# Lecture 16: Intracellular drug delivery

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**Last time:** nano- and micro-particle drug carriers
Delivery to tissues from systemic circulation

**Today**: Intracellular drug delivery

**Reading**: A.S. Hoffman et al., 'Design of "smart" polymers that can direct intracellular drug delivery,' *Polym. Adv. Technol*. **13**, 992-999 (2002)

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Ph gradients and drug delivery: cancer res. 56, 1194 (1996); adv drug deliv rev 25, 3(1997); see asokan minireview J. Pharm. Sci 2002

## Intracellular delivery of molecules

### Pathways of import into the cell

- Uptake of extracellular material by cells
  - Endocytosis
    - Size limitations: ~500 nm or less
    - Occurs in clathrin-coated pits
    - Can be triggered by receptor binding
    - Environment within endocytic vesicles:
      - PH lowered in pathway

| | | |
|---|---|---|
| Extracellular fluid | 7.4 | DNAses, proteases, peptidases |
| Endosomes | ~5.5-6.5 | Proteases |
| lysosomes | ~3.0-5.5 | Proteases (e.g. cathepsins) |
| cytosol | | |

  - macropinocytosis, phagocytosis
    - Specialized scavengers (macrophages, neutrophils) and antigen presenting cells
    - Size limitations: up to the size of the cell

**Endocytosis:**
(nearly all cells)

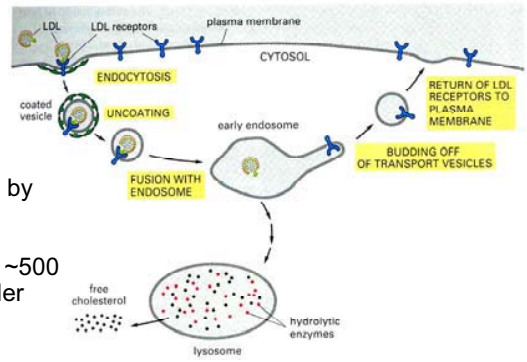



**Figure 13–33 Receptor-mediated endocytosis of LDL.** Note that the LDL dissociates from its receptors in the acidic environment of the endosome. After a number of steps (see Figure 13–34) the LDL ends up in lysosomes, where it is degraded to release free cholesterol. In contrast, the LDL receptor proteins are returned to the plasma membrane in transport vesicles that bud off from the tubular region of the endosome, as shown. For simplicity, only one LDL receptor is shown entering the cell and returning to the plasma membrane. Whether it is occupied or not, an LDL receptor typically makes one round trip into the cell and back to the plasma membrane every 10 minutes, making a total of several hundred trips in its 20-hour life-span.

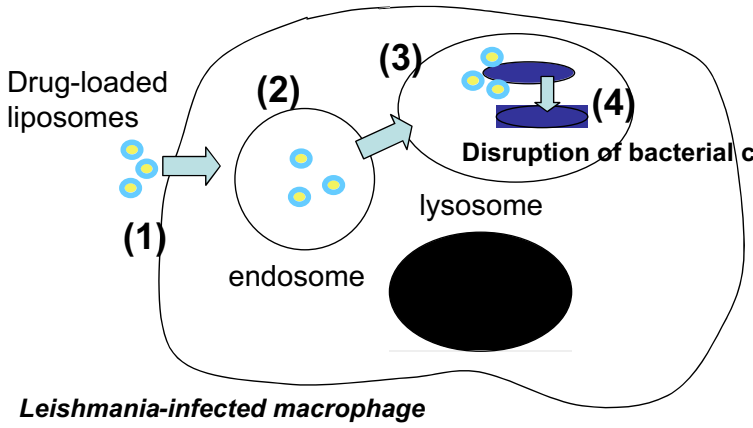
- ¥Can be triggered by receptor binding
- ¥Engulfs volumes ~500 nm diam. or smaller

**Phagocytosis:**
(macrophages, neutrophils, dendritic cells)

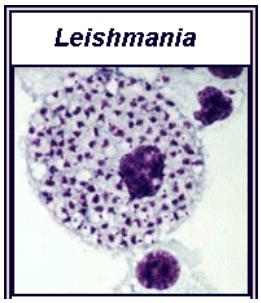
- ¥Engulf volumes up to the size of the cell

