Arrowhead Research
CORPORATION

Using Polymers as Endosomal Escape Agents for siRNA Delivery in vivo

TIDES Pre-conference Workshop

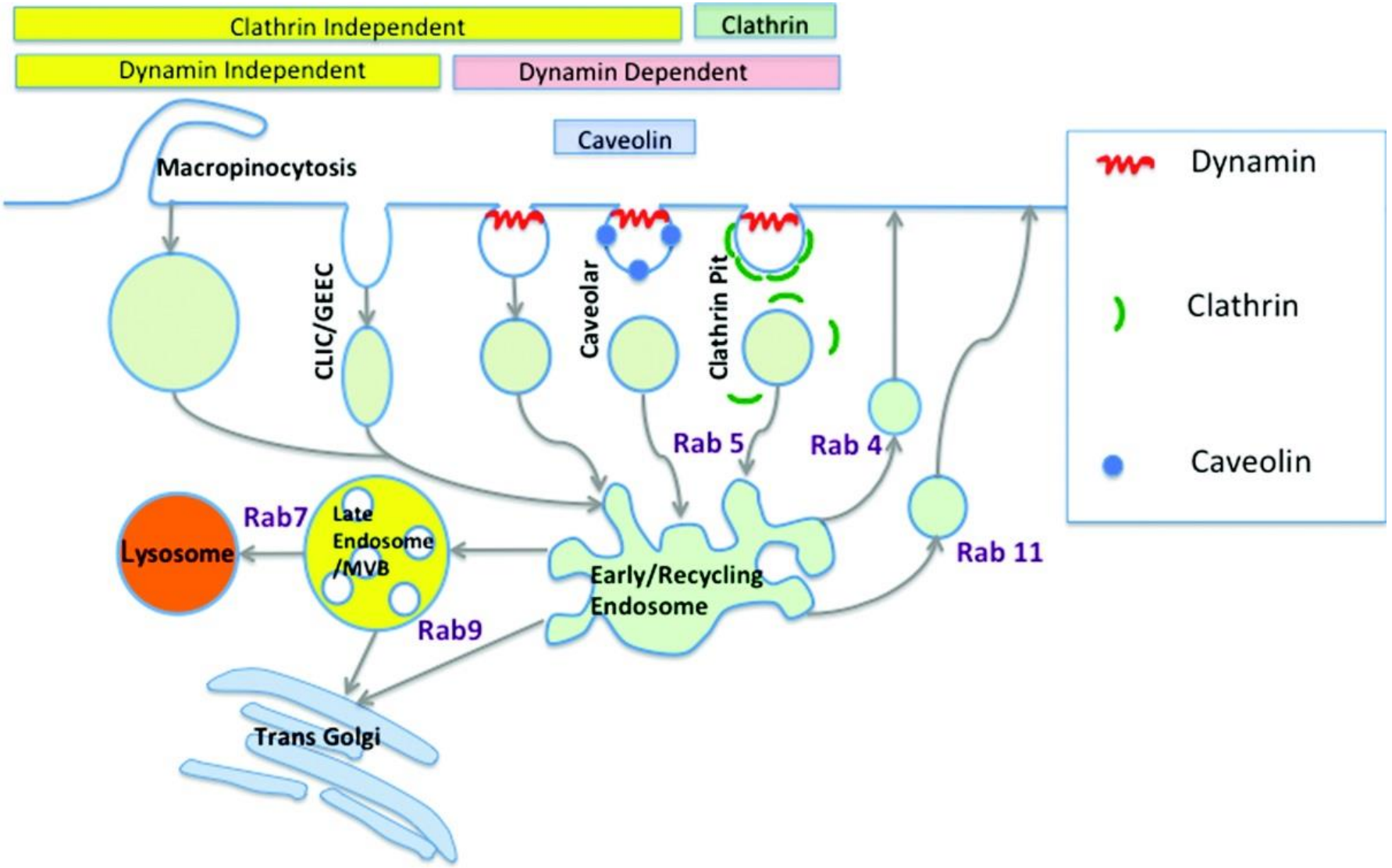
May 12, 2014

David Lewis, PhD
Chief Scientific Officer
Arrowhead Research Corporation

Presentation outline

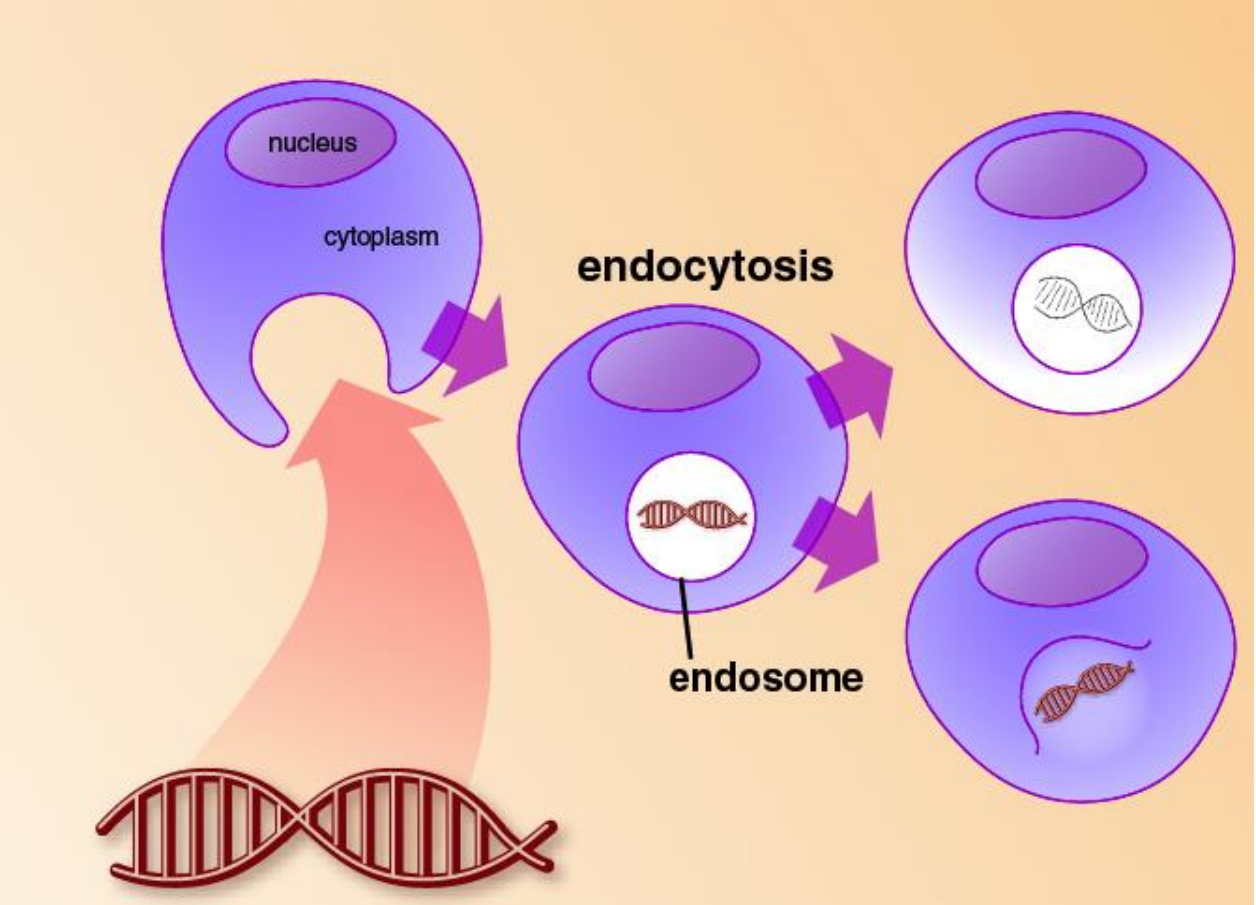
- Uptake and the endosome
- Endosomal escape mechanisms
  - Endosomal buffering agents: Proton sponges
  - pH titrable polyanions
  - Cell penetrating peptides
  - Masked membrane-lytic amphipathic polyamines

All uptake routes lead to the endosome



Juliano et al, *Nucleic Acid Therapeutics*. April 2014, 24(2): 101-113.

