50% silencing efficiency whereas incubation with pHPMA-MPPM and TMC polyplexe made at N/P ratios of 8 and 16 showed 30–40% gene silencing. Incubation of cells with branched PEI polyplexes led to 25–30% silencing of luciferase. The XTT cell viability assays show low cytotoxicity of the polymer complexes (except PE I) made at an N/P ratio of 8 (less than 15%) and by increasing the N/P ratio to 16, cytotoxicity level is increased. In the case of pDMAEMA, pHPMA-MPPM and TMC the cytotoxicity of the polyplexes at the N/P ratio of 16 is about two folds higher than the N/P ratio of 8 which is correlated to the increased concentration of the polymers in the formulations and subsequently in the culture medium (Fig. 5).To facilitate the endosomal release of the siRNA complexes, photochemical internalization (PCI) was applied. PCI is a technique based on the use of a photosensitizer that photochemically destabilizes endosomal membranes after illumination [6]. By application of PCI, the silencing efficiency of the pDMAEMA polyplexes made at N/P ratios of 8 and 16 increased up to 70% and 60% respectively whereas for both N/P ratios of 8 and 16, the silencing efficiency of the pHPMA-MPPM and TMC—33% polyplexes increased up to 70% and 80% respectively.

The gene silencing efficiency of PEI polyplexes, after application of PCI showed a slight increase (5%) which is not significant and is probably due to the intrinsic endosomolytic properties of PEI which makes it independent of PCI [7]. In the case of lipofectamine, the silencing efficiency of the complexes increased up to 60% after application of PCI (Fig. 4). These results show that biodegradable polymethacrylates and TMC polymers are much more in favor of PCI as an endosome disruptive technique. For all formulations, the observed increase in gene silencing efficiency was not due to PCI-associated cytotoxicity (Fig. 5).

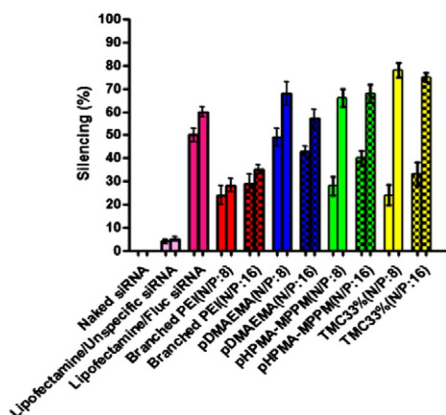

Fig. 4. Luciferase gene silencing after incubation of H1299 cells with polyplexes in medium without serum, with and without application of PCI (mean ± standard deviation (n = 3)).

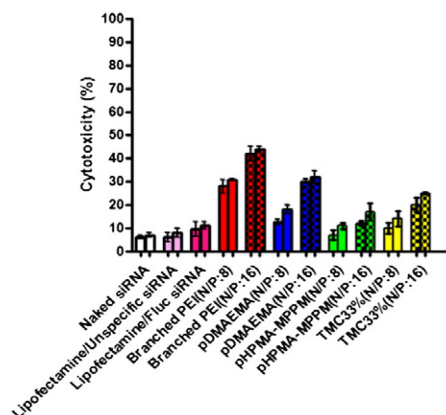

Fig. 5. Cell viability as measured by XTT assay after incubation of siRNA complexes in medium without serum with and without application of PCI (mean ± standard deviation (n = 3)). In all formulations, the left and the right bars show the silencing efficiency without and with PCI respectively.

Conclusion

Biodegradable pHPMA-MPPM and TMC (33%) polymers are efficient and safe vectors for siRNA delivery and gene silencing, which by facilitating their endosomal escape, show a promising gene silencing efficiency.

References

[1] J. Park, M. Ye, M.K. Park, Biodegradable Polymers for Microencapsulation of Drugs, Molecules 10 (1) (2005) 146–161. doi:10.3390/10010146.

[2] J. Luten, N. Akeroyd, A. Funhoff, M.C. Lok, H. Talsma, W.E. Hennink, Methacrylamide polymers with hydrolysis-sensitive cationic side groups as degradable gene carriers, Bioconjugate Chem. 17 (2006) 1077–1084.

[3] R.J. Verheul, M. Amidi, S. van de. Wal, E. van Riet, W. Jiskoot, W.E. Hennink, Synthesis, characterization and in vitro biological properties of O-methyl free N,N,N-trimethylated chitosan, Biomaterials 29 (27) (2008) 3642–3649.

[4] K. Berg, P.K. Selbo, L. Prasmickaite, T.E. Tjelle, K. Sandvig, J. Moan, G. Gaudernack, O. Fodstad, S. Kjølsrud, H. Anholt, G.H. Rodal, S.K. Rodal, A. Høgset, Photochemical internalization: a novel technology for delivery of macromolecules into cytosol, Cancer Res. 59 (1999) 1180–1183.