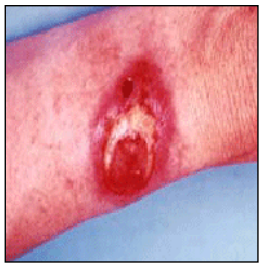

- Access to the cytosol is tightly regulated
  - Typically, internalized material DOES NOT ever reach the cytosol- confined to vesicles
    - For mouse fibroblasts, only 5% of tested protein and 20% of oligonucleotides internalized by a cell could reach the cytosol (Cancer Res. 59, 1180 (1999); Nucleic Acids Res. 25, 3290 (1997))
  - Special case: dendritic cells and (maybe) macrophages
    - Cross-priming: triggering of certain receptors by pathogens leads to delivery of antigens to the cytosol
  - Drug delivery has been attempted by using high doses to obtain a small 'leak' current into cytosol
    -

- Delivery of proteins, DNA, small-molecule drugs to the cytosol
- Example motivation: treatment of leishmania bacterial infections
  - Leishmania (Alving 1988 Adv Drug Deliv Rev 2, 107)
  - Pathway to attack intracellular bacteria:
    - Phagocytosis of carrier
    - Fusion of endome with parasite-loaded lysosomes
    - Binding of liposomal carrier to bacterial cell wall and disruption of cell wall
  - commercial product: Ambisome (Gilead, Boulder CO)
    - liposomal formulation of amphotericin B to treat leishmaniasis[1]
    - lipid-like drug inserts in liposomal wall as well as within liposomal internal aqueous compartment

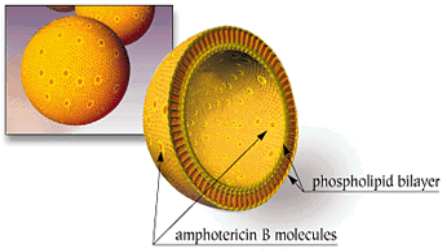



*Leishmania-infected macrophage*

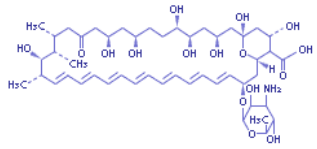


cutaneous leishmaniasis



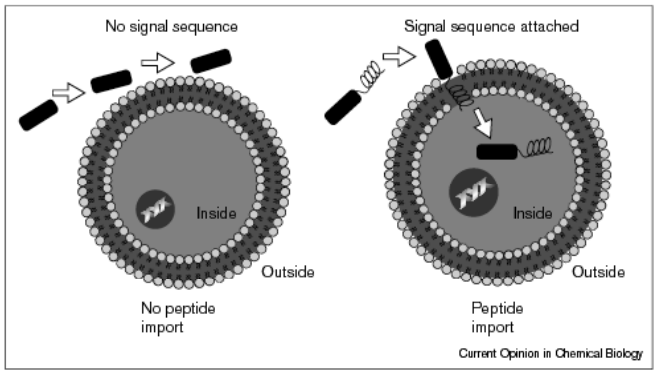

**Amphotericin B**



## Mechanisms for intracellular delivery

- ***Cross the plasma membrane***[2]
  - Direct entry to cytosol
  - Viral peptide transporters
    - Typically use highly stable hydrophobic helix peptide
      - Structural similarity to transmembrane protein tails
    - Difficult to mimic selective activation of membrane-penetration activity that viruses have- conjugates always 'on'- can't control which membranes are crossed