The general problem



oligonucleotide

Endosome maturation and degradation of oligo

Endosomolysis allowing access to cytoplasm

Endosomes acidify as they mature

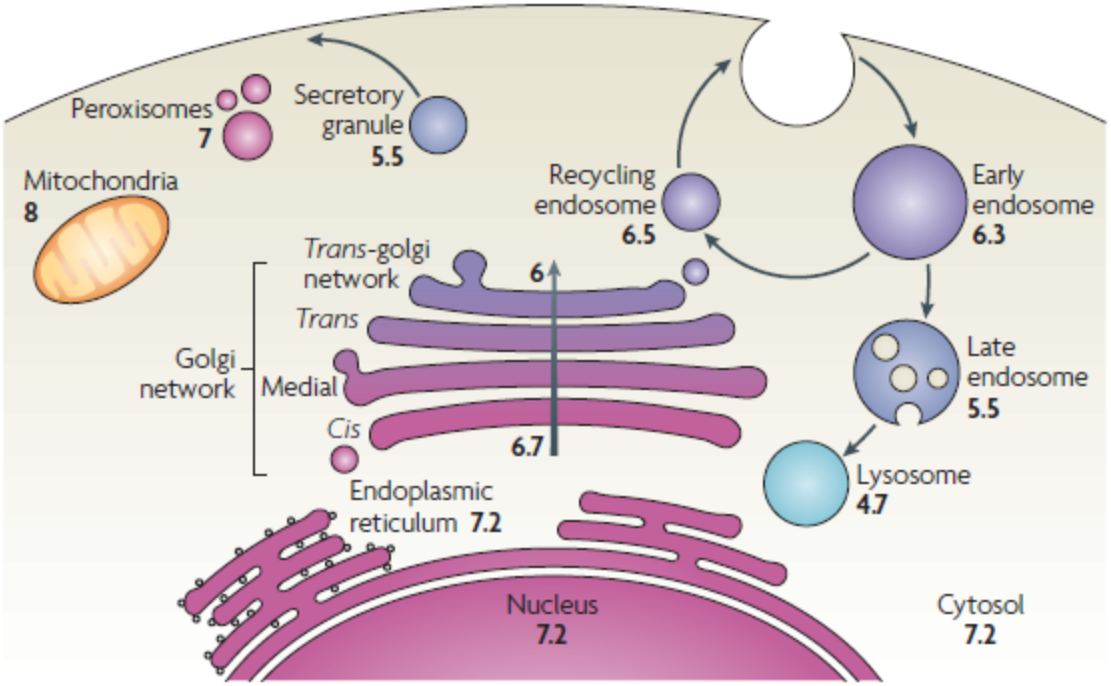


Figure 1 | **pH of the different subcellular compartments.** The pH of individual cellular organelles and compartments in a prototypical mammalian cell. The values were collected from various sources. The mitochondrial pH refers to the matrix, that is, the space contained by the inner mitochondrial membrane. Early endosomes refer to the sorting endosomal compartment. The pH of the multivesicular late endosome refers to the bulk luminal fluid; the pH of the fluid contained by the internal vesicles might differ.

Delivery agents can exploit the acidic pH of endosomes to trigger activity

Casey JR et al. Nat Rev Mol Cell Biol. 2010 Jan;11(1):50-61

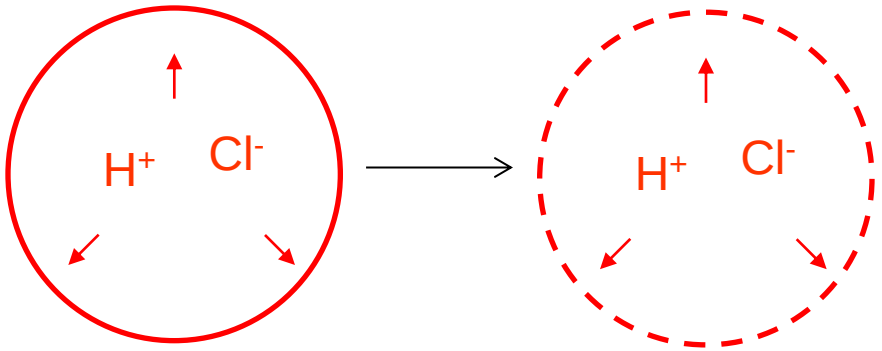
Endosomal buffering agents:

The proton-sponge hypothesis

1. The buffering capacity of the polymers prevents the acidification of the endosomes.

2. The endosome reacts to this by pumping additional protons into its lumen, accompanied by an influx of Cl^- ions.

3. The increase in ionic strength in the endosome causes an influx of water thereby increasing the osmotic pressure. This results in destabilization of the endosomal membrane.



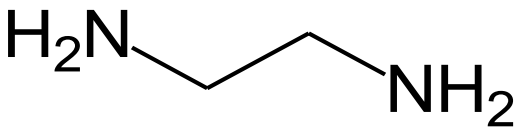
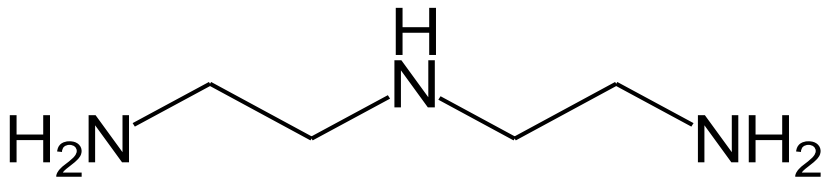
Evidence for the proton sponge mechanism

- pH of PEI-containing endosomes are 6.1, while poly-L-lysine containing endosomes are 4.6 (Akinc, Thomas, Klibanov and Langer *J Gene Med.* **2005**, *7*, 657)
- Accumulation of Cl- seen in endosomes and lysosomes of PEI-containing vesicles (Sonawane, Szoka and Verkman *JBC*, **2003**, *278*, 44826)
- Incorporation of some weak bases results in enhanced transfection activity (i.e. endosomolysis)
  - High-density polyamines with pK_a's in physiological range such as PEI and polyamidoamine dendrimers (Boussif et al PNAS, 1995, 92, 7197)
  - Imidazole

However,

- Incorporation of amines with physiologically-relevant pKa's are not always endosomolytic ('Endosomal Escape of Polymeric Gene Delivery Complexes Is Not Always Enhanced by Polymers Buffering at Low pH' Funhoff et al *Biomacromolecules*, **2004**, *5*, 32)

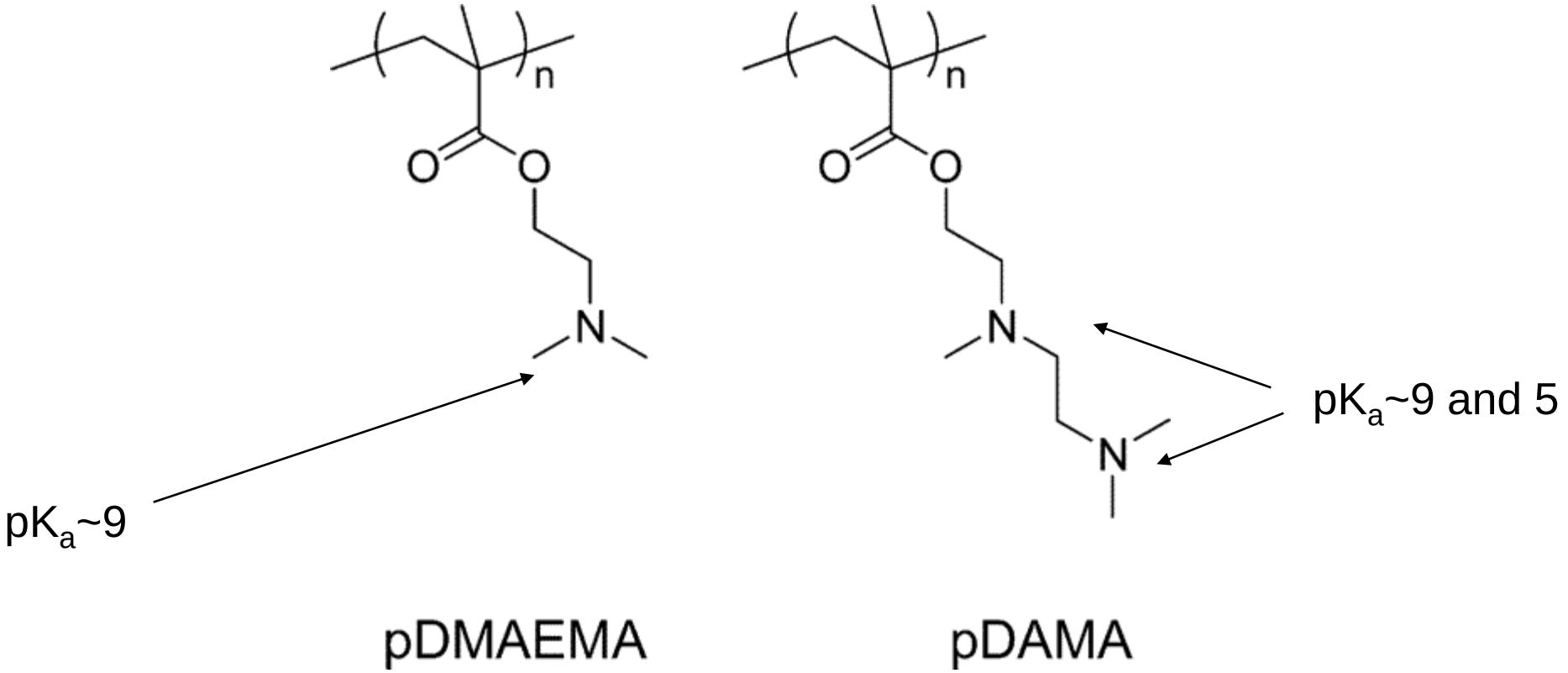
Examples of proton sponges: *Ethylene-spaced oligoamines*