[5] R.B. Arote, S.K. Hwang, M.K. Yoo, D. Jere, H.L. Jiang, Y.K. Kim, Y.J. Choi, J.W. Nah, M. H. Cho, C.S. Cho, Biodegradable poly(ester amine) based on glycerol dimethacrylate and polyethylenimine as a gene carrier, J. Gene Med. 10 (11) (2008) 1223–1235.

[6] A. Bonsted, B.O. Engesæter, A. Høgset, G.M. Mælandsmo, L. Prasmickaite, C. D'Oliveira, W.E. Hennink, J.H. van Steenis, K. Berg, Photochemically enhanced transduction of polymer-complexed adenovirus targeted to the epidermal growth factor receptor, J. Gene Med. 8 (2006) 286–297.

[7] T. Xia, M. Kovochich, M. Liong, H. Meng, S. Kabehie, S. George, J.I. Zink, A.E. Nel, Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs, ACS Nano. 3 (10) (2009) 3273–3286.

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Unraveling the cellular uptake of bioreducible poly(amido amine) — Gene complexes in cells of the retinal pigment epithelium

D. Vercauteren[1,*], M. Piest[2], M. Al Soraj[3], A.T. Jones[3], J.F.J. Engbersen[2], S.C. De Smedt[1], K. Braeckmans[1]

[1]Lab of General Biochemistry and Physical Pharmacy, Ghent University, Ghent, East Flanders, B9000, Belgium

[2] MIRA Institute for Biomedical Technology and Technical Medicine, Dept. of Biomedical Chemistry and Dept. of Polymer Chemistry and Biomaterials, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

[3] Welsh School of Pharmacy, Cardiff University, Cardiff, CF10 3XF, Wales, United Kingdom

**Corresponding author.*

E-mail: dries.vercauteren@UGent.be.

Abstract summary

In vitro endocytosis of gene complexes composed of a bioreducible polyamidoamine CBA ABOL and plasmid DNA, in cells of the retinal pigment epithelium (RPE) was studied, the latter being an interesting target for ocular gene therapy. We found that cationic CBA ABOL DNA polyplexes attach to cell surface proteoglycans of these RPE cells and get subsequently internalized via a phagocytosis-like mechanism, as well as Flotillin dependent endocytosis.

Introduction

Proper delivery of therapeutic genes to designated cells and their availability at the intracellular site of action are crucial requirements for successful gene therapy. To this end, typically sub-micron sized particles are made by combining the therapeutic genes with a carrier material, such as cationic polymers, that aid in delivering the genes to the target site. In this work, for the first time, we have evaluated the ability of the highly promising bioreducible polymer carrier cystamin bisacrylamid aminobutanol (CBA ABOL) [1] (Fig. 1) to deliver plasmid DNA in cultured cells of the retinal pigment epithelium (ARPE-19) and characterized *in vitro* the cellular interactions with these target

cells. These studies are of crucial importance since the further design and functionalization of polymeric gene carriers depend strongly on our understanding of the mechanisms involved in cellular adhesion, intracellular uptake and intracellular processing of the polyplexes.

Experimental methods

ARPE-19 cells (retinal pigment epithelial cell line; ATCC number CRL-2302) were cultured in DMEM:F12 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 2% penicillin-streptomycin. All cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. CBA ABOL gene complexes with an average hydrodynamic diameter of 130 nm and an average zeta potential of +45 mV in 20 mM HEPES buffer were obtained by adding the polymer in a mass ratio of 48/1 in 20 mM HEPES to the plasmid and vortexing the mixture for 10 min. For every transfection, fresh polyplexes were prepared and applied to the cells within 30 min after complexation. For all uptake studies, YOYO-1™ (λ_{ex} = 491 nm, λ_{em} = 509 nm, Molecular Probes, Merelbeke, Belgium) labeled pGL4.13 plasmid (Promega, Leiden, The Netherlands) was used. For all transfection studies, gWiz™GFP plasmid (Aldevron, Freiburg, Germany) was used. siRNAs were all purchased from Dharmacon and transfected in cells with the help of LipofectaminRNAiMAX (Invitrogen, Merelbeke, Belgium). Protein knockdown was assessed on Western Blot. Uptake of polyplexes or endocytic markers or GFP expression was measured on a FACS Calibur Flow Cytometer (Beckton Dickinson, Erembodegem, Belgium). For genetic labeling of endosomes, cells were transfected with GFP-fusion proteins.

For fluorescence colocalization studies with GFP labeled cellular structures, cells were transfected with GFP-fusion proteins using Lipofectamin2000 (Invitrogen, Merelbeke, Belgium) and 24 h later exposed to red fluorescent labeled polyplexes. For this, CBA ABOL was complexed with pGL4.13 plasmid, covalently labeled with Cy5 (Label IT Nucleic Acid Labeling Kit, Mirus Bio Corporation, WI, USA). Live cell fluorescence colocalization was then performed on a custom built laser epi-fluorescence microscope set-up. A Nikon Plan Apo VC 100× 1.4 NA oil immersion objective lens (Nikon Belux, Brussels, Belgium) was used for imaging. GFP and Cy5 were excited with 491 nm and 636 nm laser light and emission was detected on an EMCCD camera (Roper Scientific, Nieuwegein, The Netherlands). For live cell imaging the cells were placed in a stage top incubation chamber (Tokai Hit, Shizuoka, Japan), set at 37 °C, 5% CO_2 and 100% humidity.