### Hydrophobic sequences used by viruses to enter cells:



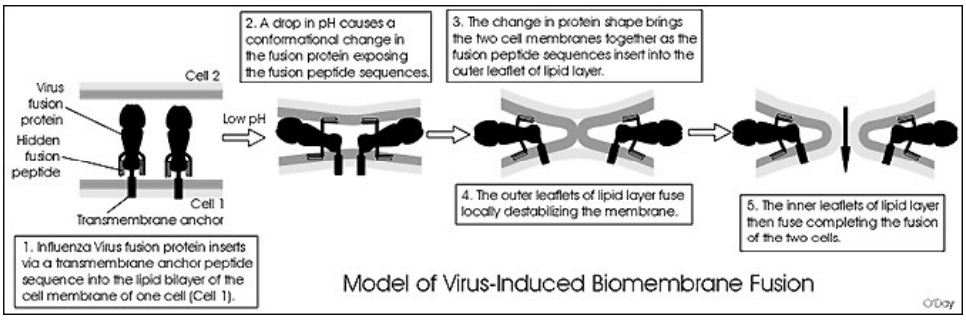

The conceptual design of peptide import based on signal sequence. Cell-permeable peptides are designed by using the h region of the signal peptide (represented by the helix-like leading portion) covalently bound to the amino terminus or the carboxyl terminus of selected sequences of intracellular proteins.

(Hawiger, 1999)

- Generally believed to be a more dangerous strategy than endosomal escape:

- Potential to destroy electrical potential gradient maintained by cell across plasma membrane causing cell death

- ***Escape from endosomes/lysosomes***
  - Enter endoycytic pathway, cargo released from vesicles once taken inside the cell
    - Dangers of the endocytic pathway (Asokan 2002[3])
      - PH: surface 7.4 -> endosomes -> 6.5-5.5 -> lysosomes ~5.0
        - Lysosomes reached in 30-60 min. typically
      - Endosomes and lysosomes contain proteases (e.g. cathepsins), lipases, glycolases, phophatases
  - Routes
    - Viral peptides evolved for endosomal escape
      - HIV-tat peptide (J. Biol Chem 276, 3254 (2001))
        - Polybasic Tat sequence[4]:
          - GRKKRRQRRRPPQC
        - Current mechanism hypothesis:
          - Positively-charged residues bind polyanionic proteoglycans, triggering rapid internalization
          - Unclear how escape from endosome occurs
      - Influenza hemagluttinin peptide
        - Undergoes conformational change at reduced pH
          - Inserts in membrane, reduced pH causes a membrane-destabilizing change in conformation
          - Source for 'model of virus-induced biomembrane fusion' graphic: http://www.erin.utoronto.ca/~w3bio315/biomembrane%20fusion.htm




    - Fusion with endosomal membranes
      - Liposomes that become unstable and fusion-competent at reduced pH[5]
      - Yatvin Fig.1 and Fig. 2
    - Disruption of endosomal compartments
      - pH-triggered membrane-destabilizing component
      - hemolysin from listeria monocytogenes bacterium[6,7]