	pK_a's
	7, 10
	5, 9, 10

Higher buffering capacity with high density amines

J. Yperman, J. Mullens, J. P. Francois, L. C. Van Poucke; *J. Phys Chem.* **1982**, *86*, 298-303.

Polymethacrylate-based polyamines



pDMAEMA

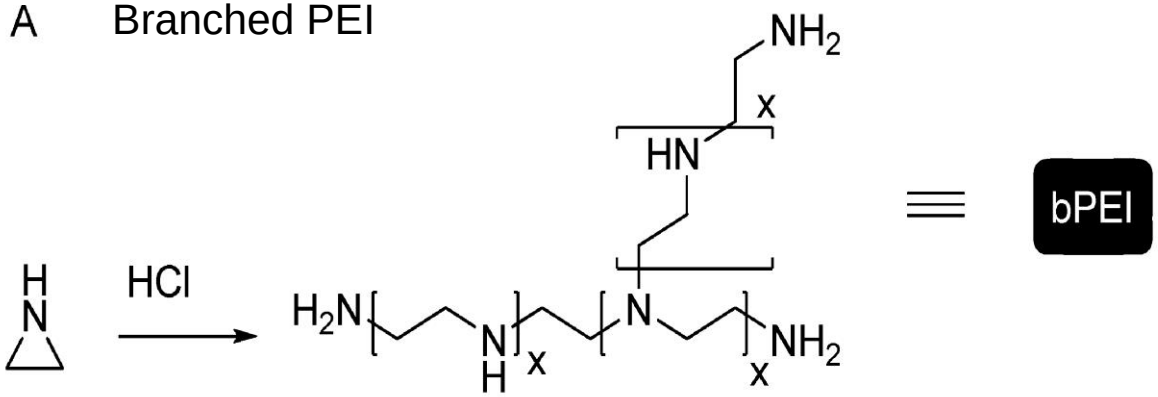
pDAMA

Transfection active

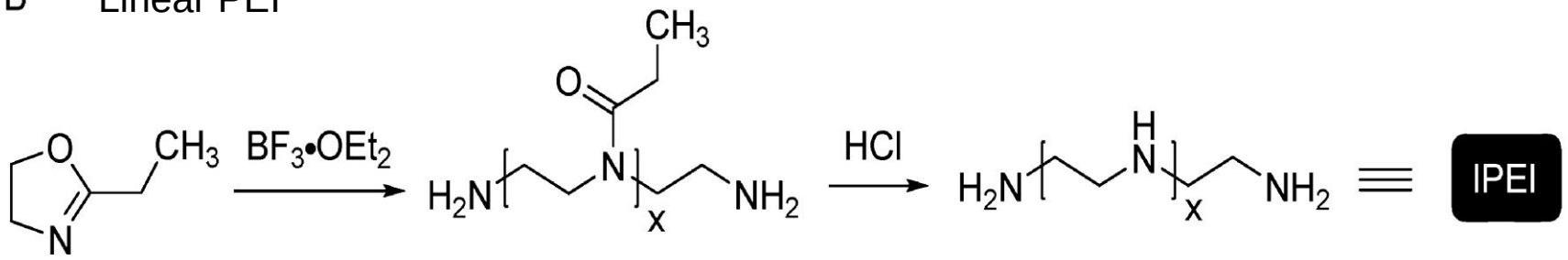
Transfection inactive

Polyethyleneimines

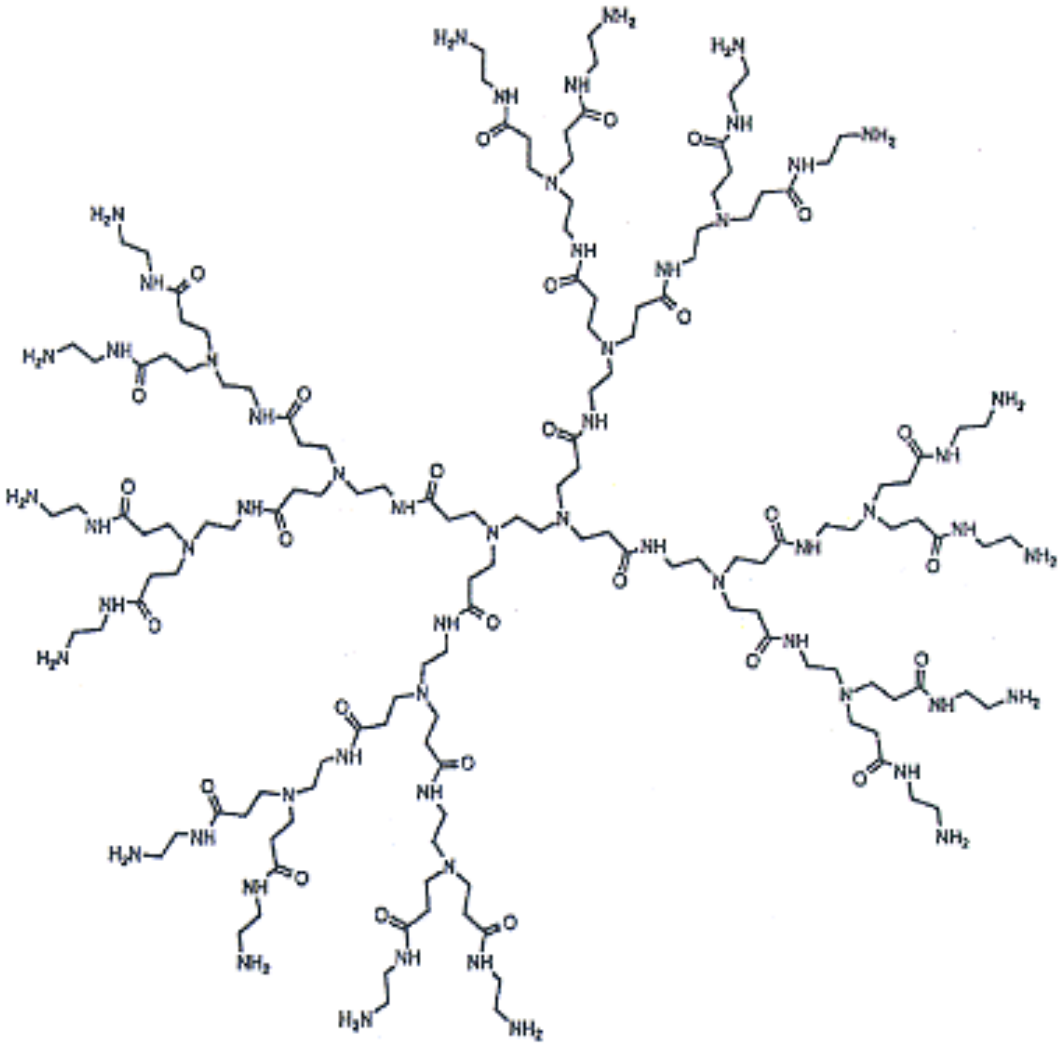
A Branched PEI



B Linear PEI

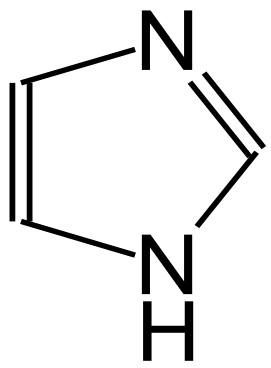


Dendrimers



PAMAM 2nd generation dendrimer

Imidazole-modified compounds



pKa 6.5-7.1

-Histidylated Polylysine: Midoux, P. and Monsigny, M.
Bioconjugate Chem. **1999**, *10*, 406-411.

-Imidazoylated Polylysine: Putnam, D.; Gentry, C.A.; Pack, D.W.; Langer, R.
Proc. Natl. Acad. Sci. **2001**, *98*, 1200-1205.

-Combinatorial Imidazoylated Polycations: Lynn, D.W.; Anderson, D.G.; Putnam, D.; Langer, R.
J. Am. Chem. Soc. **2001**, *123*, 8155-8156.

-Histidylated Lipids: Kumar, V.V.; Pichon, C.; Refregier, S.M.; Guerin, B.; Midoux, P.; Chaudhuri, A.
Gene Therapy **2003**, *15*, 1206-1215.

