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Role of dynamin-dependent and clathrin-dependent uptake pathways in nonviral gene delivery studied by chemical and genetic means

Polina Ilina [1,*], Zanna Hyvonen [2], Marjo Yliperttula [1], Marika Ruponen [2]
[1] *Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, P.O. Box 56 (Viikinkaari 5 E), FI-00014 University of Helsinki, Finland*
[2] *University of Kuopio, Finland*

*Corresponding author.
E-mail: polina.ilina@helsinki.fi (P. Ilina).

Introduction: Endocytosis is known to be a major cell uptake mechanism for non-viral gene delivery vehicles. Several mechanisms of endocytosis have been described and it seems that not all of them are equally beneficial in terms of gene delivery efficiency. According to the literature the preferential cell uptake pathway is both carrier and cell type dependent. Rational design of effective and safe gene delivery vectors requires deeper understanding of the cellular uptake mechanisms of gene delivery vehicles. The purpose of our study was to clarify the role of dynamin-dependent cell uptake pathways, including both clathrin-dependent and caveolae-dependent endocytosis, in non-viral gene delivery. *Methods*: The studies were performed with three widely used non-viral gene delivery systems: cationic polymer branched polyethyleneimine (PEI), cationic lipid N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethyl ammonium methylsulfate (DOTAP) and calcium phosphate (CaP) precipitates. The internalization pathways of these gene delivery vehicles were studied by using genetically modified cell lines: HeLaK44A cells with inducible block of dynamin-dependent endocytosis and BHK21-tTA cells with inducible block of clathrin-dependent endocytosis. As an alternative approach chemical blockers chlorpromazine, dansylcadaverine, nystatin and dynasore were used to inhibit specific endocytic pathways. Relevant concentration of each inhibitor was determined by MTT cell viability assay. Size of the complexes was measured, and expression of marker protein at different timepoints from 0 to 72 hours after exposure to complexes was determined in intact cells and cells with blocked endocytic pathway(s). Results: The obtained data indicated that in both HeLaK44A and BHK21-tTA cell lines for DOTAP-based nanoparticles clathrin-dependent endocytic pathway seemed to be predominantly responsible for successful gene delivery, whereas for efficient PEI-mediated transfection caveolae-mediated pathway was important. In HeLaK44A cells block of dynamin-dependent endocytosis resulted only in moderate (40–50%) decrease of transfection efficiency of both PEI and DOTAP complexes. This suggests that other pathways, not dependent of dynamin, participate in the uptake of both PEI- and DOTAP-based nanoparticles in this cell line. In HeLaK44A cells blockage of dynamin-dependent endocytosis by genetic means increased transfection efficiency of Ca-phosphate precipitates 4-fold whereas chemical blockage of dynamin-dependent pathway by dynasore reduced transfection efficiency of Ca-phosphate precipitates almost completely. However, in general, the results obtained by using genetic means were comparable with results obtained by using chemical inhibitors.

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Enhanced intracellular delivery by guanidinium functionalized ROMP-polymers

A. Ozgul Tezgel [1,*], Janice C. Telfer [2], Gregory N. Tew [1]
[1] *Polymer Science and Engineering Department, University of Massachusetts, Amherst MA, 01003, United States*
[2] *Veterinary and Animal Science Department, University of Massachusetts, Amherst MA, 01003, United States*

*Corresponding author.
E-mail: atezgel@mail.pse.umass.edu (A.O. Tezgel).

