

Chapter 1

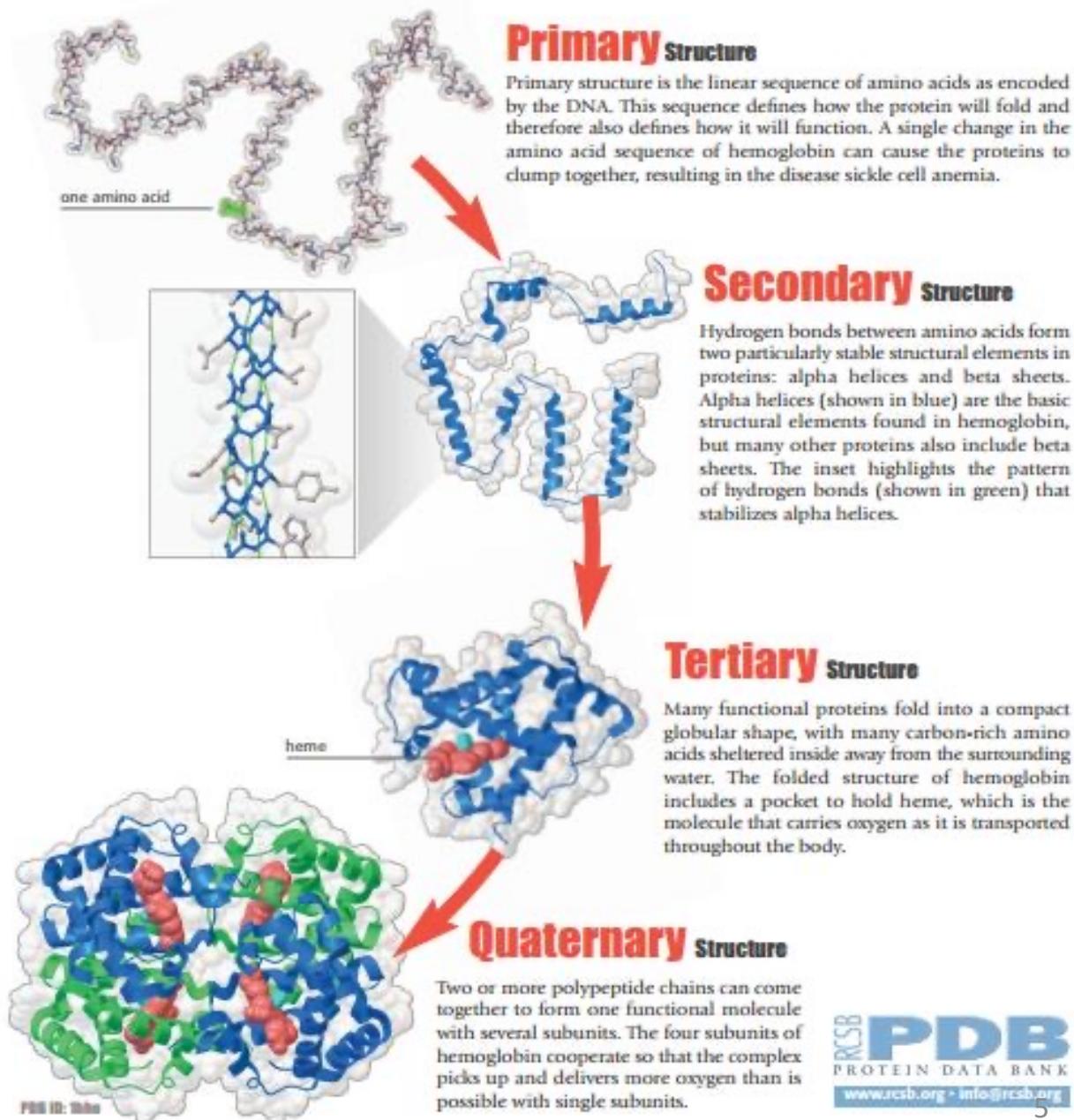
Peptide/Protein-Based Theranostics

What is a Protein?

Seeing proteins!



John Kendrew (left) and Max Perutz shared the 1962 Nobel prize in Chemistry for determining the first 3D structures of proteins.

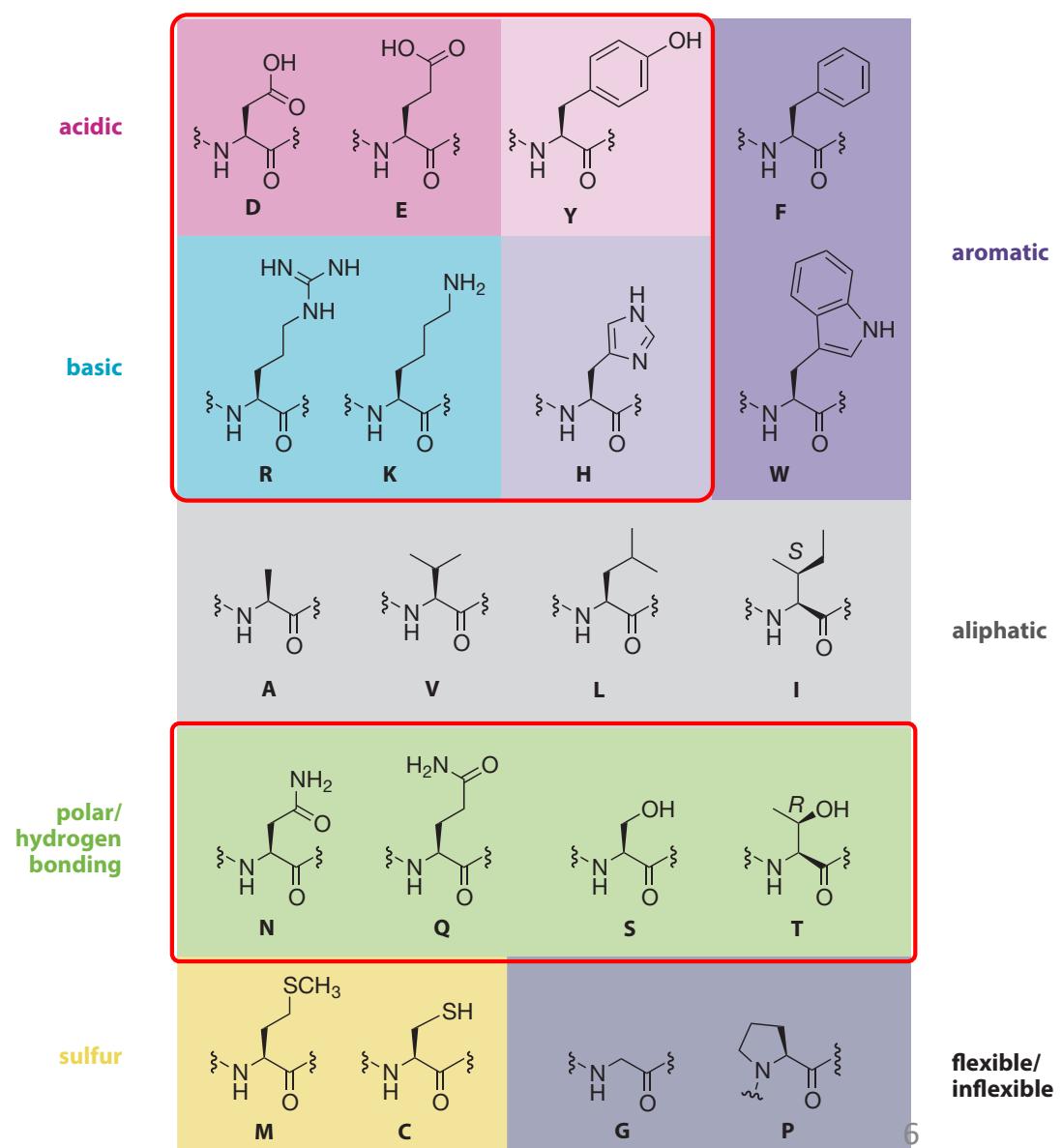
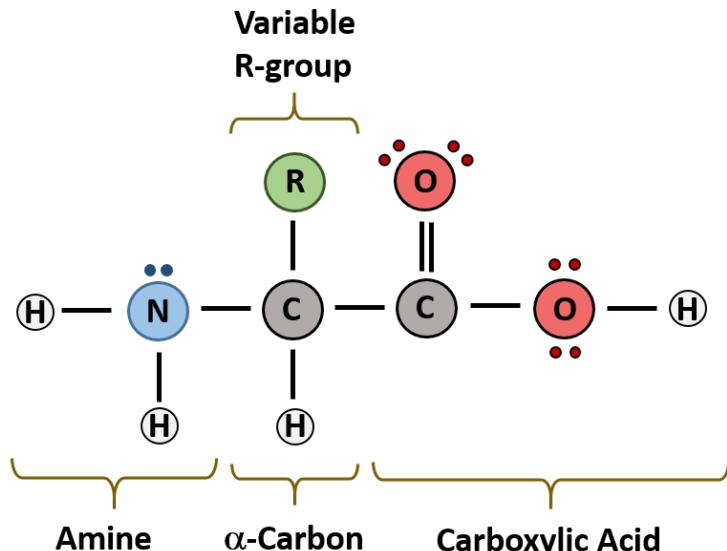


Amino Acids

The 20 ribosomally incorporated amino acids!