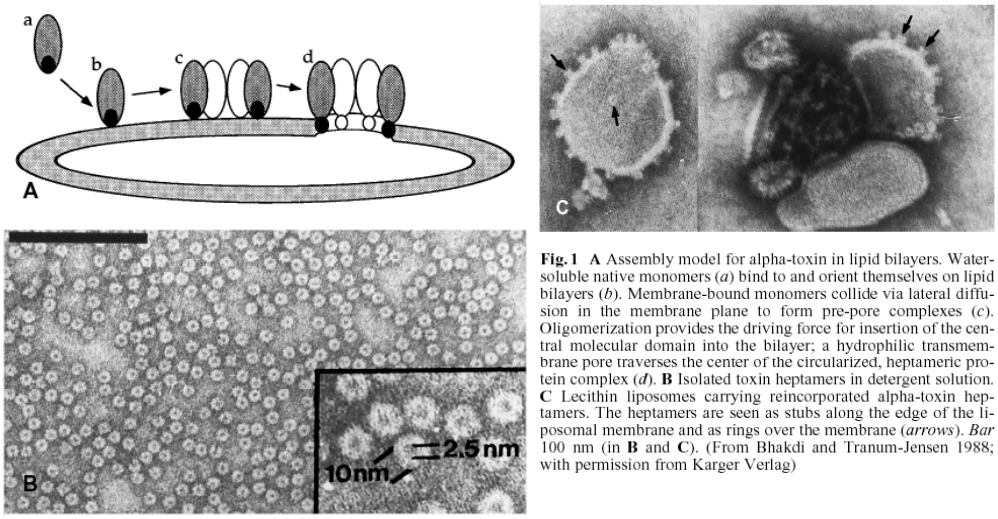



**Fig. 1** **A** Assembly model for alpha-toxin in lipid bilayers. Water-soluble native monomers (*a*) bind to and orient themselves on lipid bilayers (*b*). Membrane-bound monomers collide via lateral diffusion in the membrane plane to form pre-pore complexes (*c*). Oligomerization provides the driving force for insertion of the central molecular domain into the bilayer; a hydrophilic transmembrane pore traverses the center of the circularized, heptameric protein complex (*d*). **B** Isolated toxin heptamers in detergent solution. **C** Lecithin liposomes carrying reincorporated alpha-toxin heptamers. The heptamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (*arrows*). *Bar* 100 nm (in **B** and **C**). (From Bhakdi and Tranum-Jensen 1988; with permission from Karger Verlag)

(Bhakdi 1996)

- Targeting to antigen presenting cells that cross-prime[8,9]
  - Mechanism not yet known

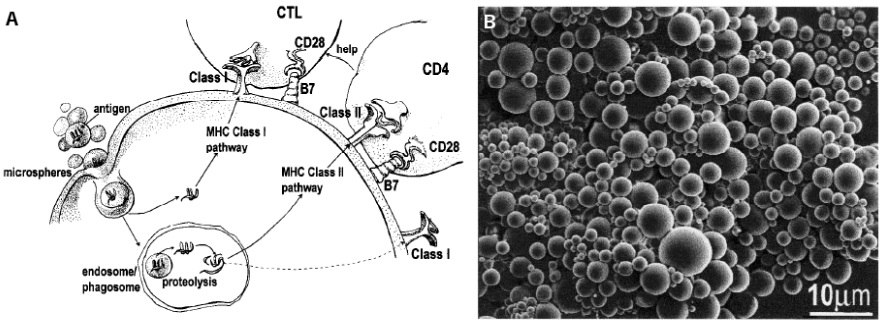



Figure 2. Presentation of exogenous antigens on MHC class I and II molecules. (A) In dendritic cells and macrophages, particulate antigens such as microspheres are internalized into phagosomes. A portion of the antigen is released into the cytoplasm where it becomes available to the MHC class I molecules. These APCs express all of the molecules, such as B7, needed to stimulate T cells and also may recruit CD4 T-cell help for CD8 T cells by bringing these two cells into close proximity. (B) A scanning electron micrograph of PLG microspheres. These preparations can efficiently deliver antigens into the exogenous class I and class II pathways and induce protective immune responses. Bar = 10 microns.

## Example Approach: 'smart' release from endosomes

- Pat Stayton and Allan Hoffman U. Washington- Murthy et al.[10]
- 'encrypted' polymers
  - concept: mask membrane-disruptive moieties on a drug-carrying polymer until endosomes are reached