Endosomal buffering agents

- Pros
  - Membrane activity limited to endosomal compartment
- Cons
  - Inherently cationic (limits in vivo use)
  - Requires huge excess for transfection (N:P >10)
  - Buffering capacity alone does not predict effectiveness

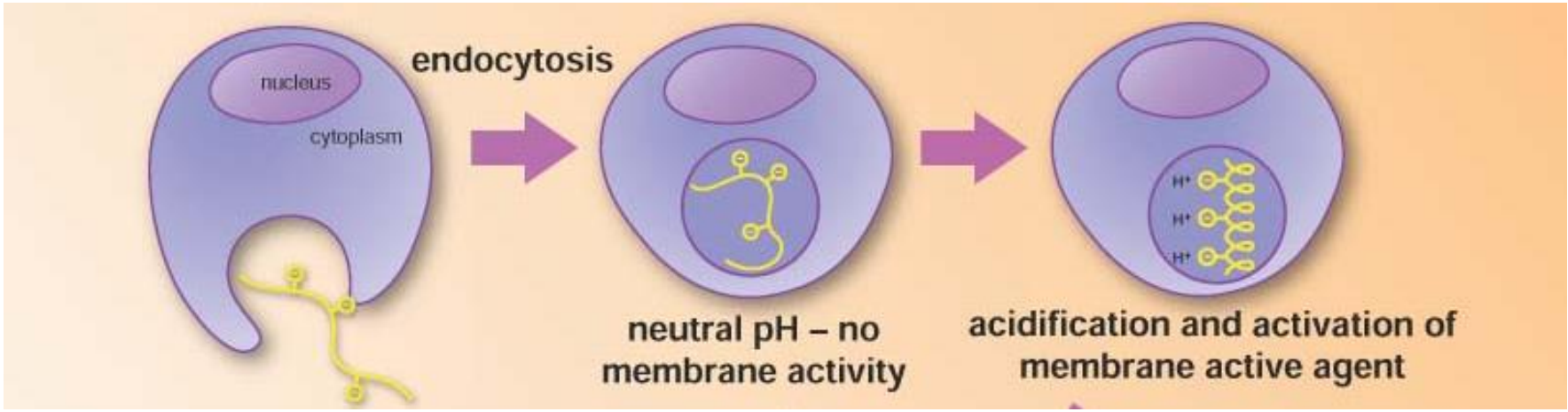
pH-titratable amphipathic polyanions

Arrowhead Research CORPORATION

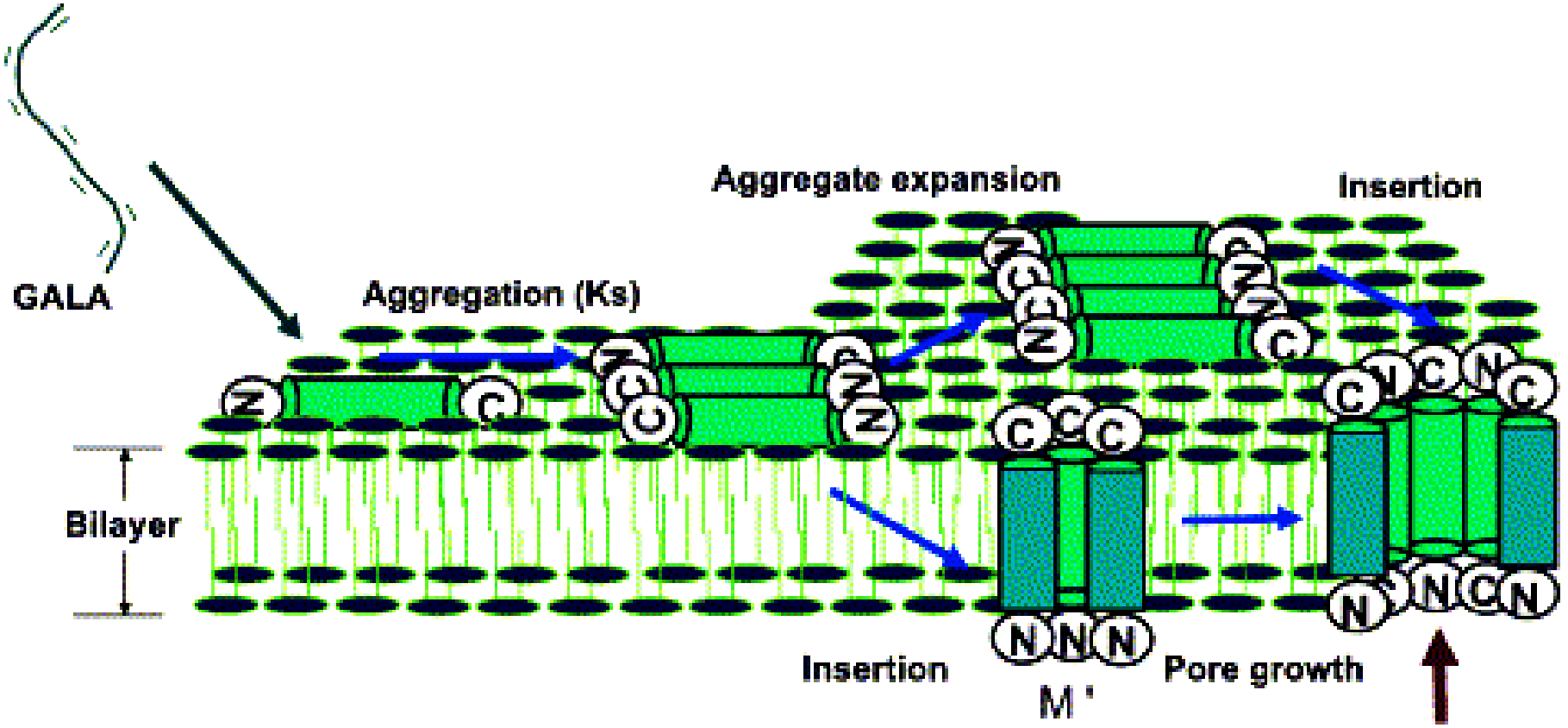
Polymers whose solubility and/or structure is pH-dependent in such a way that at acidic pH they are hydrophobic and membrane interactive.

- EXAMPLES
  - Peptides derived from viral coat proteins, such as the N-terminus of influenza hemagglutinin subunit HA-2, and synthetic analogues containing the GALA (Glu-Ala-Leu-Ala) motif
    - WEAALAEALAEALAEHLAEALAEALEALAA
  - Amphipathic polyanions such as polyalkylacrylic acid and styrene-maleic anhydride copolymer (ethyl-Thomas, Devlin and Tirrell *BBA*, **1996**, *1278*, 73; propyl-Hoffman et al *Journal of Controlled Release,* 1999*, 61,* 137-143;
    and styrene-MA-Henry et al *Biomacromolecules* **2006**, *7*, 2407)

pH titratable polyanions: mechanism of action



Proposed Mechanism of GALA



Li, Nicol and Szoka *Adv. Drug Del. Rev.* **2004**, *56*, 967.

pH-titratable polyanions

- Pros
  - Anionic polymers are inherently less toxic than cationic polymers
- Cons
  - Activity may be affected by conjugation
    - GALA-mAb conjugates were “invariably..significantly less active than unconjugated” see Kuehne and Murphy *Bioconjugate Chem* **2001**, *12*, 742.
  - Large amounts of material required in vitro (and presumably even more in vivo)
    - ~ 200 µg/mL (10 µM) for delivery of calcein by polyalkylacrylic acids see Jones et al *Biochem J.* **2003**, *372*, 65