Intracellular delivery of therapeutic molecules has always been a challenge due to the poor permeability of cell membrane to large, negatively charged macromolecules and their restricted biodistribution. In the past decades, cell penetrating peptides (CPPs) are shown to improve the intracellular delivery of bioactive molecules and among the CPPs, arginine-rich peptides are highlighted as the most effective subclass. In the light of this information, we designed and synthesized guanidinium functionalized polyoxanorbornenes which can adopt cell penetrating activity and show superior uptake properties compared to peptide analogues (i.e. nonaarginine, R9). The structure–activity relationship was studied by mono-guanidinium and di-guanidinium functionalized monomers and a specific trend was observed for each cell line studied. In addition to intracellular uptake profiles of molecules, their exceptional ability to deliver bioactive cargo, such as DNA, siRNA and intact proteins, into both adherent and suspension cell lines, as well as in primary cells has been demonstrated. A non-covalent complexation approach was utilized for the delivery of bioactive molecules, instead of covalent attachment. Non-covalent interactions are highly favored over covalent attachment of cargo, in terms of simplicity, efficiency of delivery and stability of bioactive cargo. Furthermore, structural requirements and optimal experimental conditions have been investigated for an efficient intracellular delivery agent.

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Engineering functional chitosan for delivery of drugs or RNAs

Xiudong Liu [1,2,*], Yan Yang [2], Huofei Zhou [2], Weiting Yu [2], Jiani Zheng [2], Demeng Zhang [2], Xiaojun Ma [2,*]
[1] *College of Environment & Chemical Engineering, Dalian University, Dalian 116622, China*
[2] *Laborotary of Biomedical Materials Engineering, Dalian Institute of Chemcial Physics, Chinese Academy of Sciences, Dalian 116023, China*

*Corresponding author.
E-mails: liuxd@dicp.ac.cn (X. Liu), maxj@dicp.ac.cn (X. Ma).

In the last decade, considerable studies on preparation of nanocarriers with cationic liposomes or polymers have been reported for intracellular delivery of DNA and siRNA [1]. Particle uptake has been proven through several kinds of endocytosis pathways, but the uptake efficiency varies depending on the property of carrier materials, particle size, and cell types. Using biocompatible and biodegradable chitosan (CTS) as carrier material, we designed and synthesized functional chitosan derivatives (such as amphiphilic CTS, ligand-targeted CTS), and then developed different technologies to prepare CTS nanoparticles for the potential application of loading, delivering and releasing anti-cancer drugs or RNA therapeutics (siRNA and microRNA). In one system, we initially conjugated a fatty acid (LA) to CTS to obtain amphiphilc CTS-LA, and then synthezised CTS-LA-TM by quaternization. Subsequently nanoparticles with size less than 200 nm can be easily formed by self-assembly of CS-LA-TM in biological solution or neutral solution [2]. These loaded PTX with encapsulation efficiency of 60–90% and showed sustained release in 1 week without burst release. Alternatively,

we formulated CTS-RNAs (siRNA or microRNA) nanoparticles by direct complexation. The nanoparticles with sizes of 120–200 nm and surface charge of ~20 mV showed complex stability and efficiency of protecting RNAs from RNase degradation. These nanoparticles can both transfer RNAs into cells and protect entrapped intracellular RNAs, in 2–4 hours without apparent critical cytotoxicity. Moreover, cell adhesive peptide GRGDY has been grafted to CTS by photosensitive crosslinker [3], and PEGylation has been carried out for target transportation to tumor cells with overexpressed integrin receptors and for efficient delivery of drugs or RNA therapeutics.

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A13
Incorporation of 2,3-diaminopropionic acid in linear cationic amphipathic peptides produces pH sensitive vectors

Yun Lan [1], Bérangère Langlet-Bertin [2], Vincenzo Abbate [1], Louic S. Vermeer [1], Xiaole Kong [1], Kelly E. Sullivan [1], Christian Leborgne [2], Daniel Scherman [2], Robert C. Hider [1], Alex F. Drake [1], Sukhvinder S. Bansal [1], Antoine Kichler [2], A. James Mason [1,*]

[1] *King's College London, Pharmaceutical Science Division, 150 Stamford Street, London, SE1 9NH, UK*

[2] *CNRS FRE 3087-Généthon, 1 rue de l'Internationale, F-91002, Evry, France*

*Corresponding author.

E-mail: james.mason@kcl.ac.uk (A.J. Mason).