Result and discussion

First, we found evidence that these net positively charged CBA ABOL polyplexes adhere to the negatively charged heparan sulfate proteoglycans (HSPGs) at the cell surface and that polyplex internalization is blocked by antibodies against Toll-like receptor 9.

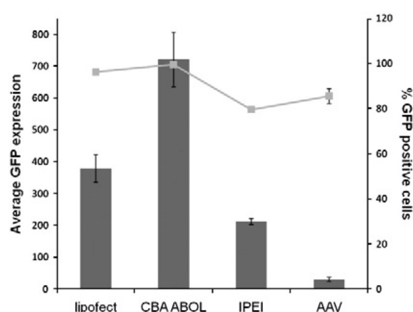

Fig. 1. Comparison of transfection efficacy of different gene delivery vehicles carrying the GFP reporter gene in ARPE-19 cells. Cells were incubated for 2 h in OptiMEM with different gene carriers: Lipofectamin2000, CBA ABOL, Exgen500 (linear PEI) and Adeno Associated Virus (AAV). Transfection analysis was performed 24 h later with flow cytometry. The square data points represent the percentage of transfected (GFP expressing) cells, the grey bars represent the amount of expression (average GFP fluorescence intensity) per cell. It appears that the performances for CBA ABOL in RPE cells are in these conditions competitive to Exgen500 and AAV mediated transfection and even with Lipofectamin2000 lipofection.

Second, in an extensive study on the characterization of the endocytic pathways that are involved in the cellular internalization of these polyplexes, we learned by the use of endocytic inhibitors [2] that polyplex uptake is for a large part very similar to the uptake of *Escherichia coli*, which indicates a substantial contribution by phagocytosis. We also learned from these studies that clathrin dependent endocytosis is not involved and that the internalization of polyplexes is highly dependent on actin, dynamin, cholesterol and tyrosine kinase activities. In a complementary study, where we used RNAi to knock down specific key endocytic proteins, not only we could confirm that polyplex uptake is independent of clathrin, but also of Caveolin-1, Arf6, PAK1 and GRAF-1. On the other hand we did find a dependency on Dynamin-2 and Flotillin-1. This indicates that, next to phagocytosis, probably a second endocytic pathway dependent on Flotillin-1 [3] is involved, which is in agreement with what has been reported for gene complexes based on the polymer polyethylenimine [4]. Finally, using live-cell dual colour fluorescence colocalization microscopy, we could confirm the involvement of Flotillin during polyplex internalization at early time-points while no colocalization was found with Caveolin-1 containing vesicles.

Conclusion

In conclusion, we learned by combining three different methodological approaches that these polyplexes stimulate their own cellular uptake and are rapidly internalized in RPE cells via two endocytic pathways, being Flotillin mediated endocytosis and phagocytosis. We would like to note that this conclusion was only possible by combining the use of chemical inhibitors, siRNA downregulation of essential endocytic proteins and live cell colocalization microscopy. By getting a detailed insight into the uptake and subsequent intracellular processing of these polyplexes, we want to provide the knowledge necessary for a further rational optimization of the design and composition of this polymer carrier.

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References

[1] C. Lin, Z.Y. Zhong, M.C. Lok, X.L. Jiang, W.E. Hennink, J. Feijen, J.F.J. Engbersen, Novel bioreducible poly(amido amine)s for highly efficient gene delivery, Bioconjugate Chem. 18 (2007) 138–145.
[2] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The Use of Inhibitors to Study Endocytic Pathways of Gene Carriers: Optimization and Pitfalls, Mol. Ther. 18 (3) (2009) 561–569.
[3] O.O. Glebov, N.A. Bright, B.J. Nichols, Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells, Nat. Cell Biol. 8 (2006) 46-U16.
[4] C.K. Payne, S.A. Jones, C. Chen, X.W. Zhuang, Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands, Traffic 8 (2007) 389–401.

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Sustained delivery of oncolytic adenovirus in alginate gel for local tumor virotheraphy

Pyung-Hwan Kim[1], Eunah Kang[2], Jung Woo Choi[2], Tae Jin Yun[2], Hong-Kwan Park[3], Jung Hyun Kim[c], Chae-Ok Yun[1,2,*]
[1]Graduate Program for Nanomedical Science, Yonsei University 1661 College of Medicine, Seoul, South Korea
[2] Brain Korea 21 Project for Medical Sciences and Institute for Cancer Research, Yonsei University College of Medicine, Seoul, South Korea
[3] Department of Chemical and Biomolecular Engineering, Yonsei University, 134 Shinchon-Dong, Seodaemun-Gu, Seoul, South Korea
**Corresponding author.*
E-mail: chaeok@yuhs.ac.