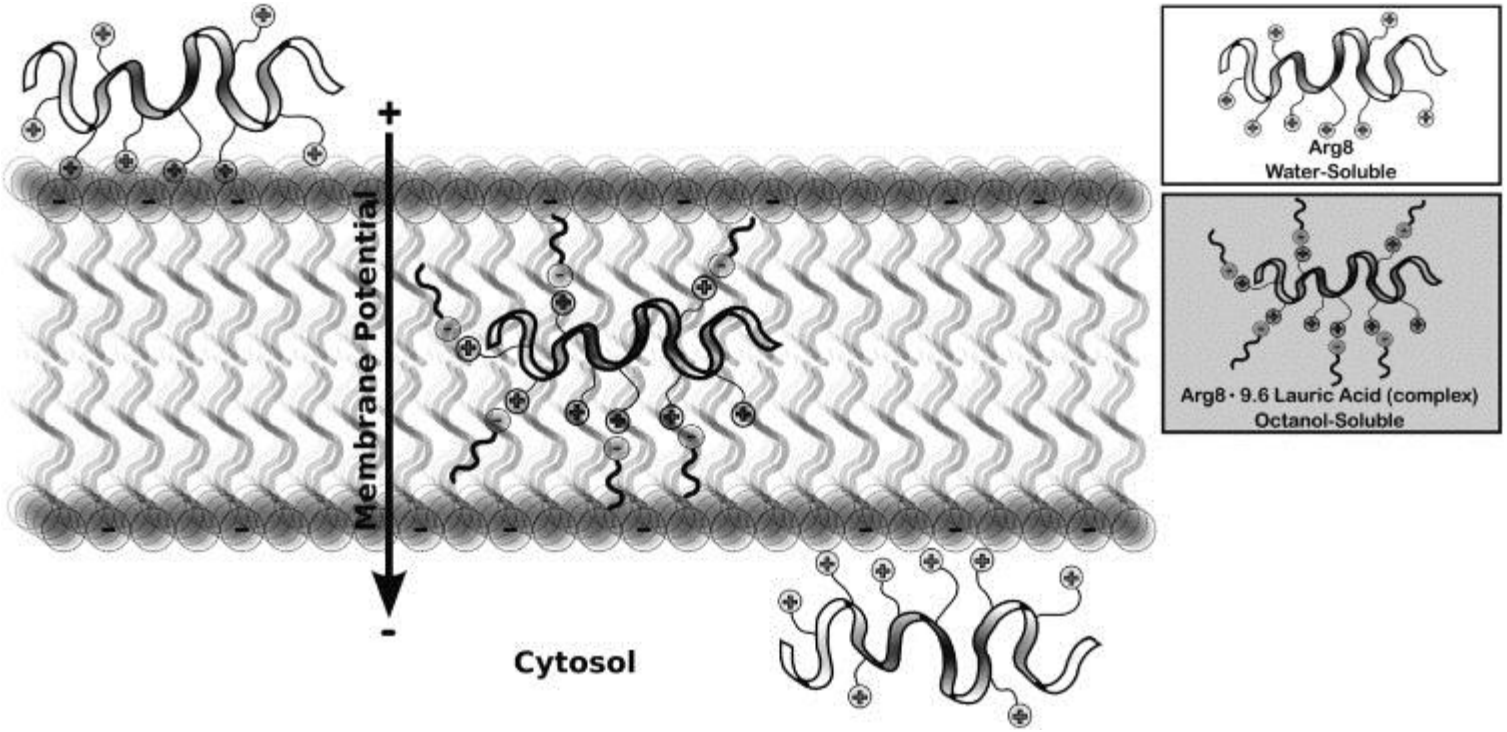
Cell Penetrating Peptides

Peptides (generally cationic) that enter cells purportedly without membrane lysis.

- EXAMPLES
  - Transportan (GWTLNSAGYLLGKINLKALAALAKKIL; Pooga, Hallbrink, Zorko and Langel *FASEB*, **1998**, 12,67.)
  - TAT peptide (YGRKKRRNRRR; Vives, Brodin, and Lebleu *JBC*, **1997**, *272*, 16010)
  - Antp peptide (RNIKIWFQNRRMKWKK; Derossi, Joliot, Chassaing, Prochiantz *JBC*, **1994**, *269*, 10444)
  - Oligoarginine (R_8; Goun et al *Bioconjugate Chem.* **2006**, *17*, 787)

Proposed mechanisms:

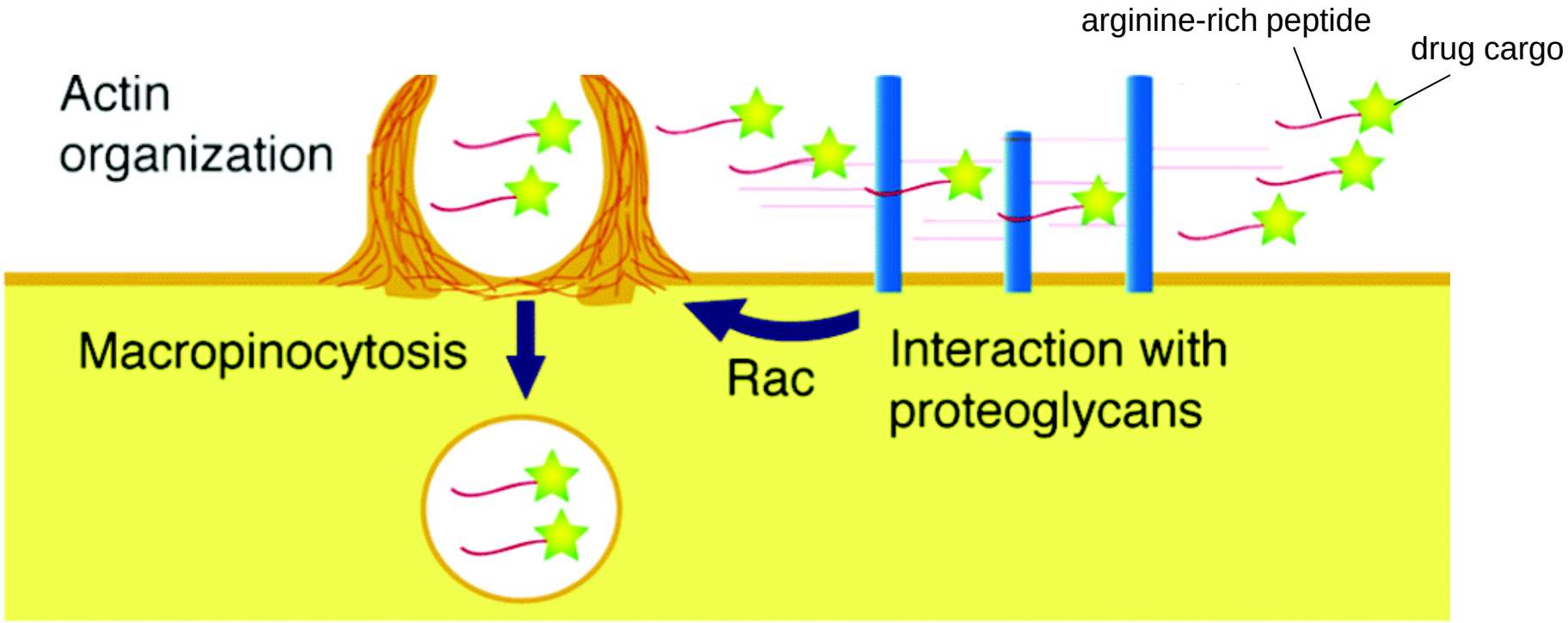
1. Membrane translocation



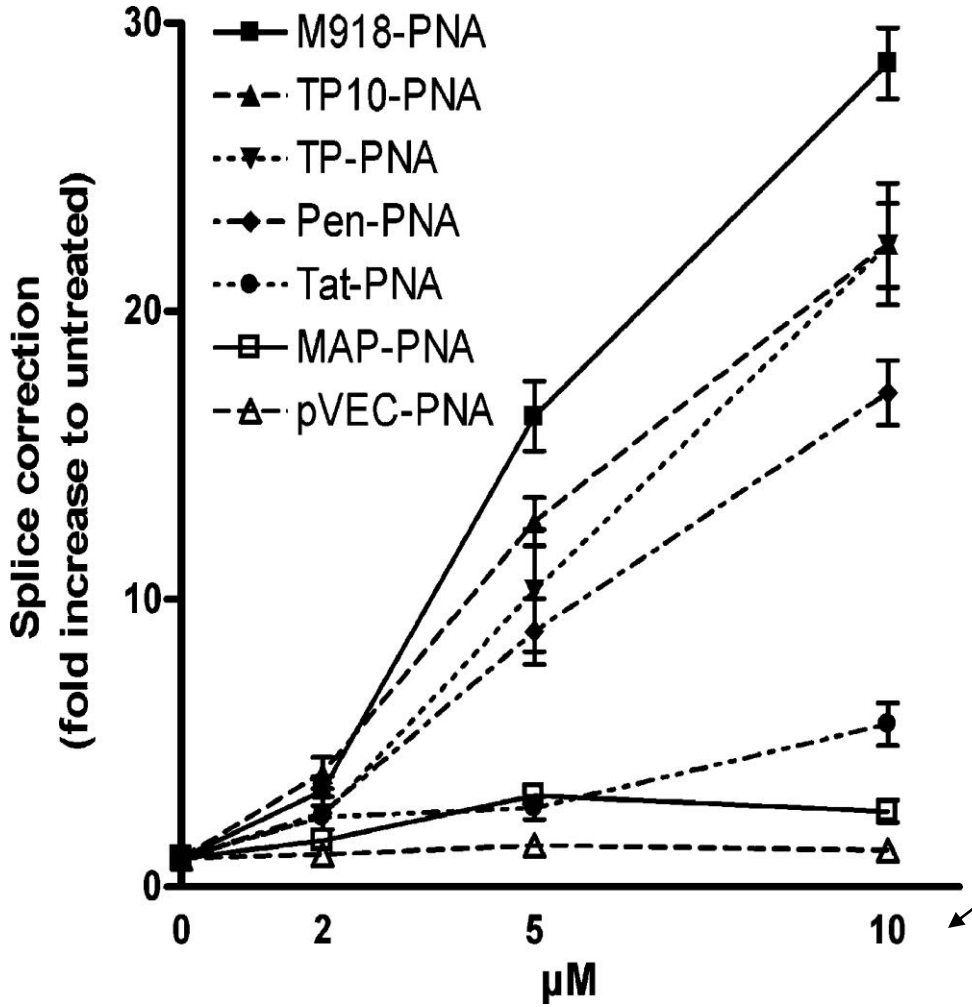
Adaptive Translocation Wender et al Advanced Drug Delivery Reviews 2008, 60, 452.