Non-viral vectors that harness the change in pH in endosomes are increasingly being used to deliver cargoes, including nucleic acids, to mammalian cells. Here we present evidence that the pK_a of the β-NH2 in 2,3-diaminopropionic acid (Dap) is sufficiently lowered, when incorporated in peptides, that its protonation state is sensitive to the pH changes that occur during endosomal acidification. The lowered pK_a around 6.3 is stabilised by the increased electron withdrawing effect of the peptide bonds by inter-molecular hydrogen bonding and from contributions arising from the peptide conformation, including mixed polar/apolar environments, Coulombic interactions and inter-molecular hydrogen bonding. Changes of the charged state are therefore expected between pH 5 and 7 and large-scale conformational changes are observed in Dap rich peptides, in contrast with analogues containing lysine or ornithine, when the pH is altered through this range. These physical properties confer a robust gene delivery capability on designed cationic amphipathic peptides that incorporate Dap. Recent results investigating the link between hydrophobicity, number of charges, Coulombic interactions and side chain pK_a are considered in terms of the efficiency of gene delivery.

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Octaarginine mediated delivery of fluorescent cargo to human smooth muscle cells

Michele Sweeney [1], Catherine L. Watkins [2], Arwyn T. Jones [2], Michael J. Taggart [1,*]

[1] *Reproductive and Vascular Biology Group, Institute of Cellular Medicine, Newcastle University, UK*

[2] *Welsh School of Pharmacy, Cardiff University, UK*

*Corresponding author.

E-mail: michael.taggart@ncl.ac.uk (M.J. Taggart).

The high incidence and severity of diseases involving smooth muscle dysfunction, which include cardiovascular diseases and premature labour, dictates the need for our continued search for novel therapeutic strategies to treat these conditions. Cell penetrating peptides (CPP) are a class of non-viral vectors that show considerable promise for drug delivery purposes yet their suitability for uptake, and delivery of biologically active cargo, to human native cells and tissues remains unresolved. For any new drug delivery strategy, including the use of CPPs, to reach fruition this needs to be elucidated. We have begun to explore this issue for CPPs applied to human uterine cells and tissues (including myometrium and blood vessels) obtained from biopsies collected, following LREC-approved written informed consent, from patients undergoing elective Caesarean section at the end of pregnancy. Primary cultured human myometrial cells were prepared on glass-bottomed culture dishes, grown to 80–90% confluence and exposed to serum-reduced conditions overnight before exposure to CPP (or, separately, were methanol-fixed for subsequent immunofluoresence staining of protein localisation). Cellular uptake of fluorescently labelled (Alexa 488) D-Octaarginine (R8, 2 μM) was assessed in the first series of experiments for 24, 48 and 72 hours ($n = 2$). At each time point, z-section confocal microscopy revealed punctate intracellular fluorescence (indicative of vesicular compartmentalisation) particularly dense in the perinuclear area. A second series of experiments assessed the time-course of intracellular delivery up to 24 hours. Punctate intracellular loading was observed by 4 hours. More dense perinuclear and plasma membrane-localised fluorescence was observed at later time points. Immunofluoresence labelling revealed that human myometrial cells possessed expected cytoskeletal (α-smooth muscle actin, tubulin), plasma membranous and perinuclear localised components of endocytotic pathways (Caveolin-1, Clathrin Heavy Chain, Early Endosomal Antigen-1, Lysosomal Associated Membrane Protein-1 and 2 and Flotillins). Next, small segments of native (non-cultured), human uterine tissue were incubated with 2 μM D-R8 and nuclear dye Hoechst 33342 (1 μM) for 4 hours. Confocal microscopic examination revealed peptide entry into smooth muscle cells of both the myometrium and uterine blood vessels with homogenous intracellular fluorescence in many cells but some with more punctate perinuclear/nuclear fluorescence. In uterine tissues incubated with a similar, putatively cell-impermeant, Alexa488 control peptide $(GS)_4GC$, no intracellular fluorescence was observed. These preliminary investigations illustrate that an octameric cationic CPP can successfully enter primary cultured and native human smooth muscle cells and tissues. This opens up a new avenue for targeted delivery of cellular therapeutics in human tissues and in particular to human smooth muscles.

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