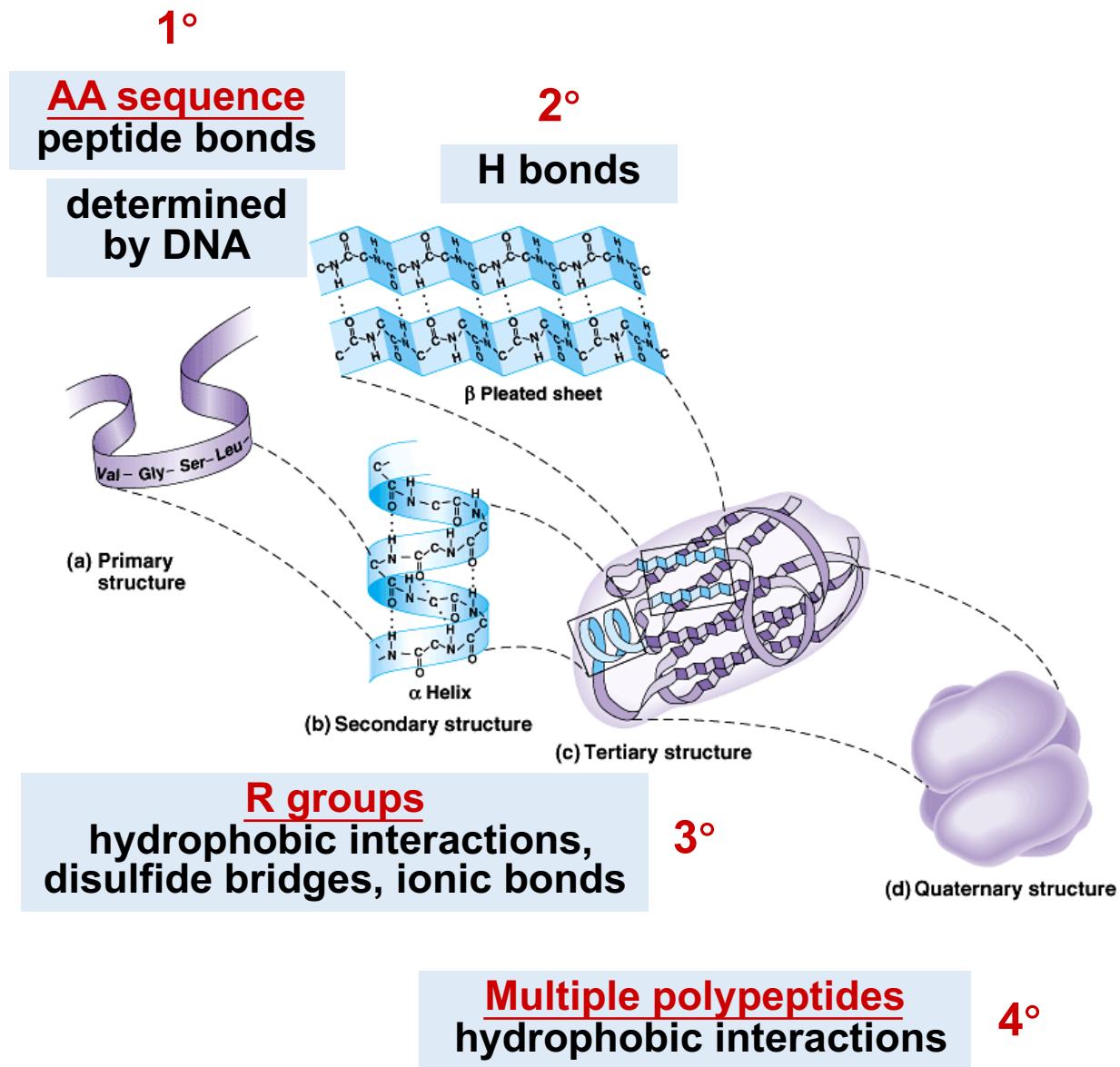
Structure of Amino Acids:

- central carbon (α carbon)
- amino group
- carboxyl group (acid)
- R group (side chain)
 - variable group
 - confers unique chemical properties

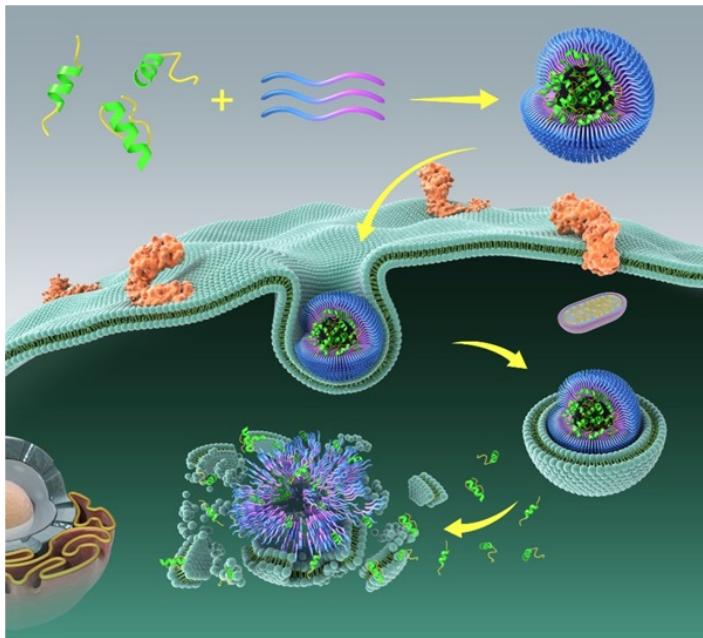


Levels of Organization

- **Primary structure**
 - Amino acid sequence of the protein
- **Secondary structure**
 - H bonds in the peptide chain backbone
 - α -helix and β -sheets
- **Tertiary structure**
 - Non-covalent interactions between the R groups within the protein
- **Quaternary structure**
 - Interaction between 2 polypeptide chains



1.1. Peptide-Based Therapeutics



Peptide-based anticancer agents that require intracellular delivery into two major classes based on target cell:

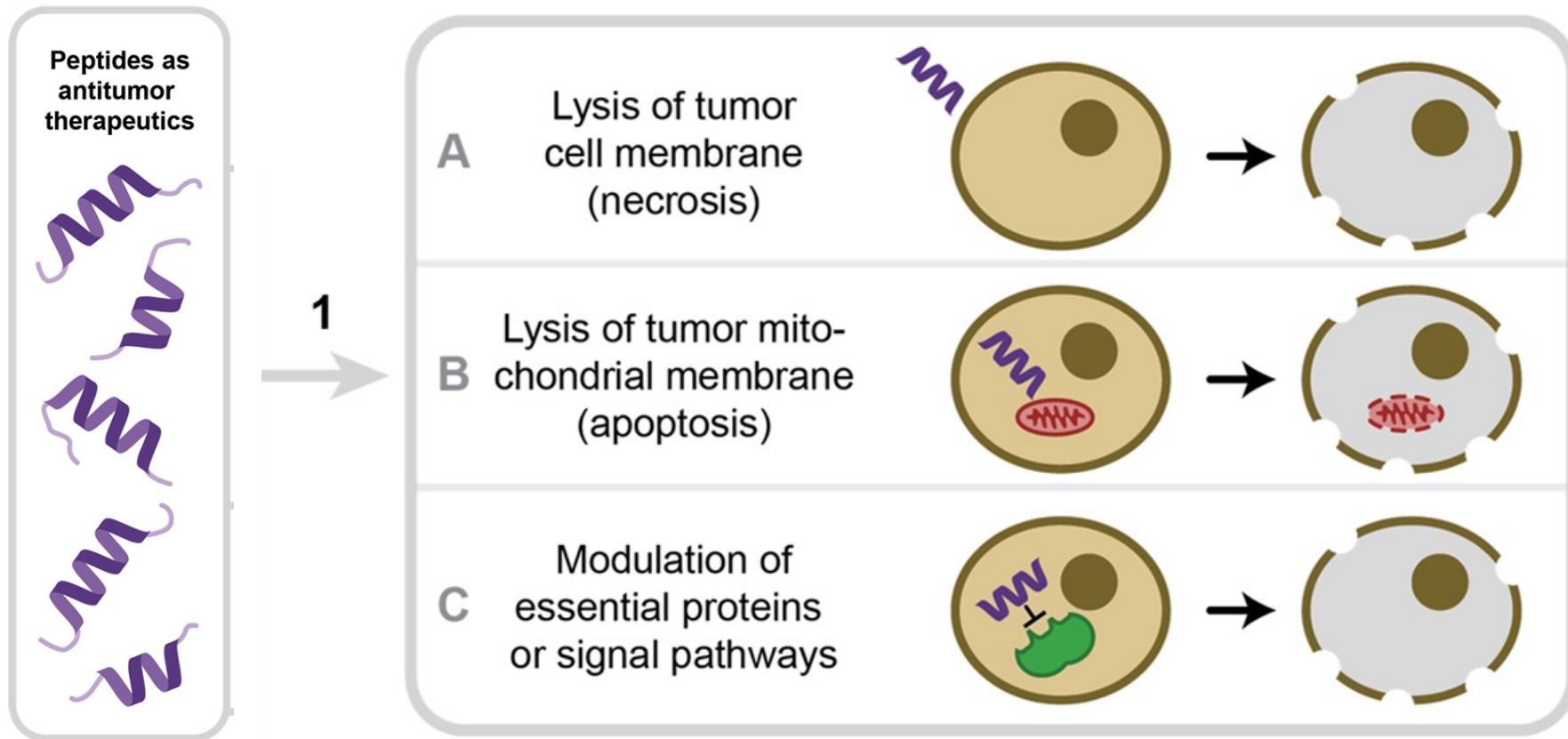
- (1) **therapeutic peptides** which display **inhibitory activities directly in tumor cells**;
- (2) **peptide antigens** that **activate immune cells to eradicate tumor cells**.

Table 1. Summary of Advantages and Disadvantages of Peptide-Based Therapeutics

advantages	disadvantages
broad chemical diversity	poor <i>in vivo</i> stability
wide range of targets	rapid clearance
high selectivity and potency	lack of membrane permeability
ease of synthesis and modification	low metabolic stability
good biocompatibility and safety	aqueous solubility

1.1.1. Therapeutic Anticancer Peptides

➤ Antitumor peptides with different mechanisms

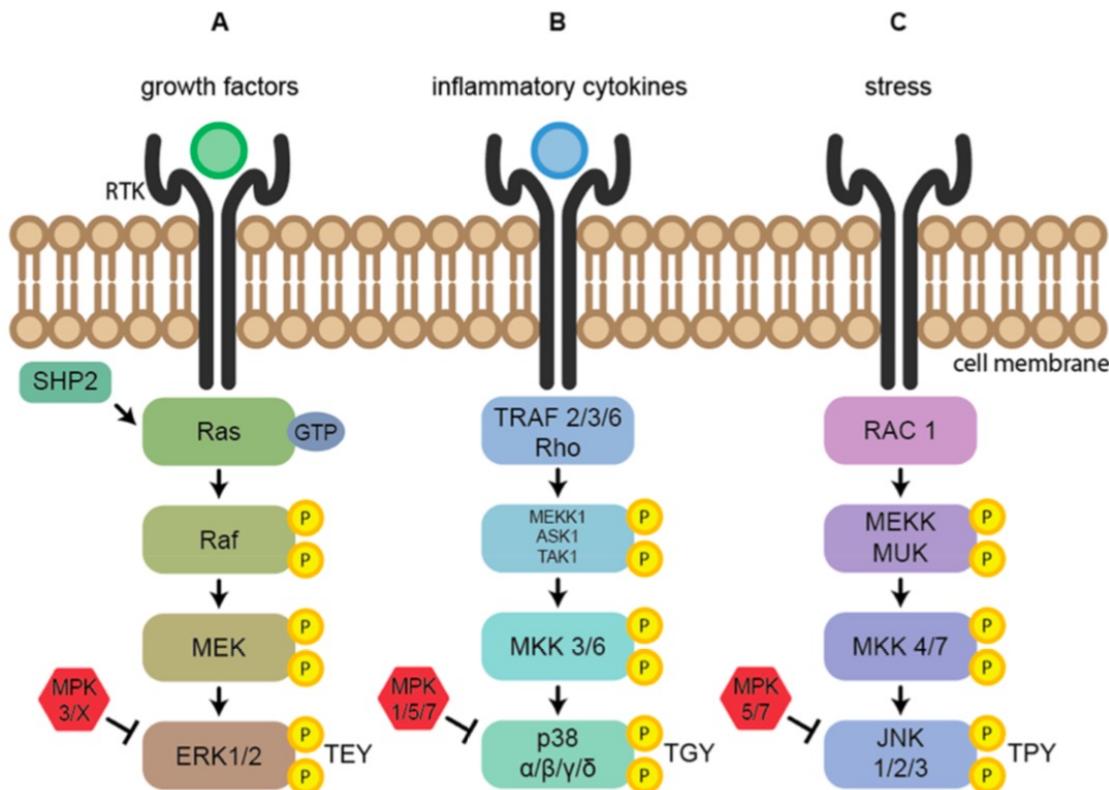


Anticancer peptides (ACPs) are a broad class of anticarcinogens with various and distinctive mechanisms of antineoplastic activity. On the basis of these mechanisms, ACPs can be classified into three groups:

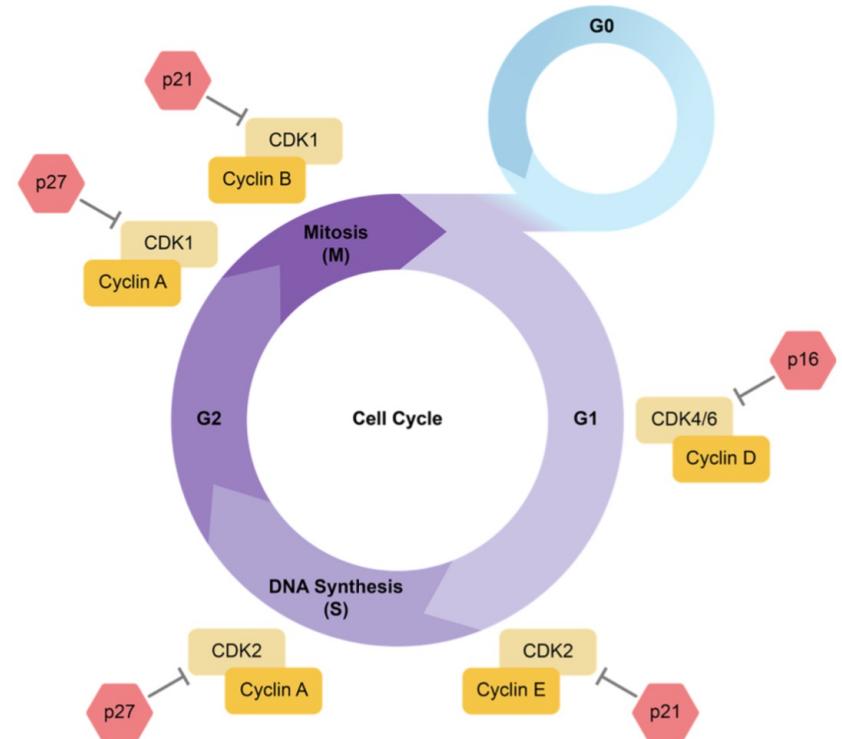
- A**) membranolytic ACPs that induce necrosis by lysing the tumor cell membrane;
- B**) membranolytic ACPs that induce apoptosis by disrupting the mitochondrial membrane;
- C**) ACPs that target intracellular protein-protein interactions.

1.1.1. Nonmembranolytic ACPs

- ACPs can target the essential protein-protein interactions, suppress tumor growth by **activation of tumor suppressor proteins**, or **inhibit oncogenic signaling pathways**.
(1) signal transduction pathways, (2) cell cycle regulation, or (3) cell death pathways



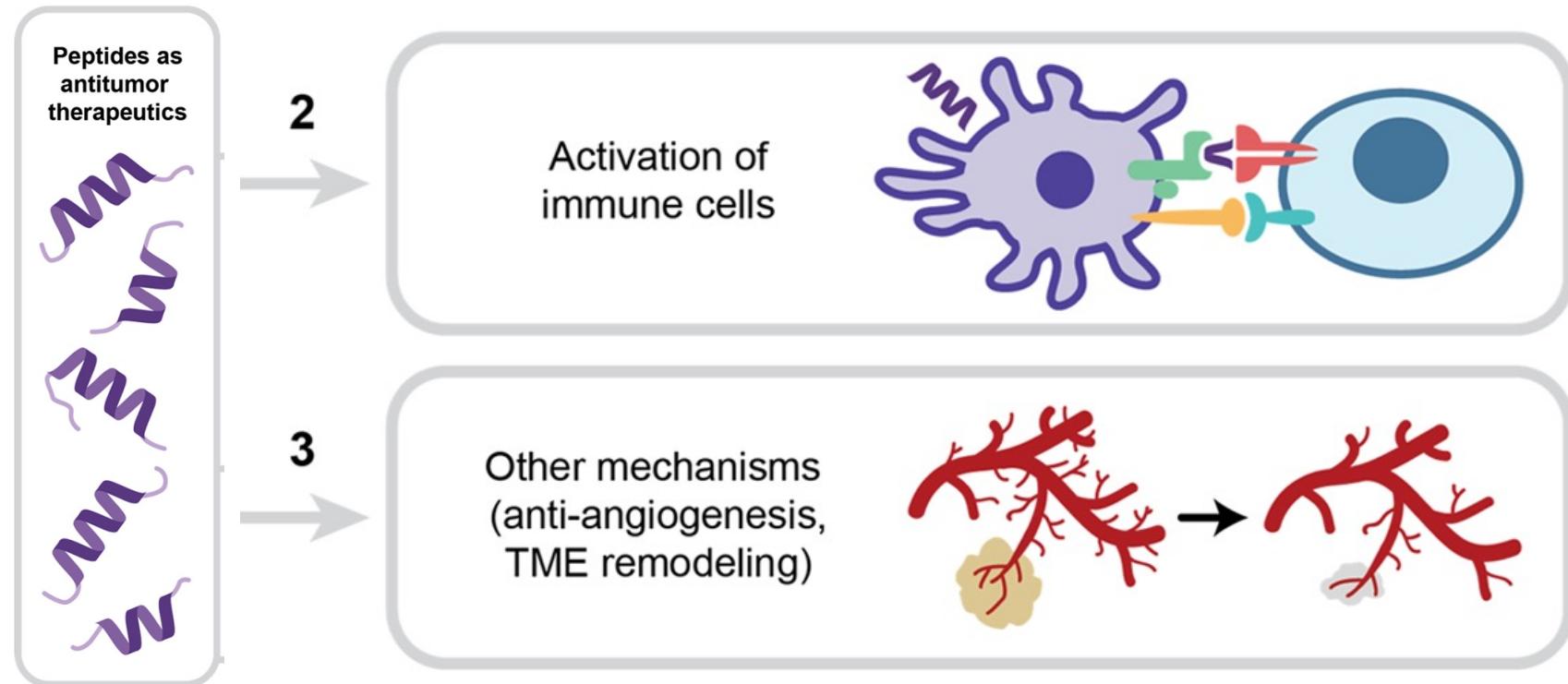
MAPK signaling pathways. (A) ERK1/2 pathway; (B) p38 α , β , δ , and γ pathways; (C) JNK 1, 2, and 3 pathways.



Schematic view of the cell cycle. Cell cycle progression is regulated by CDKs and their regulatory partner proteins, the cyclins, and CDK inhibitors.

1.1.2. Peptide Tumor Antigens

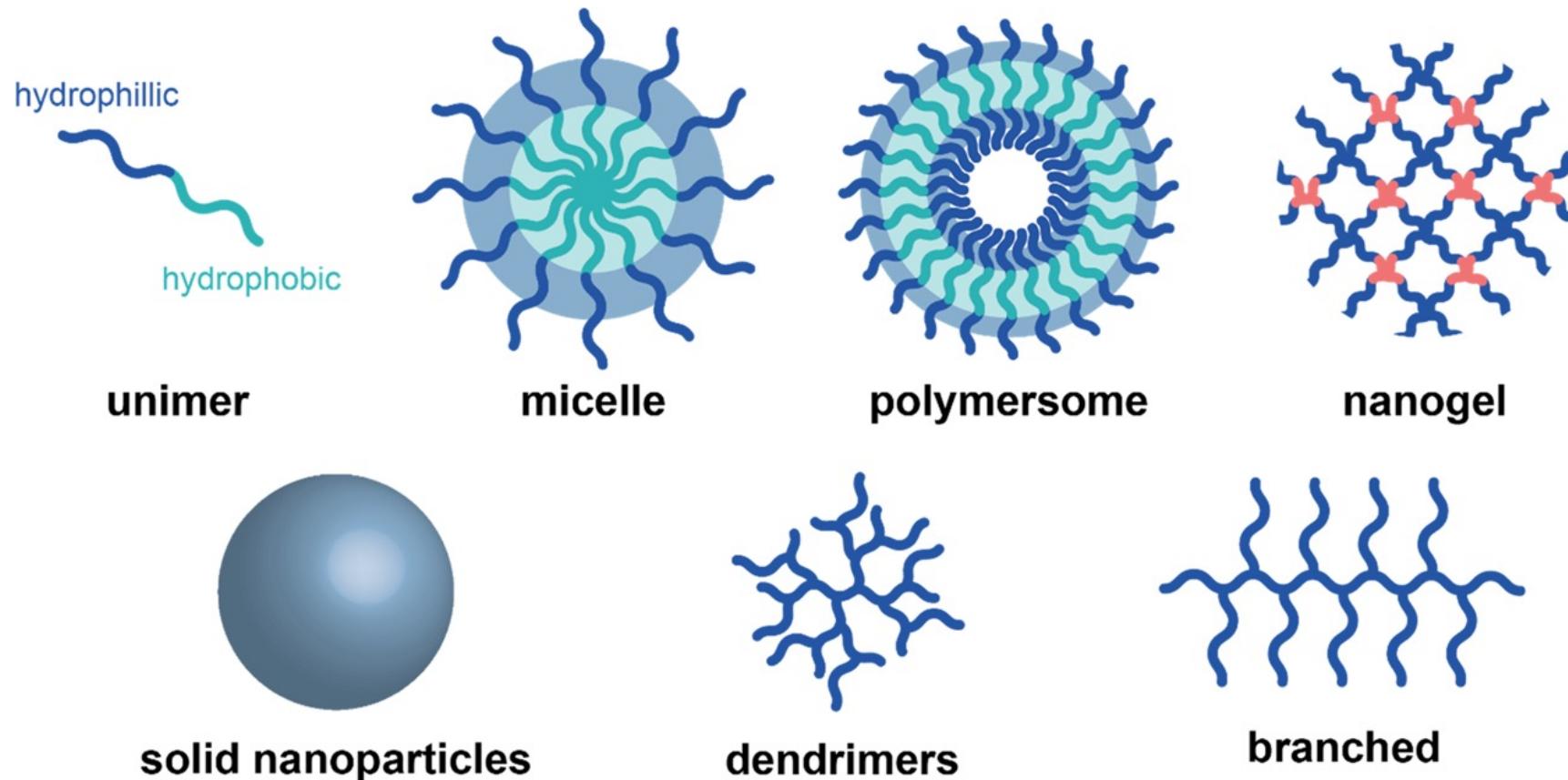
➤ Antitumor peptides with different mechanisms



- **Peptides can also serve as antigens in cancer vaccine formulations.** Cancer vaccines elicit tumor-specific responses from the patient's own immune system to treat tumors and/or provide sustained protection against recurrence.
- **Peptide antigens**, as chemically produced products, offer comparable storage stability, manufacturing ease, and low cost.
- **The target cells for peptide antigens are professional APCs, especially dendritic cells (DCs).** Because both cytotoxic and helper T cells are required for a full immune response, peptide antigen delivery systems must facilitate not only **DC targeting** but also **cytosolic delivery**.