**Multi-function molecular carriers:**

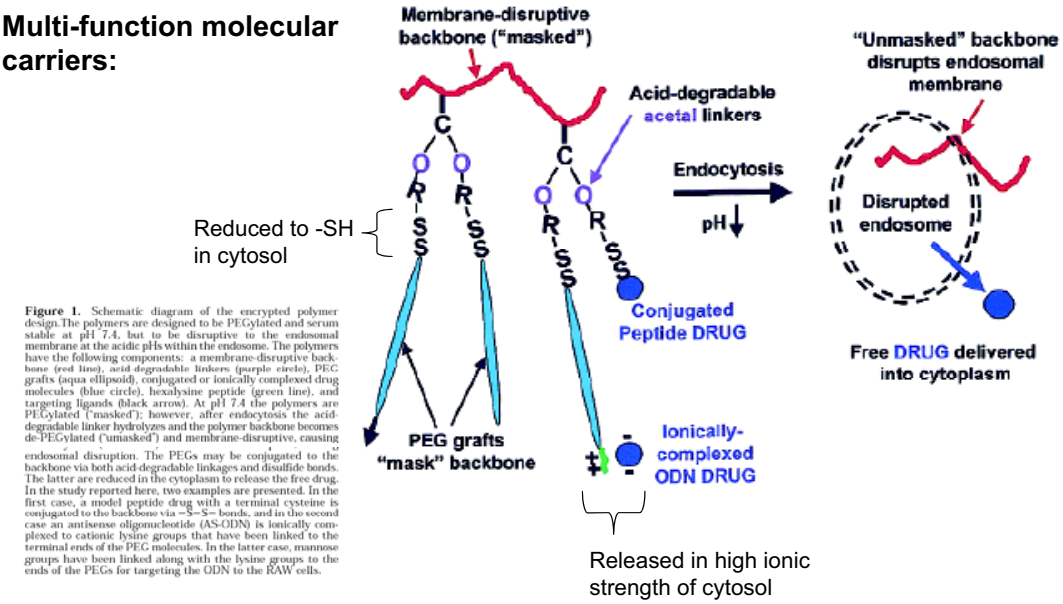
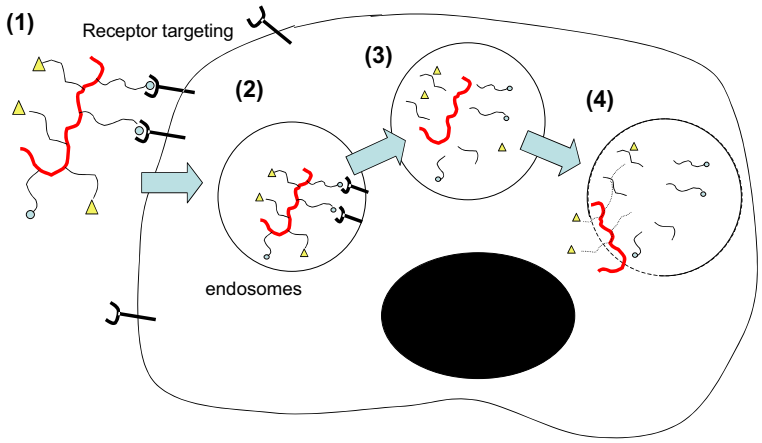



**Figure 1.** Schematic diagram of the encrypted polymer design. The polymers are designed to be PEGylated and serum stable at pH 7.4, but to be disruptive to the endosomal membrane at the acidic pHs within the endosome. The polymers have the following components: a membrane-disruptive backbone (red line), acid-degradable linkers (purple circle), PEG grafts (aqua ellipsoid), conjugated or ionically complexed drug molecules (blue circle), hexalysine peptide (green line), and targeting ligands (black arrow). At pH 7.4 the polymers are PEGylated ("masked"); however, after endocytosis the acid-degradable linker hydrolyzes and the polymer backbone becomes de-PEGylated ("unmasked") and membrane-disruptive, causing endosomal disruption. The PEGs may be conjugated to the backbone via both acid-degradable linkages and disulfide bonds. The latter are reduced in the cytoplasm to release the free drug. In the study reported here, two examples are presented. In the first case, a model peptide drug with a terminal cysteine is conjugated to the backbone via –S–S– bonds, and in the second case an antisense oligonucleotide (AS-ODN) is ionically complexed to cationic lysine groups that have been linked to the terminal ends of the PEG molecules. In the latter case, mannose groups have been linked along with the lysine groups to the ends of the PEGs for targeting the ODN to the RAW cells.

(Murthy et al., 2003)




- 3 functionalities of polymer carrier:
  1. targeting ligand for receptor-mediated endocytosis
  2. pH-responsive element for endosomal membrane disruption, exposed only when endosomes are reached
  3. therapeutic drug attached, released in endosomes

- pH-responsive element: acetal linkages
  - degradation rate of acetal linkages sensitive to identity of *para* group on attached benzene ring
    - N -> O $t_{1/2}$ drops by 60-fold (*JACS* **77**, 5590 (1955))
  - $t_{1/2}$ = 15 min at pH 5.4 for the given structure
    - hydrolysis rate 100X at pH 5.4 compared to pH 7.4
  -