Proposed mechanisms:

2. Cationic peptides induce macropinocytosis



From Nakase et al *Biochemistry* **2007**, *46*, 492.



Approximately 125 ug/ml

Splice correction by CPP−PNA conjugates. M918, TP10, transportan, and Pen conjugates efficiently corrected splicing while MAP and Tat conjugates displayed minor effects. pVEC conjugate was unable to induce splice correction. HeLa pLuc 705 cells were incubated with indicated conjugates for 4 h in serum-free media followed by exchange to full growth media for an additional 16 h. Cells were lysed and luminescence was normalized to the amount of protein. The values represent the mean of at least three independent experiments done in triplicate (mean ± SEM, *n* = 3).

Published in: Per Lundin; Henrik Johansson; Peter Guterstam; Tina Holm; Mats Hansen; Ülo Langel; Samir EL Andaloussi; *Bioconjugate Chem.* **2008,** 19, 2535-2542.
DOI: 10.1021/bc800212j

Cell Penetrating Peptides

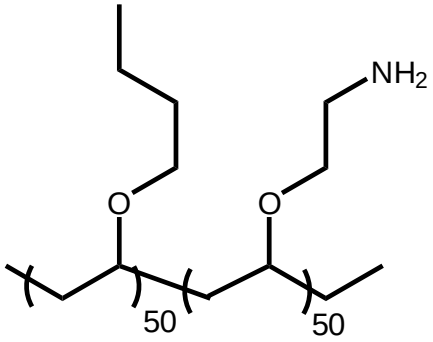
- Pros
  - Delivery is apparently unimolecular
- Cons
  - Inherently cationic (non-specific interactions problematic)
  - Low efficiency for delivery

Membrane-lytic amphipathic polycations

Polymers containing both positively charged and hydrophobic monomers making them inherently membrane-lytic.

EXAMPLES

– Primary amine-containing polyvinylethers (Wakefield et al *Bioconjugate Chemistry* **2005,** *16*, 1204-1208.

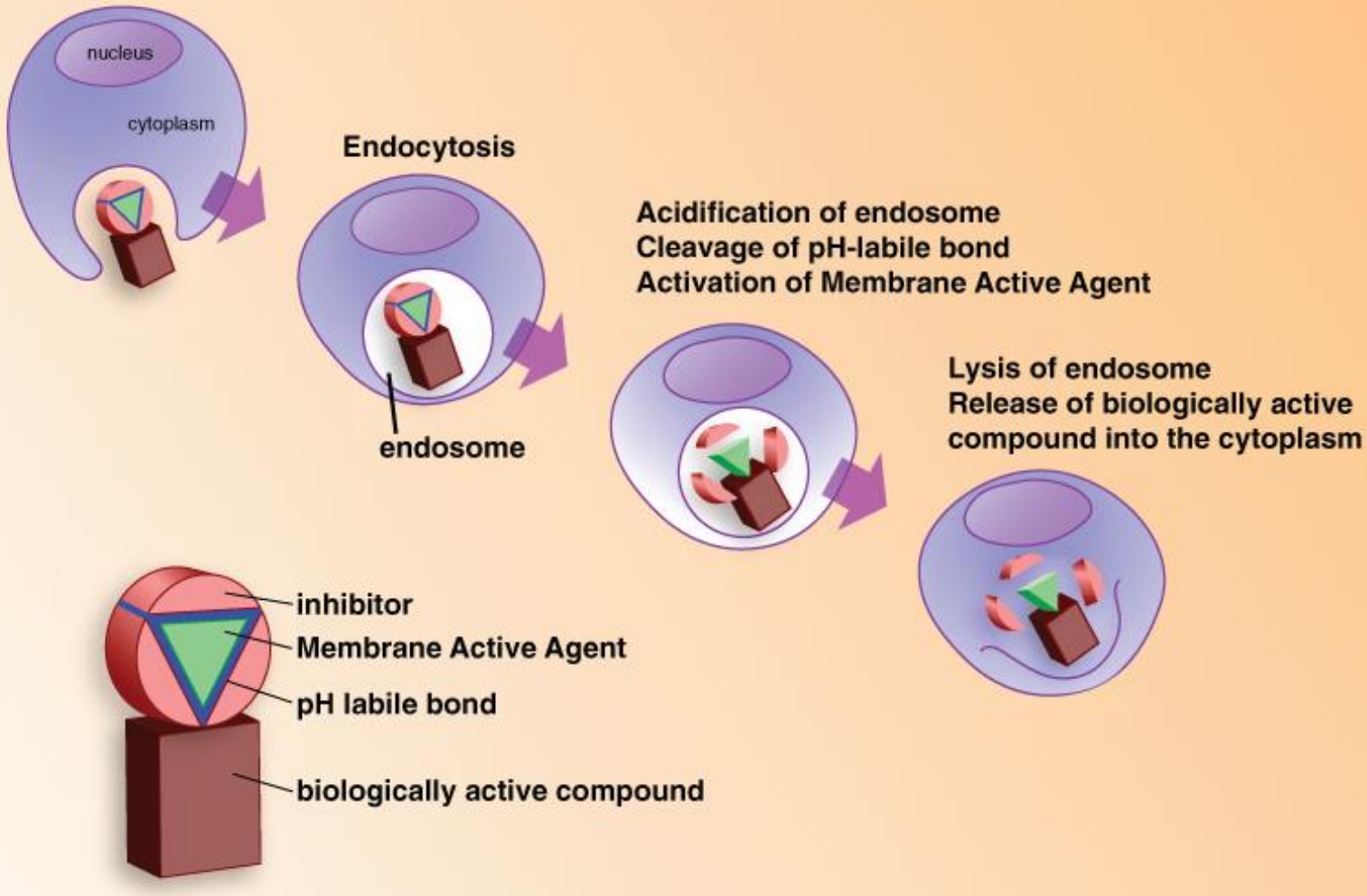


"PBAVE"

– Naturally occurring amphipathic peptides
  - Melittin
  - Pardaxin

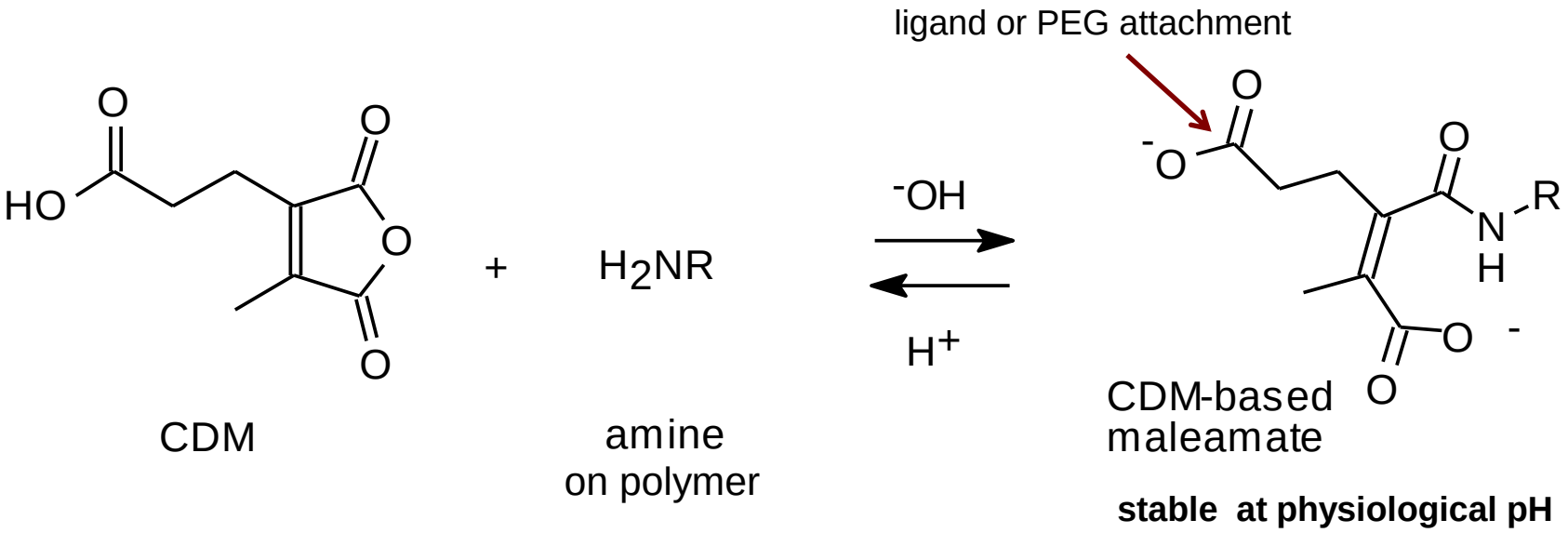
Key to the use of ploycations is to limit their membrane-lytic activity to the endosome