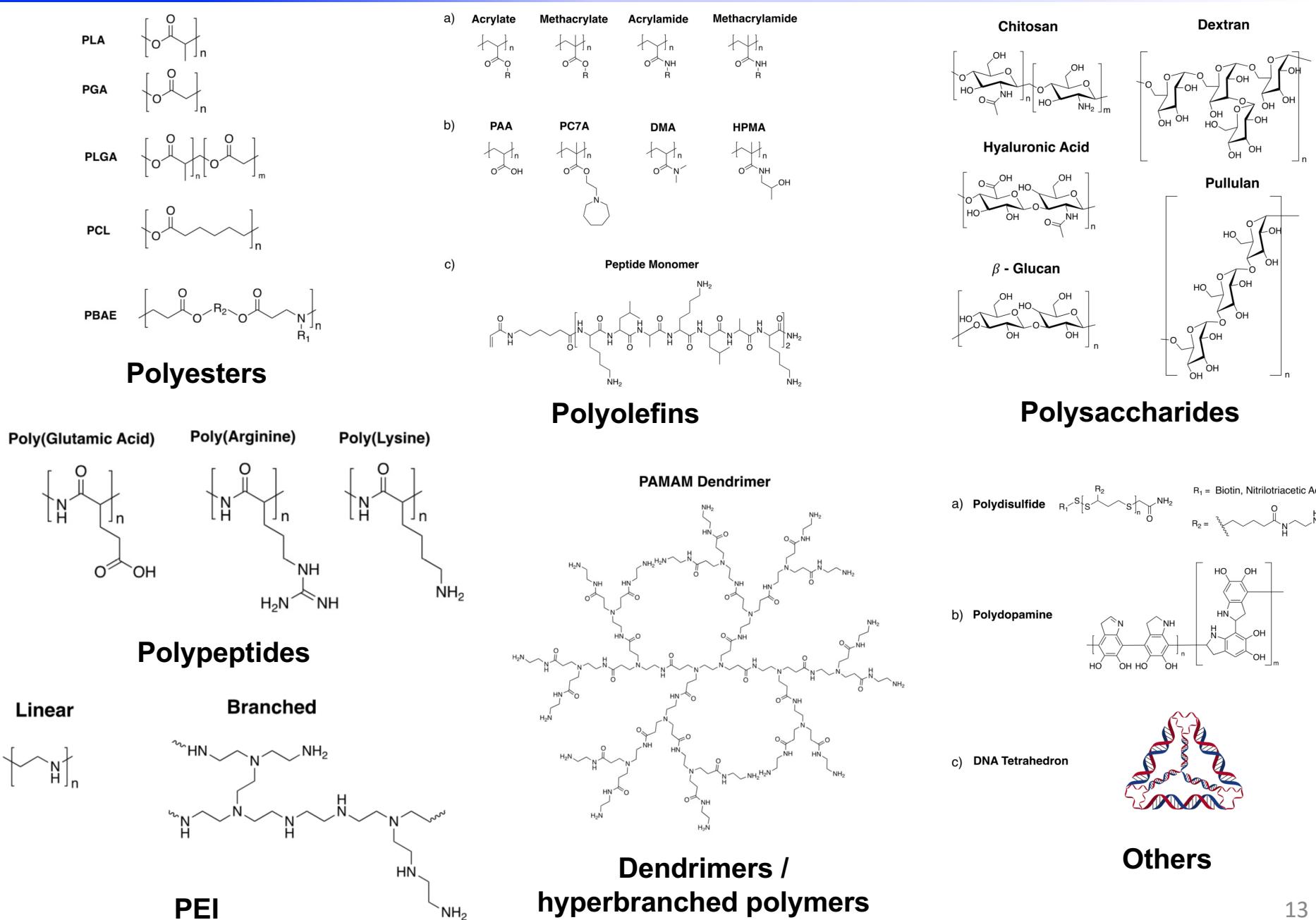
1.2. Polymers in Peptide Delivery

- Illustration of polymer architectures and self-assembled structures

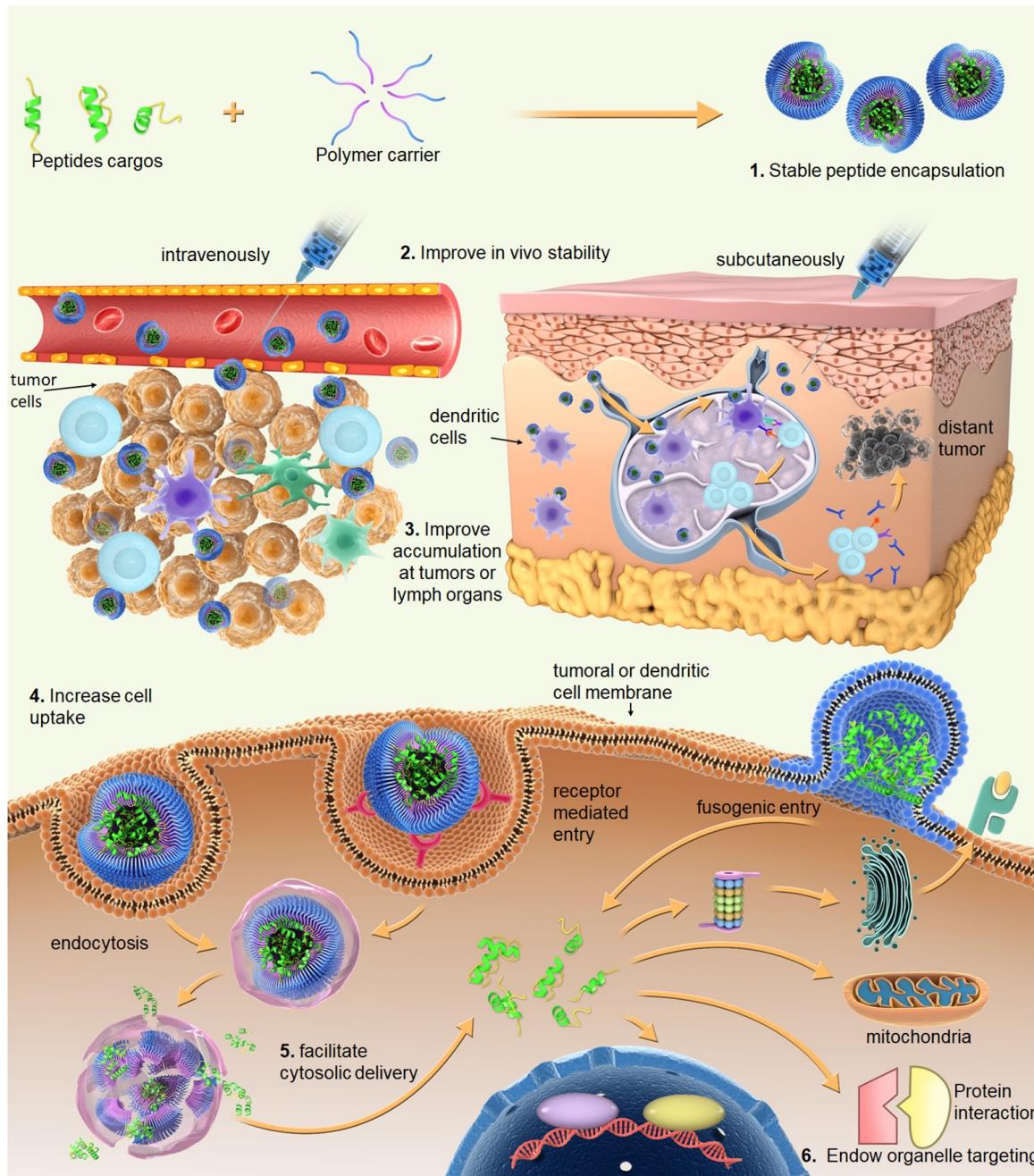


Nanomaterials are therefore **small enough to circulate in the bloodstream** but **large enough to avoid rapid renal clearance**. The same unique properties of nanomaterials have been applied for delivery of ACPs and peptide antigens.

1.2. Polymeric Carriers for Peptide Delivery



1.3. Peptide Delivery In Vitro or In Vivo



Successful delivery of anticancer agents after absorption into the bloodstream requires the following four sequential steps:

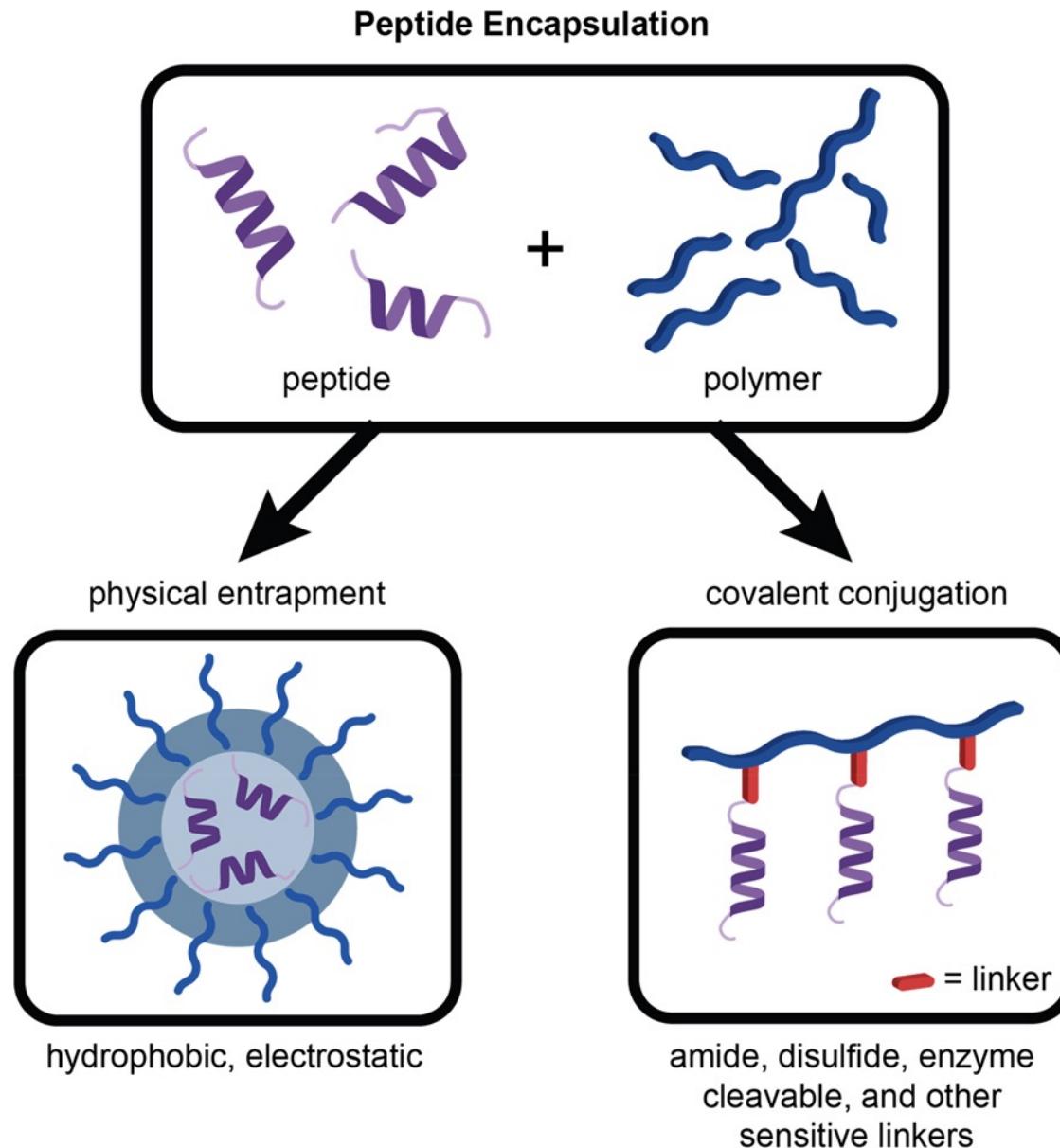
- (1) circulation in the blood compartment;
- (2) extravasation from blood vasculature and subsequent diffusion and penetration into the tumor tissue;
- (3) internalization by tumor cells;
- (4) trafficking of free drug to target intracellular sites.

