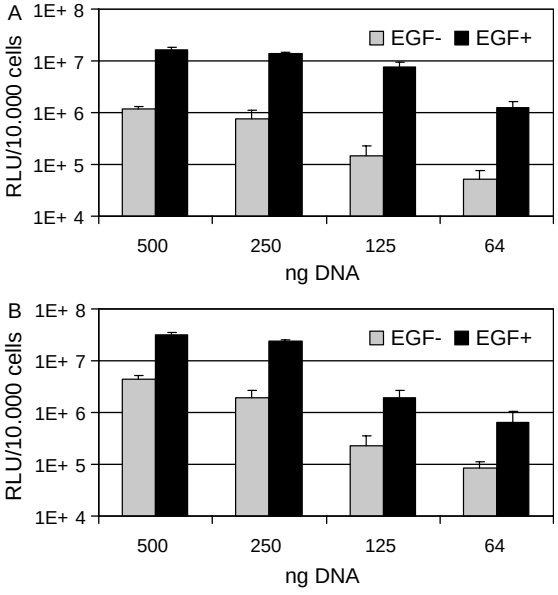

Fig. 4. Luciferase reporter gene expression on (A) HUH-7 and (B) Renca-EGFR cells. Cells were transfected with the indicated amounts of luciferase plasmid DNA, and luciferase activity was measured 24 h after transfection. Polyplexes with or without EGF (EGF^+, EGF^-) were generated as described in Section 2.

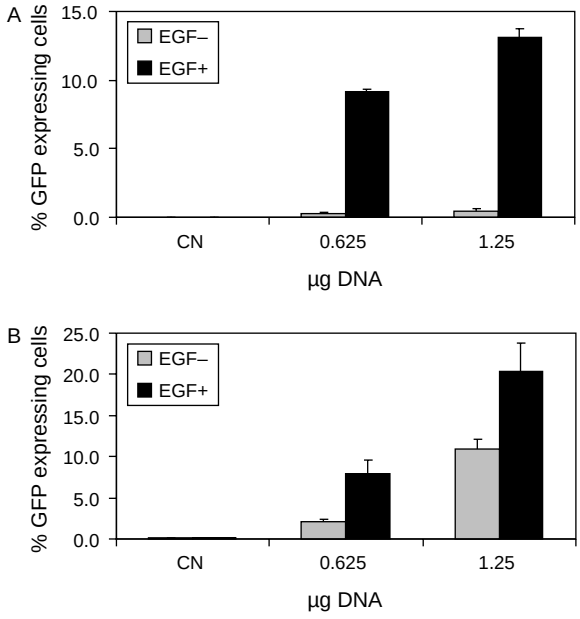

Fig. 5. Green Fluorescent Protein (GFP) reporter gene expression on (A) HUH-7 and (B) Renca-EGFR cells. Cells were transfected with the indicated amount of GFP plasmid DNA using polyplexes with or without EGF (EGF^+, EGF^-). The percentage of GFP expressing cells in comparison to untreated control cells was measured 48 h after transfection by flow cytometry analysis.

neither by the process of polyplex formation nor by cryoconservation. These capacities make EGFR-targeted polyplexes highly useful for tumor-targeted delivery of therapeutic nucleic acids in vivo [18].

However, the analyzed specifity and kinetics of EGF^+ polyplex binding to the target cells is only one key step in the process of polyplex-mediated gene transfer. Subsequent steps after the cell association, such as endosomal escape and nuclear translocation may be as well important bottlenecks in the transfection process. Therefore, novel strategies in the synthesis of tissue-targeted and PEG-shielded polyplexes are required to overcome additional key barriers of transfection.

3.5. Future strategies towards highly effective non-viral gene transfer

Endosomal escape might be limiting for PEG-shielded polyplexes of small size. PEI has an intrinsic ability to facilitate endosomal release [26–28] and mediates high gene transfer in vitro when aggregated, non-shielded polyplexes are used. Both the small size of targeted polyplexes [29] and the stable PEG shield [7,16] are known to be disadvantageous for endosomal release. Nevertheless, small and shielded formulations are indispensable for pharmacological and pharmaceutical reasons. To overcome the lower efficiency related to low endosomal release, we and others have followed several different strategies.

One possibility recently developed in our lab is based on the attachment of membrane-active melittin-derived peptides [24] into the shielded EGF^+ polyplex which resulted in strongly (10–100 fold) enhanced gene expression (Sabine Boeckle, Manfred Ogris, Ernst Wagner, unpublished data). A second alternative has been the combination with a novel technique termed 'PCI, photochemical internalization' [30,31] which was developed to improve endosomal release by light-induced photochemical rupture of endocytotic vesicles. A further possibility to improve EGFR-targeted shielded polyplexes is to incorporate PEG into polyplexes in a bioreversible fashion, resulting in endosomal removal of the PEG shield and re-activation of the endosomal escape activity (Greg Walker, Jaroslav Pelisek, Carolin Fella, Julia Fahrmeir, Sabine Boeckle, Manfred Ogris, Ernst Wagner, manuscript submitted).

In summary, we conclude that the cryoconserved EGFR-targeted polyplexes mediated improved cellular uptake and gene transfer in comparison to non-targeted polyplexes. Association of EGF^+ but not EGF^- polyplexes with EGFR-positive cells resulted in clustering of the polyplexes. Cryoconservation did not impair the biological activity of the targeting ligand. Since the current polyplexes lack efficient functional domains to overcome additional key barriers of the transfection process including endosomal release, we need further modifications to render polyplexes more effective.

Acknowledgements

We would like to thank Wolfgang Rödl for conjugate synthesis. The authors also would like to thank Sabine Boeckle and Carsten Culmsee for the critical review of the manuscript, and Olga Brück for skilful assistance in preparing the text. This work was in part funded by the Deutsche Forschungsgemeinschaft, SFB 486 'Nanoman'.

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