Masking of Membrane-Active Agents



Masking strategies:

2. Acid labile modification of amphipathic polyamines using CDM

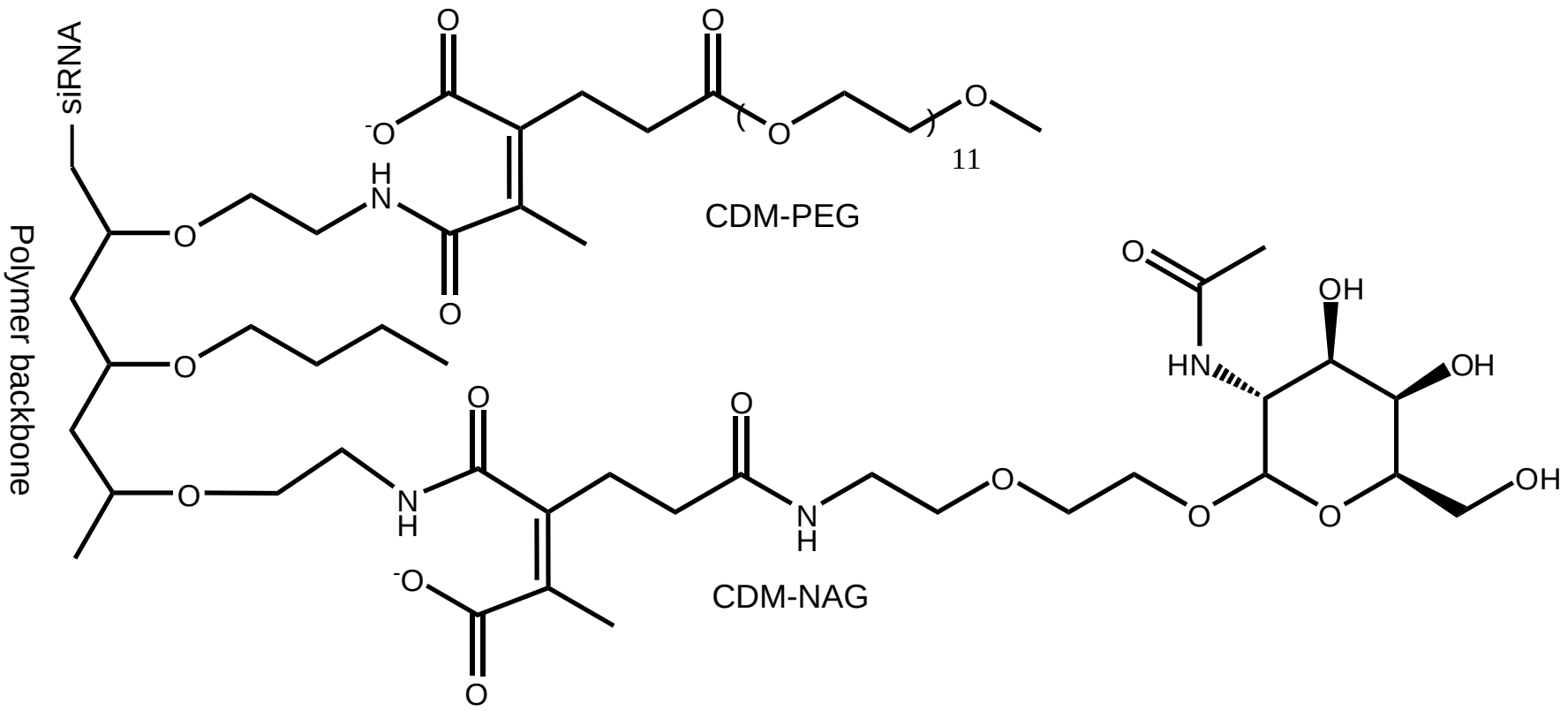


- CDM = Carboxy Dimethyl Maleic Anhydride
- Maleamate bond is stable at physiological pH, reversible under acidic conditions such as those found in the endosome (t1/2 = 5 minutes)
- Reduces postivie surface charge to neutral or slightly negative
- Carboxyl group available for attachment of other groups (e.g. PEG, targeting ligands)

*Rozema et al Bioconjugate Chemistry **2003**, 14, 51-57.*

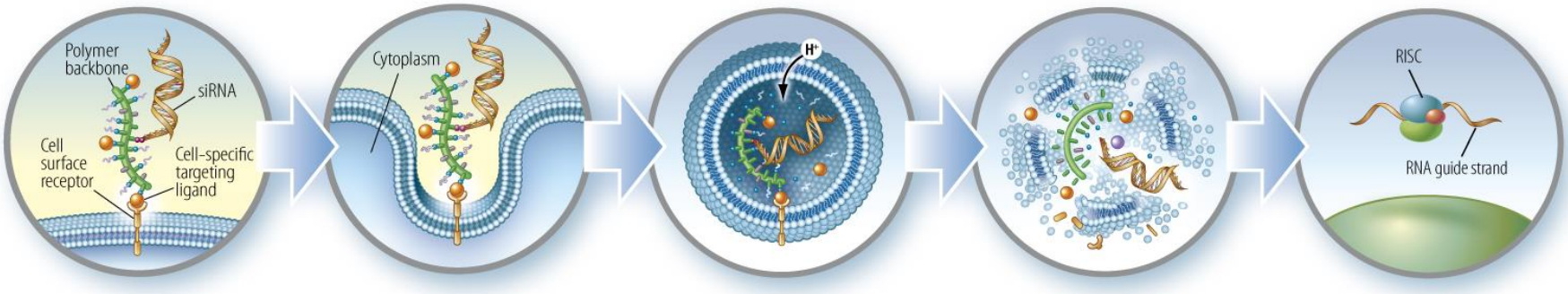
PBAVE modified with CDM-PEG and CDM-NAG

Dynamic Polyconjugate (DPC)



- Membrane-lytic activity of PBAVE inhibited at physiological pH
- CDM-PEG and net negative charge inhibits non-specific interactions in bloodstream
- Attachment of the ligand, CDM-NAG, allows for hepatocyte targeting

Mechanism of DPC-mediated siRNA delivery



DPC conjugated targeting ligand attaches to cell surface

DPC and siRNA are taken up

DPC/siRNA complex is enclosed in an endosome. Low pH results in polymer unmasking.

Polymer induces endosomolysis and release of siRNA payload into the cell cytoplasm.

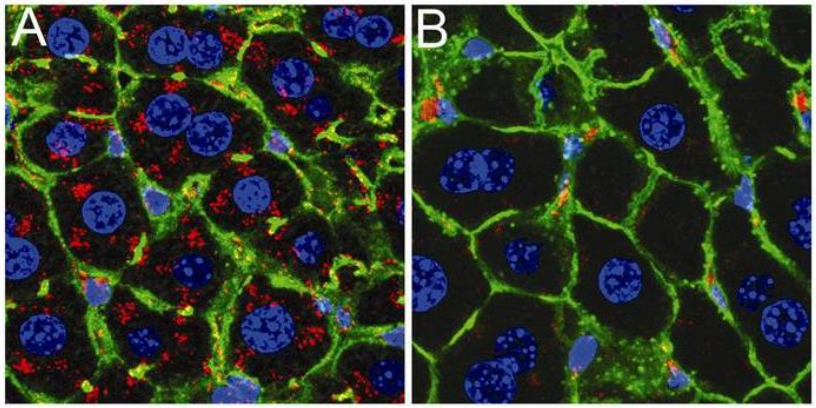
siRNA engages the cell's interference machinery, resulting in knockdown of target gene expression

DPCs for targeted siRNA delivery to hepatocytes

Ligand: N-acetyl galactosamine ligand (NAG)

NAG is a ligand for the asialoglycoprotein receptor on hepatocytes

ICR mice, t=60'