For vaccine applications, intradermal delivery of peptide antigens also requires stable encapsulation to prevent degradation by interstitial proteases, enhance drainage to LNs, and facilitate cell uptake and presentation by APCs

Challenges in polymer-mediated in vivo and intracellular delivery of peptide therapeutics for cancer treatment.

1.4. Encapsulation of Peptides

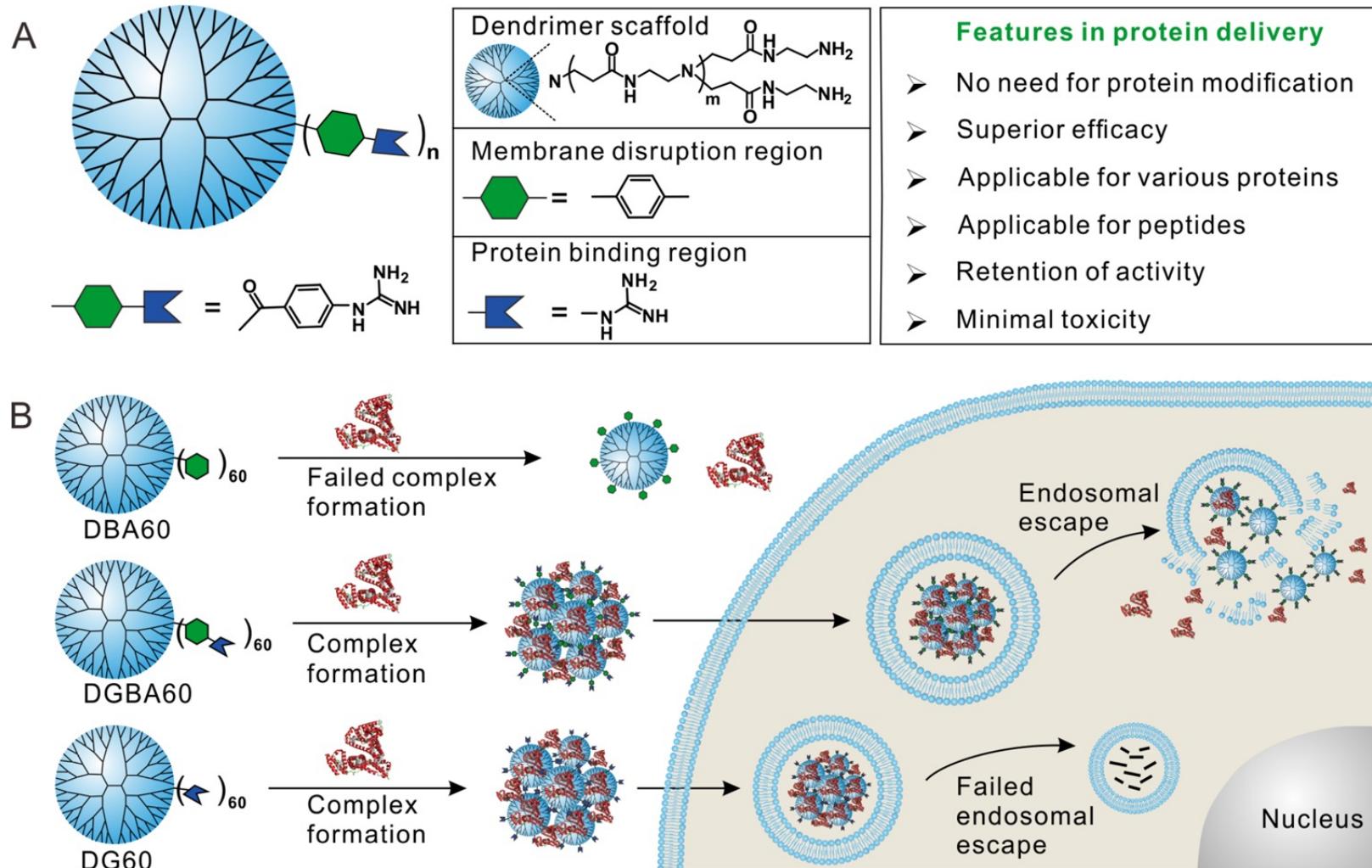
➤ Peptide encapsulation by polymers



- ✓ A well-designed polymeric carrier should enable high peptide loading efficiency and subsequent cargo release with temporal/spatial control.
- ✓ In general, peptide loading into polymeric nanocarriers is achieved through **physical entrapment** or 2) **covalent conjugation**.
- ✓ 1) **Physical entrapment:** Hydrophobic, electrostatic interactions and multiple interaction forces are the driving forces behind the formation of peptide-encapsulated NPs.
- ✓ 2) **Covalent conjugation:** Peptide cargos are commonly conjugated to the polymer chain through amide bonds, disulfide bond or thiol ether bond, etc.

1.4.1. Encapsulation by Electrostatic Interactions

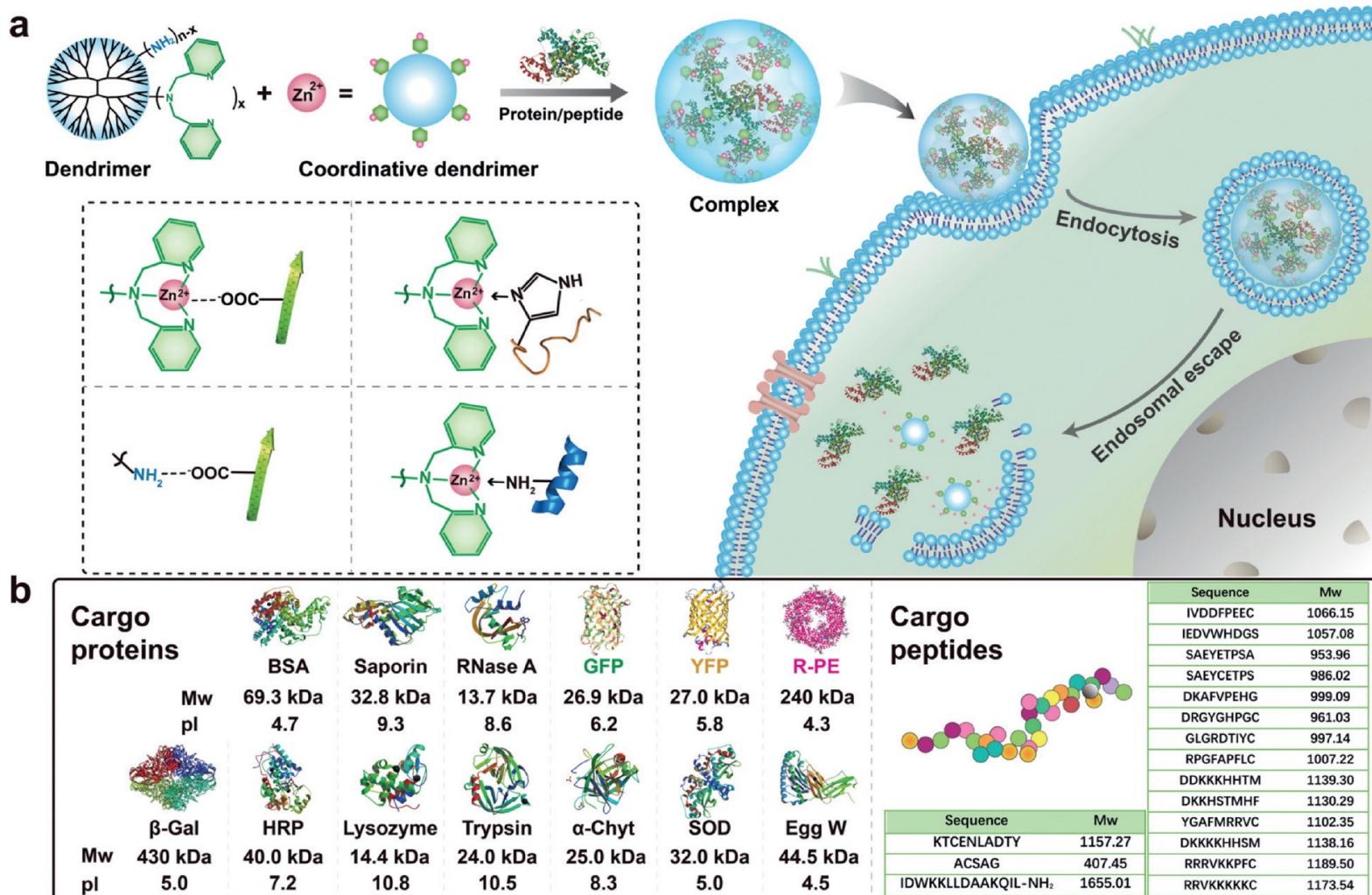
➤ Dendrimer scaffold for intracellular protein delivery



A dendrimer scaffold with a hydrophobic membrane-disruptive region and multivalent protein binding surface, conferring efficient protein/peptide binding through electrostatic interactions, endocytosis, and endosomal disruption. This platform efficiently delivered various bioactive proteins and anionic pro-apoptotic peptides into the cytosol of living cells.