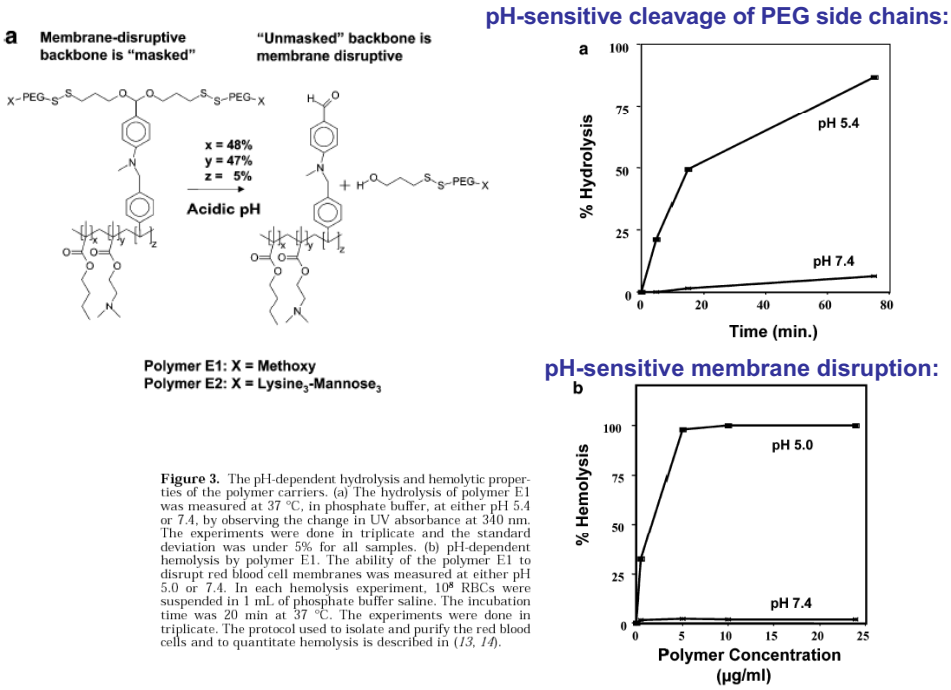



**Figure 3.** The pH-dependent hydrolysis and hemolytic properties of the polymer carriers. (a) The hydrolysis of polymer E1 was measured at 37 °C, in phosphate buffer, at either pH 5.4 or 7.4, by observing the change in UV absorbance at 340 nm. The experiments were done in triplicate and the standard deviation was under 5% for all samples. (b) pH-dependent hemolysis by polymer E1. The ability of the polymer E1 to disrupt red blood cell membranes was measured at either pH 5.0 or 7.4. In each hemolysis experiment, $10^8$ RBCs were suspended in 1 mL of phosphate buffer saline. The incubation time was 20 min at 37 °C. The experiments were done in triplicate. The protocol used to isolate and purify the red blood cells and to quantitate hemolysis is described in (*13, 14*).

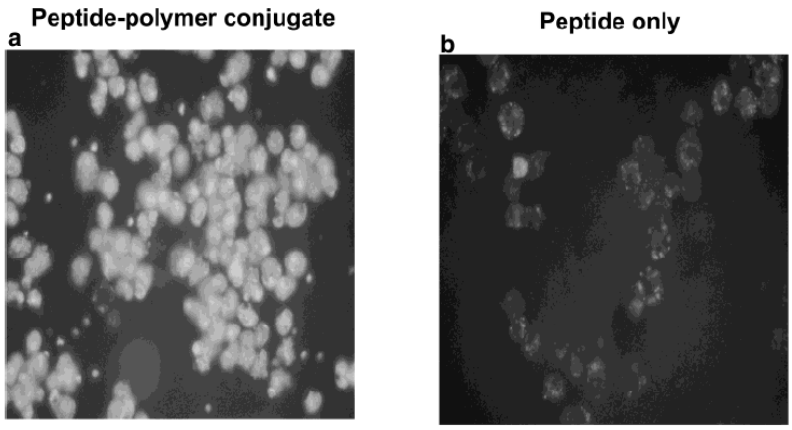



**Figure 7.** Cytoplasmic delivery of peptides with polymer E3. (a) Fluorescence microscopy (40X magnification) of RAW cells treated overnight with the peptide Cys-$(Gly)_4$-$(His)_6$-FITC conjugated to polymer E3. (b) Lysosomal localization of the peptide Cys-$(Gly)_4$-$(His)_6$-FITC. The peptide Cys-$(Gly)_4$-$(His)_6$-FITC was incubated with RAW cells overnight and visualized by fluorescence microscopy at 40× magnification.

## References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