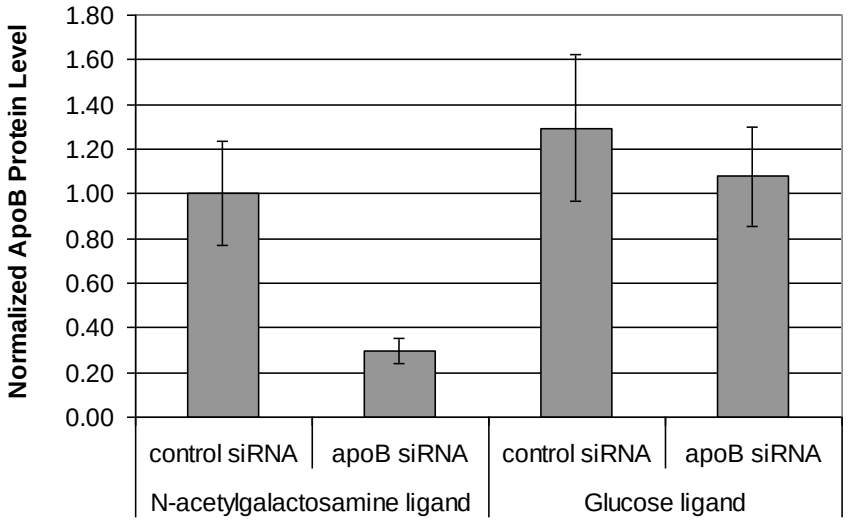


NAG ligand
(hepatocyte targeted)

glucose ligand
(non-targeted)

DPC-siRNA
nucleus
cell membrane

Hepatocyte-uptake of DPCs is ligand dependent



Target gene knockdown is ligand dependent

Rozema, Lewis et al. Proc Natl Acad Sci U S A. 2007 Aug 7;104(32):12982-7

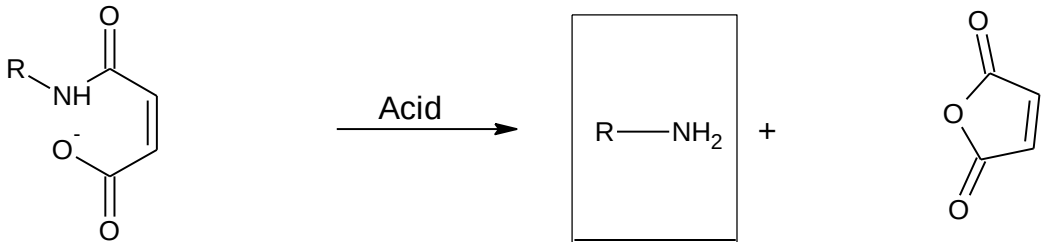
Masking strategies:

3. Protease-sensitive modification

Frist generation acid-lable CDM masking

- Polymer unmasked under acidic conditions in the endosome

R=polymer

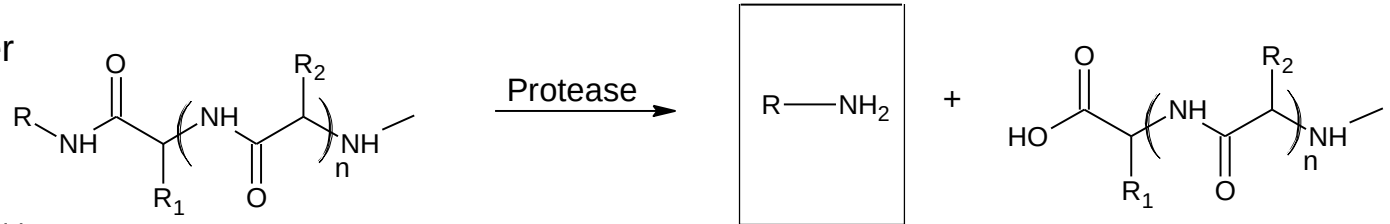


maleamate

Protease-sensitve masking

- Polymer unmasked by action of proteases in endosome/lysosome

R=polymer

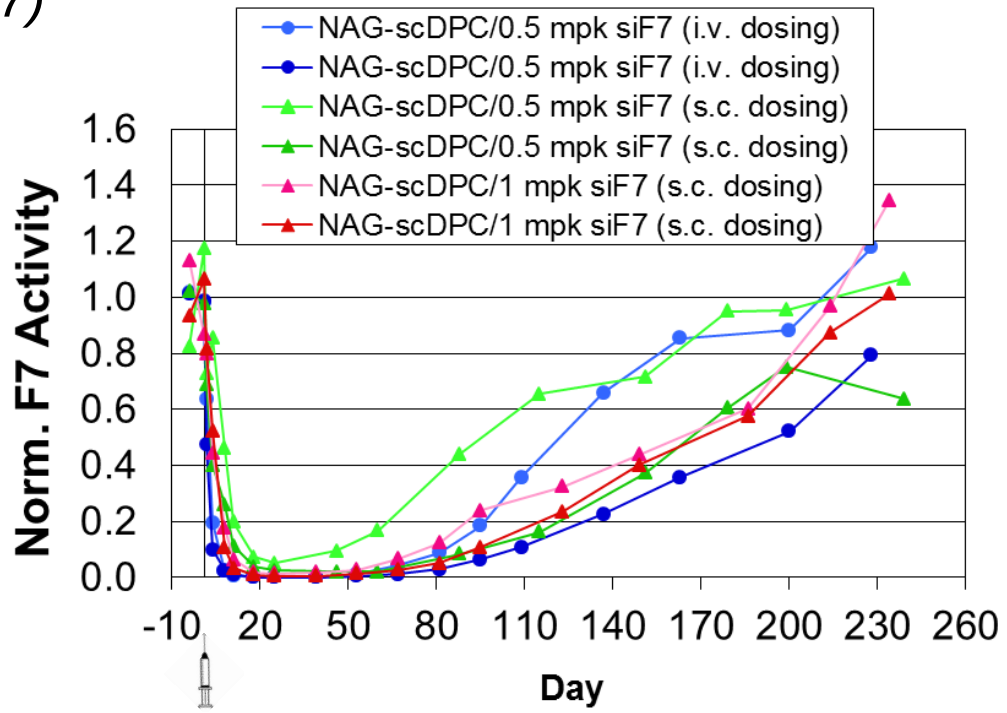


peptide

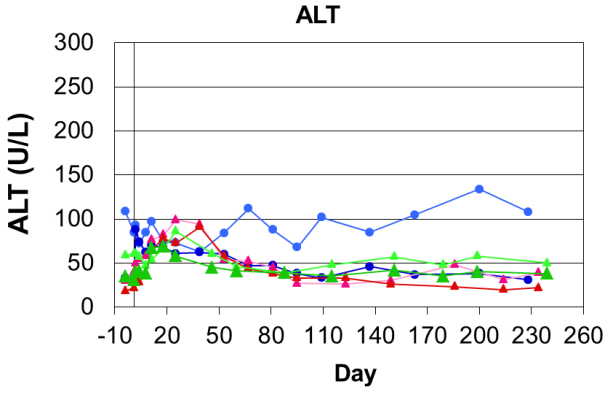
Efficacy/toxicity of protease-masked DPCs in monkeys

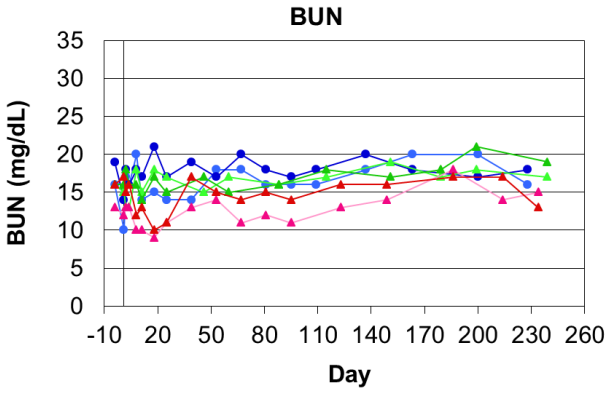
Target: Coagulation Factor 7 (F7)

- **Highly efficacious**
  - Single dose
  - i.v. and s.c. efficacy
  - >99% F7 KD at 1 mg/kg siRNA
  - **~3 month duration of effect (>80% KD) after a single dose**



- **Toxicity not observed**
  - No changes in clin chem markers
  - No changes in hematology





Amphiphathic polycations

- Pros
  - Delivery is highly efficient (ED99 <1 mg/kg siRNA)
- Cons
  - Cationic, membrane-lytic activity not necessarily limited to endosome
  - Require “masking” to prevent interaction with non-endosomal membranes

Take home messages

- Major hurdle for efficient delivery of oligonucleotides is endosomal escape.
- A wide variety of polymers types have been used to facilitate delivery.
- Various mechanisms have been invoked for endosomal release.
  - Disruption of endosomal maturation (pH buffering, eg. PEI)
  - Membrane translocation (eg. penetratin)
  - Direct destabilization of endosomal membrane
    - pH-induced structural changes in polymer resulting in membrane activity (eg. amphipathic polyanions)
    - Membrane-lytic amphipathic polycations (eg. PBAVE)
      - masking used so that membrane-lytic property is only activated within endosome


Arrowhead Research
CORPORATION