1.4.2. Encapsulation by Ionic and Coordination Interactions

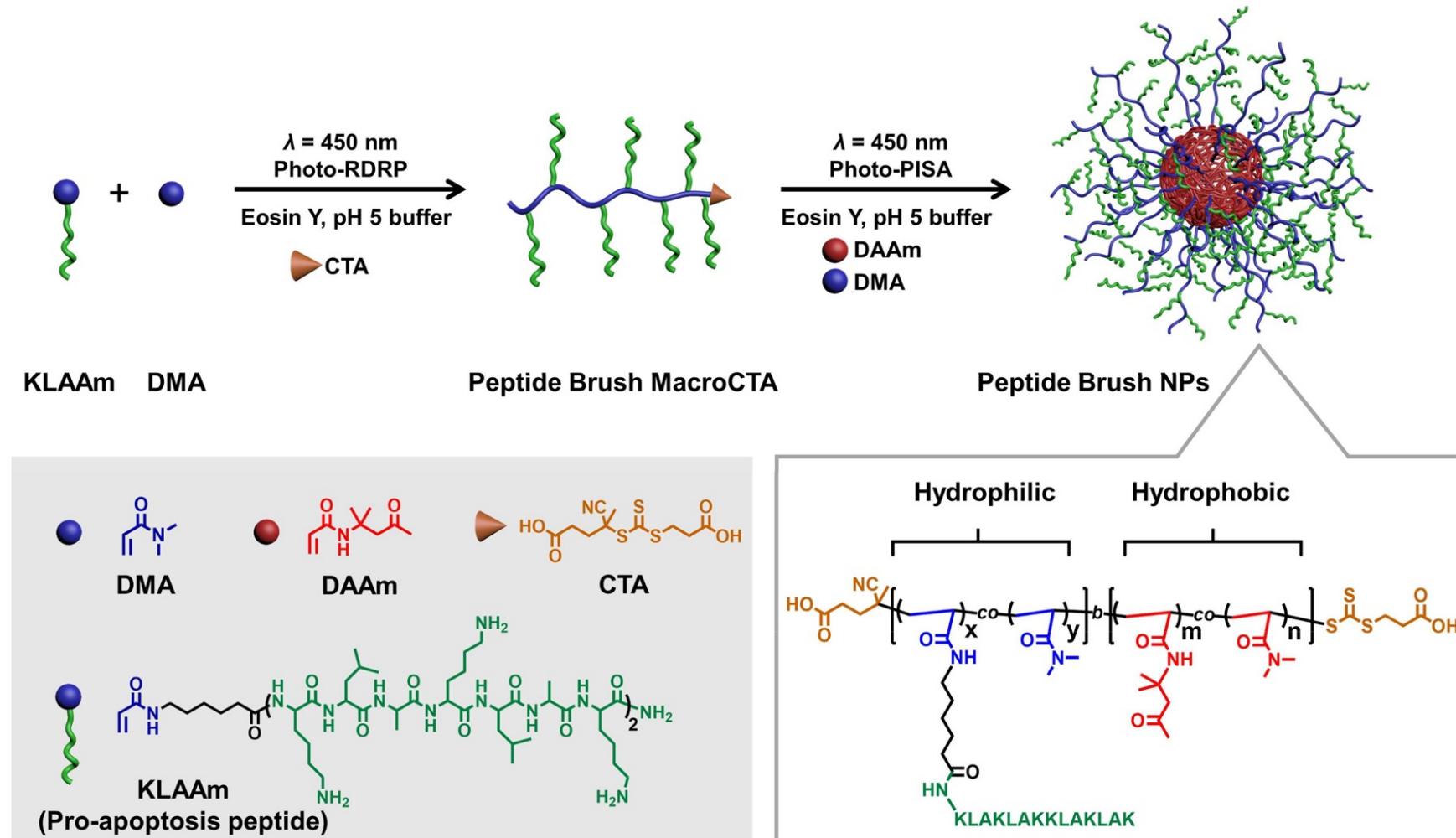
➤ Zn²⁺-modified dendrimer in cytosolic protein and peptide delivery



The coordinative dendrimer bound peptides or proteins through a combination of ionic and coordination interactions, which significantly increased the interactions between the polymer carrier and cargos. The best-performing polymer was capable of efficiently delivering 30 cargo¹⁷ proteins and peptides into the cytosol, while maintaining their bioactivity after intracellular release.

1.4.3. Covalent Conjugation by RAFT Copolymerization

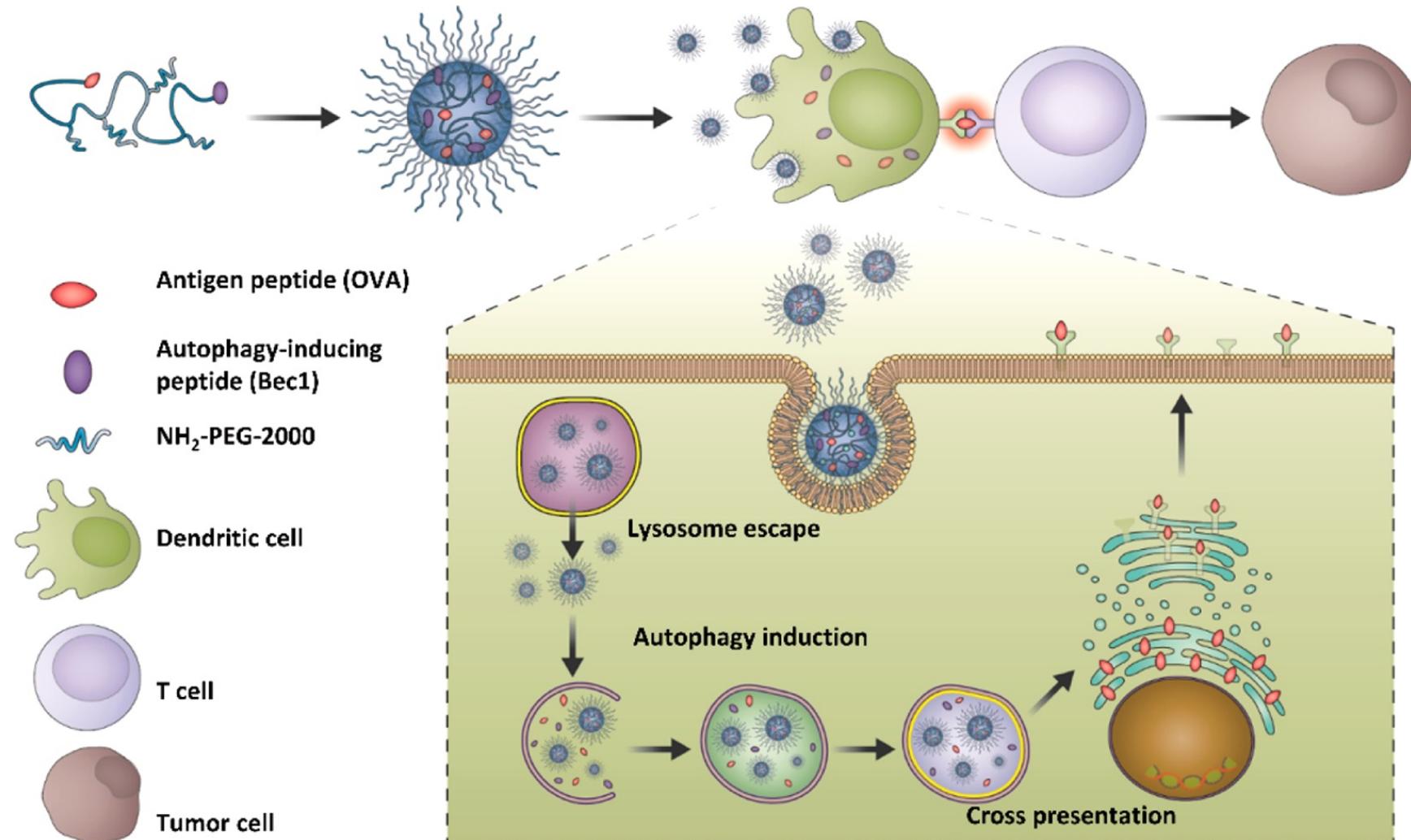
➤ One-pot approach synthesis of proapoptotic peptide brush polymer NPs



The **one-pot synthetic approach** yielded micellar NPs at high conc. and at scale, with tunable peptide loadings up to 48 wt%. The multivalent display of KLA peptide brushes significantly **enhanced cell uptake and penetration and proteolytic resistance** of the NPs, highlighting the importance of nanostructure for both stabilization and cell uptake.

Codelivery of Dual Peptide Therapeutics

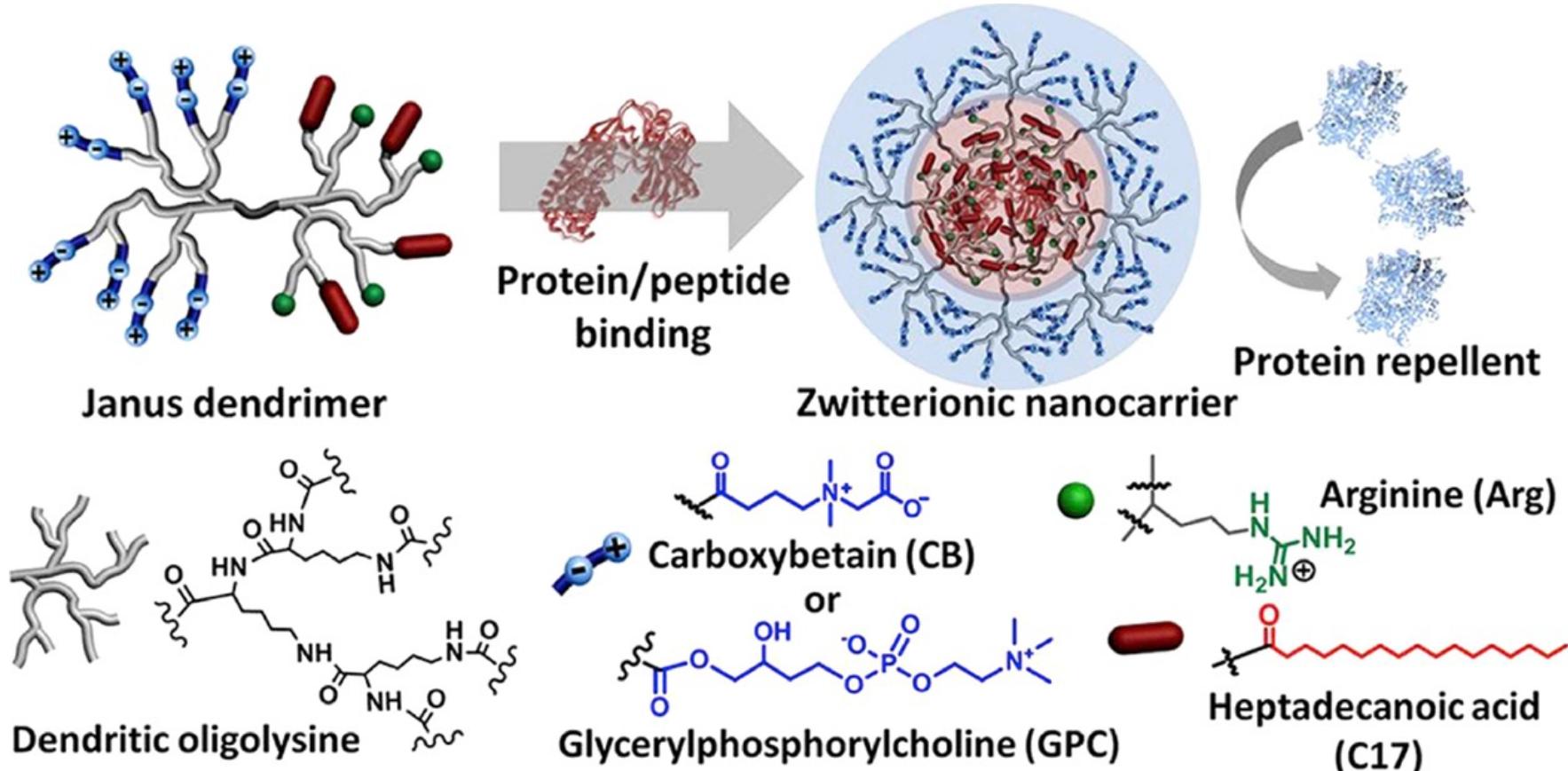
➤ Dual peptides loaded nanoactivators for cancer immune therapy



A poly(β -amino ester) (PBAE) polymer covalently conjugated to the antigen-peptide OVA₂₅₇₋₂₆₄ and the autophagy-inducing peptide beclin-1 on both terminals of the backbone. The polymers were internalized by DCs and induced autophagy, which facilitated antigen presentation and subsequent T cell activation.

Increase In Vivo Stability

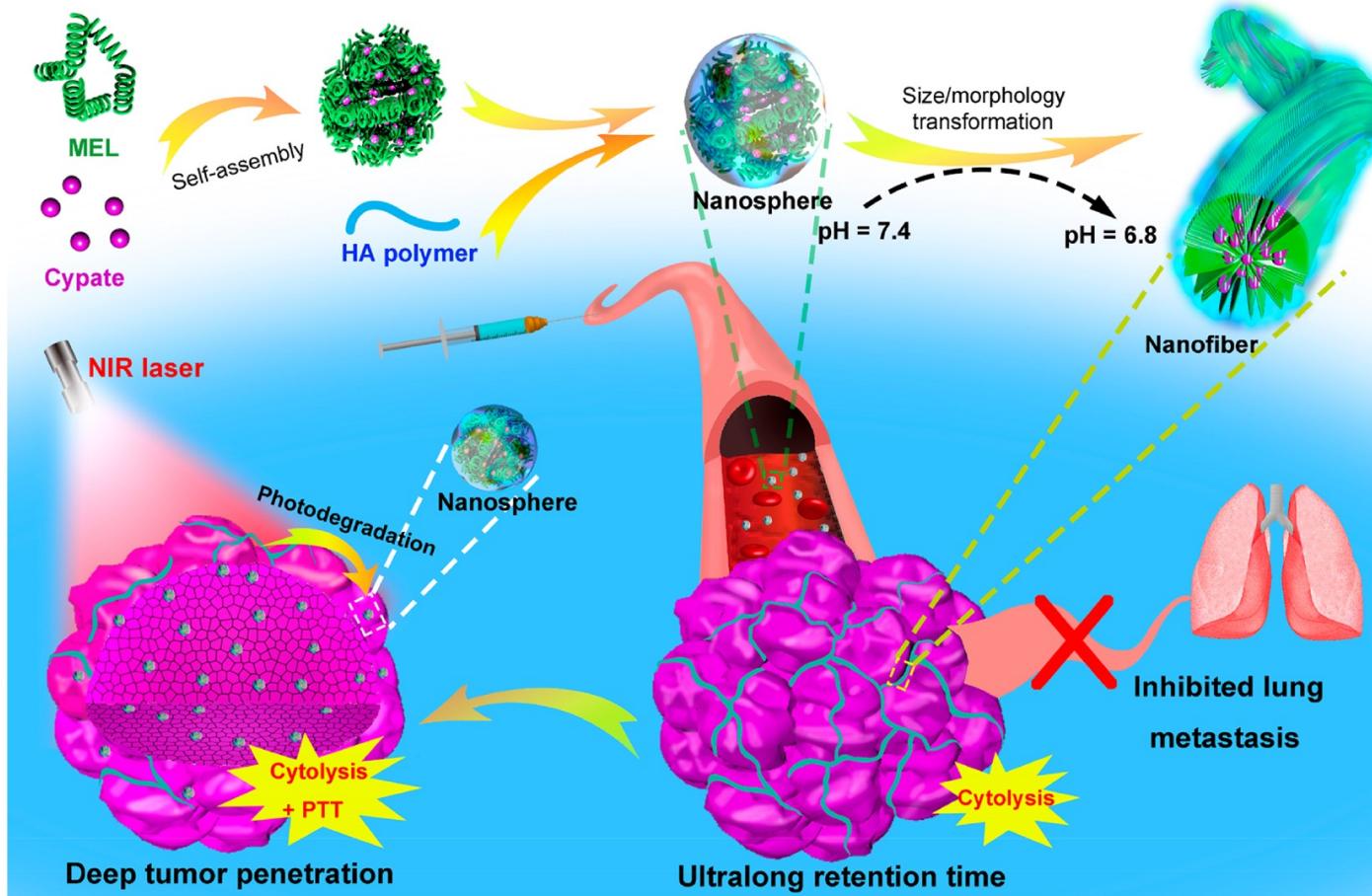
- Zwitterionic Janus dendrimer with the protein binding and antifouling features



The **in vivo stability of NPs can be improved by tuning the surface properties**. When most peptide–polymer NPs enter a biological environment (e.g., blood or extracellular matrix), the particle surface is rapidly coated by absorption of various biomolecules (typically proteins), leading to the formation of a “corona”. **PEGylation** is widely used to reduce nonspecific interactions between NPs and serum proteins, increasing in vivo stability and prolonging circulation.

Tumor Accumulation and Penetration

➤ The mechanism for tumor accumulation / penetration

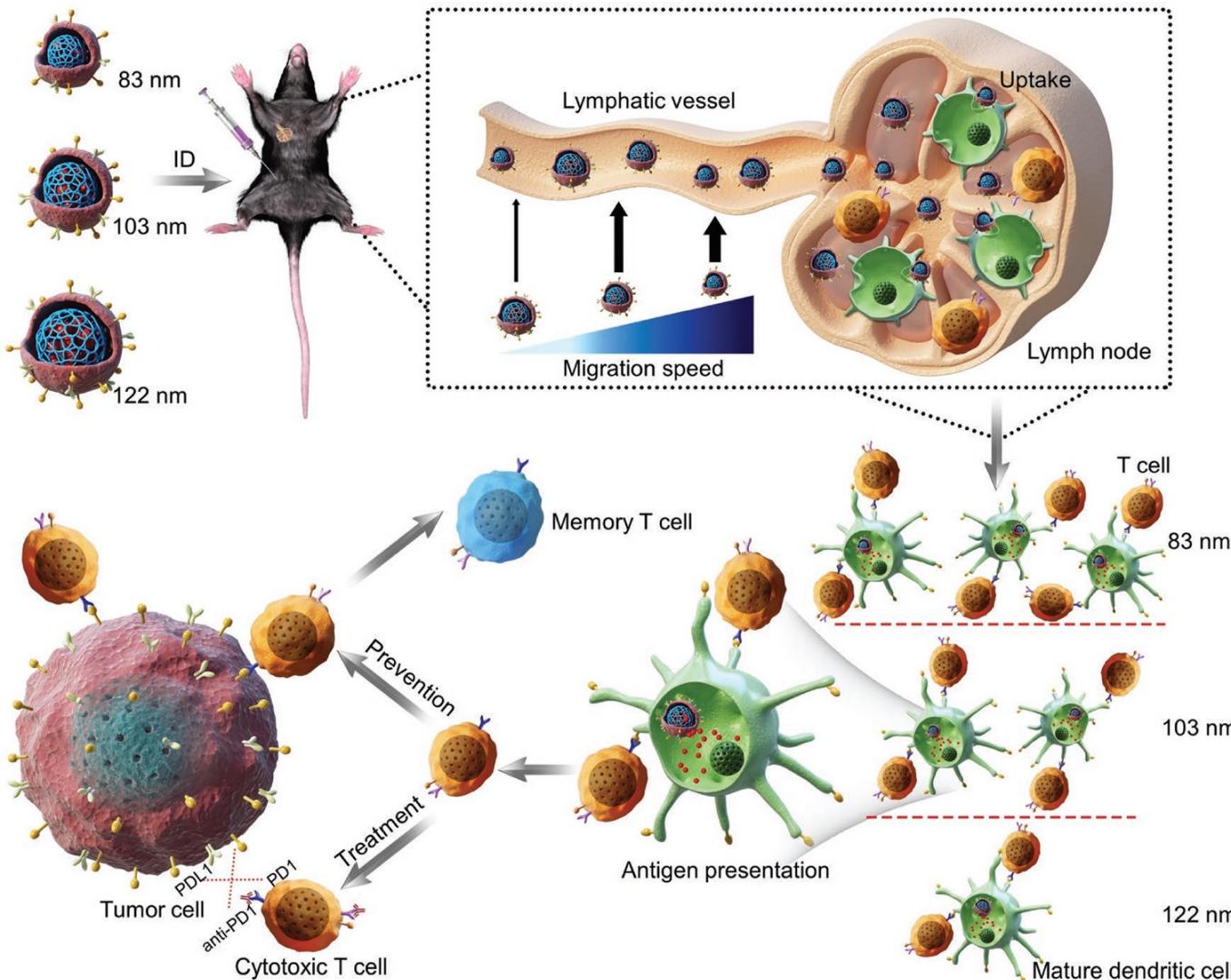


EPR principle is one of the key concepts for cancer nanotechnology; carriers with a hydrodynamic diameter larger than ~20 nm avoid rapid kidney filtration, and carriers smaller than the tumor-dependent interendothelial spacing can extravasate into the tumor tissue.

A nanocomplex of a **cytolytic peptide**, melittin; an **NIR-absorbing molecule**, cypate; and a **tumor-targeting polymer**, HA, to overcome the size paradox of nanomedicines. At pH 7.4, the complexes were negatively charged nanospheres (~50 nm), which were suitable for long- term systemic circulation. At the tumor site, the weakly acidic tumor microenvironment triggered an *in situ* transformation of the nanospheres to net-like nanofibers.

Lymph Node Accumulation

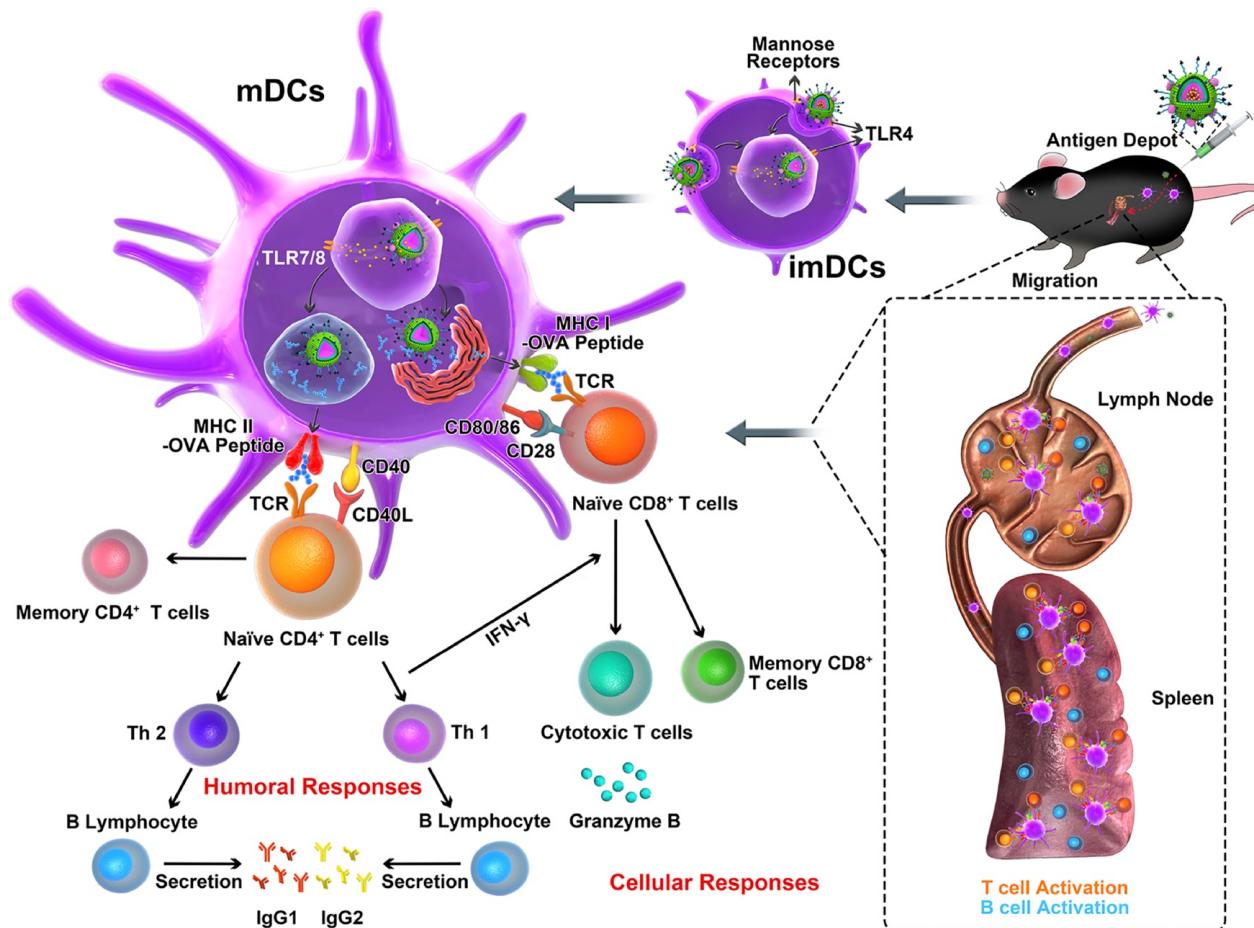
➤ Anticancer mechanism of mannose targeting NPs with varying sizes



- ✓ The target site for peptide vaccines are the dendritic cells in the lymph nodes, where tumor antigen presentation and resulting antitumor immunity is coordinated.
- ✓ The trafficking and cellular uptake of particles is largely dependent on physicochemical properties such as **size and charge**.
- ✓ Immunoadjuvant-loaded multiantigenic NPs MANPs / R837 with three different diameters (i.e., **83, 103, and 122 nm**) and utilized a cancer cell membrane coating as a source of multiple antigens onto the imiquimod R837-loaded PLGA NPs.

Cell Uptake

➤ Receptor-mediated cell uptake



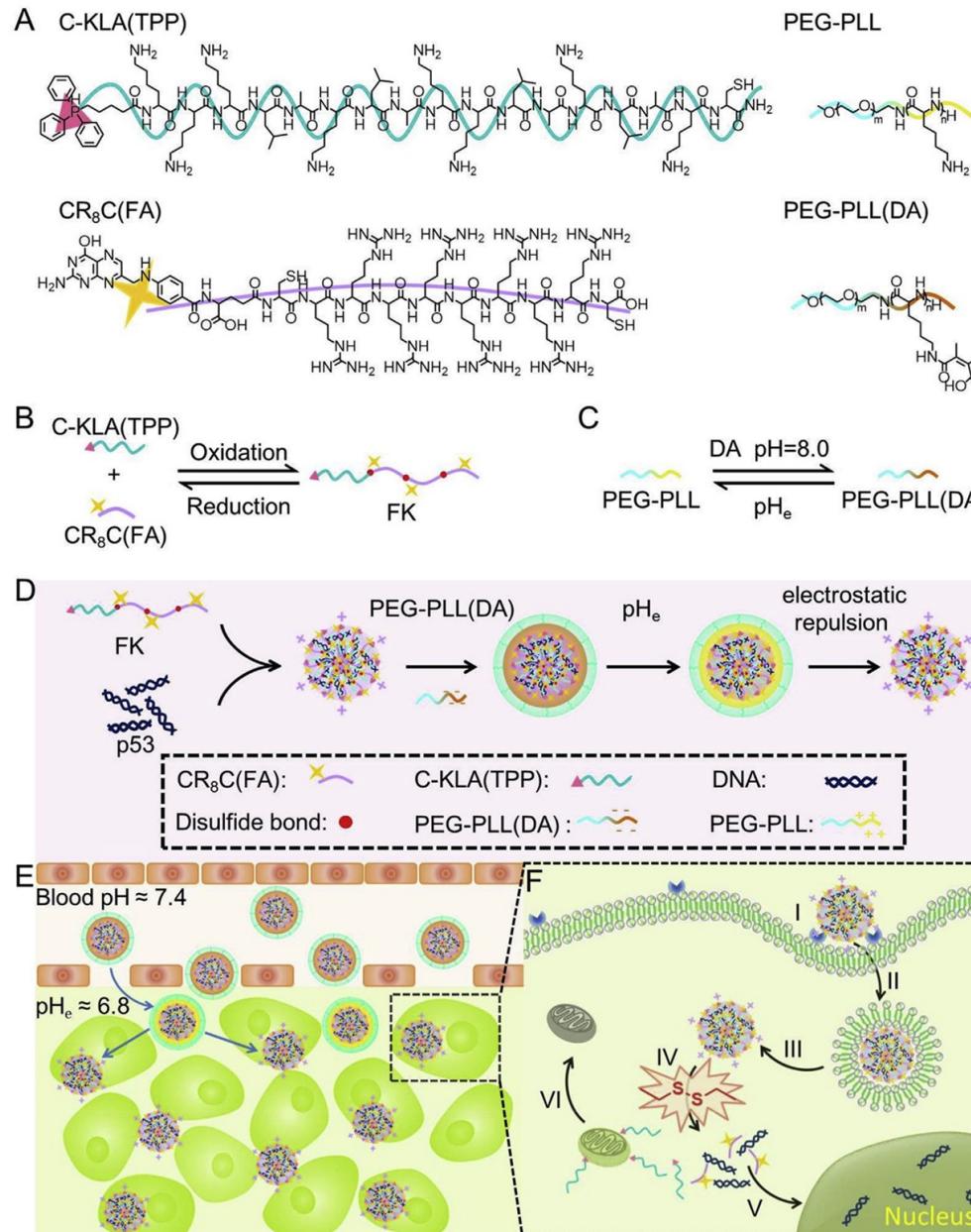
Peptide drugs or nanosized carriers are typically internalized into cells through **energy-mediated, vesicular transport**. Endocytosis of peptides or peptide-loaded carriers is induced by interaction with the cell surface either through **nonspecific charge interactions** or through **specific ligand-receptor interactions**.

Mannose-functionalized PLGA particles were loaded with cancer antigen peptides and two Toll-like receptor (TLR) agonists, which accumulated more in lymph nodes and were more effective in increasing survival and reducing tumor growth rate.

Targeted codelivery of an antigen and dual agonists by mannose targeting NPs.

Environment-Triggered Uptake

➤ Surface charge-switchable system for codelivery of proapoptotic peptide and plasmid

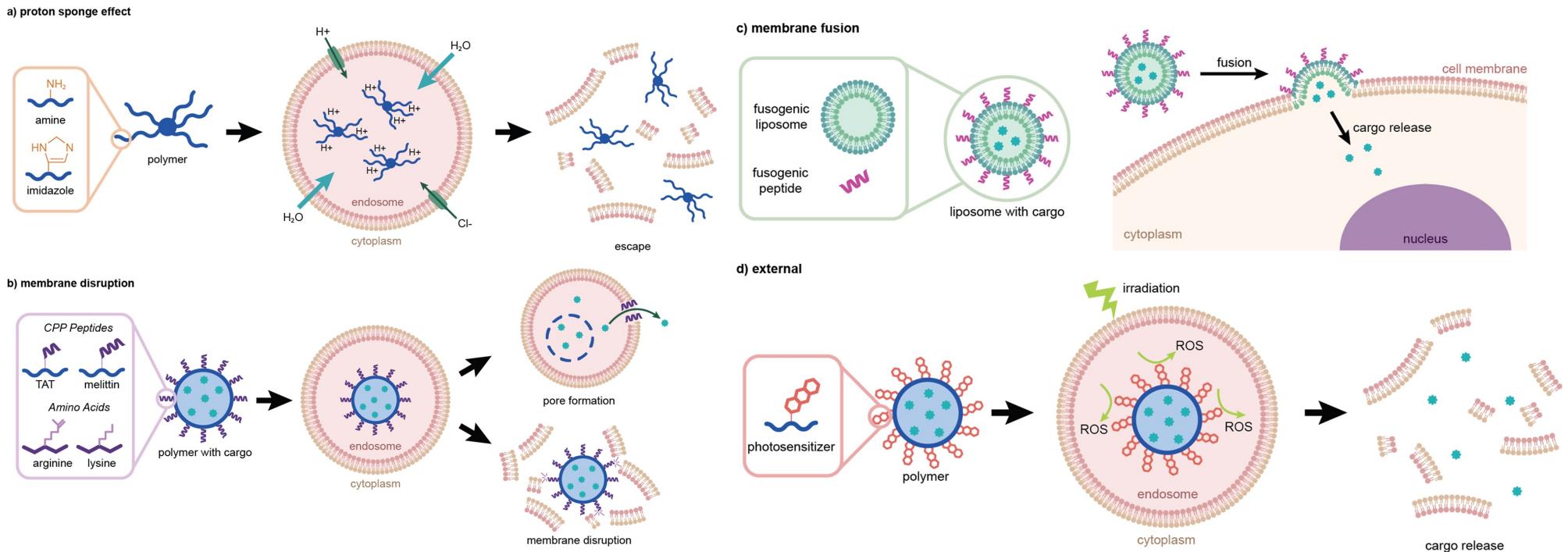


The **extracellular pH in solid tumors is often slightly acidic** ($\text{pH} 6.5\text{--}7$) because of the production of acidic metabolites and slightly hypoxic (1–2% lower O}_2 because of high oxygen demand by rapidly proliferating tumor cells.

A **surface charge-switchable system** for codelivery of proapoptotic peptide KLA and p53 plasmid. The charge-switchable PEG-shield (PEG-PLL(DA)) was used to coat the NPs by electrostatic interaction. At the physiological pH 7.4 in the bloodstream, PEG-PLL(DA) could extend the circulating time. After the accumulation at tumor sites, a **tumor-acidity-triggered charge switch** led to the detachment of PEG-PLL(DA) from the complex, resulting in **efficient tumor cell entry by folate-mediated uptake and electrostatic attraction**.

Strategies to Facilitate Cytosolic Delivery of Peptides

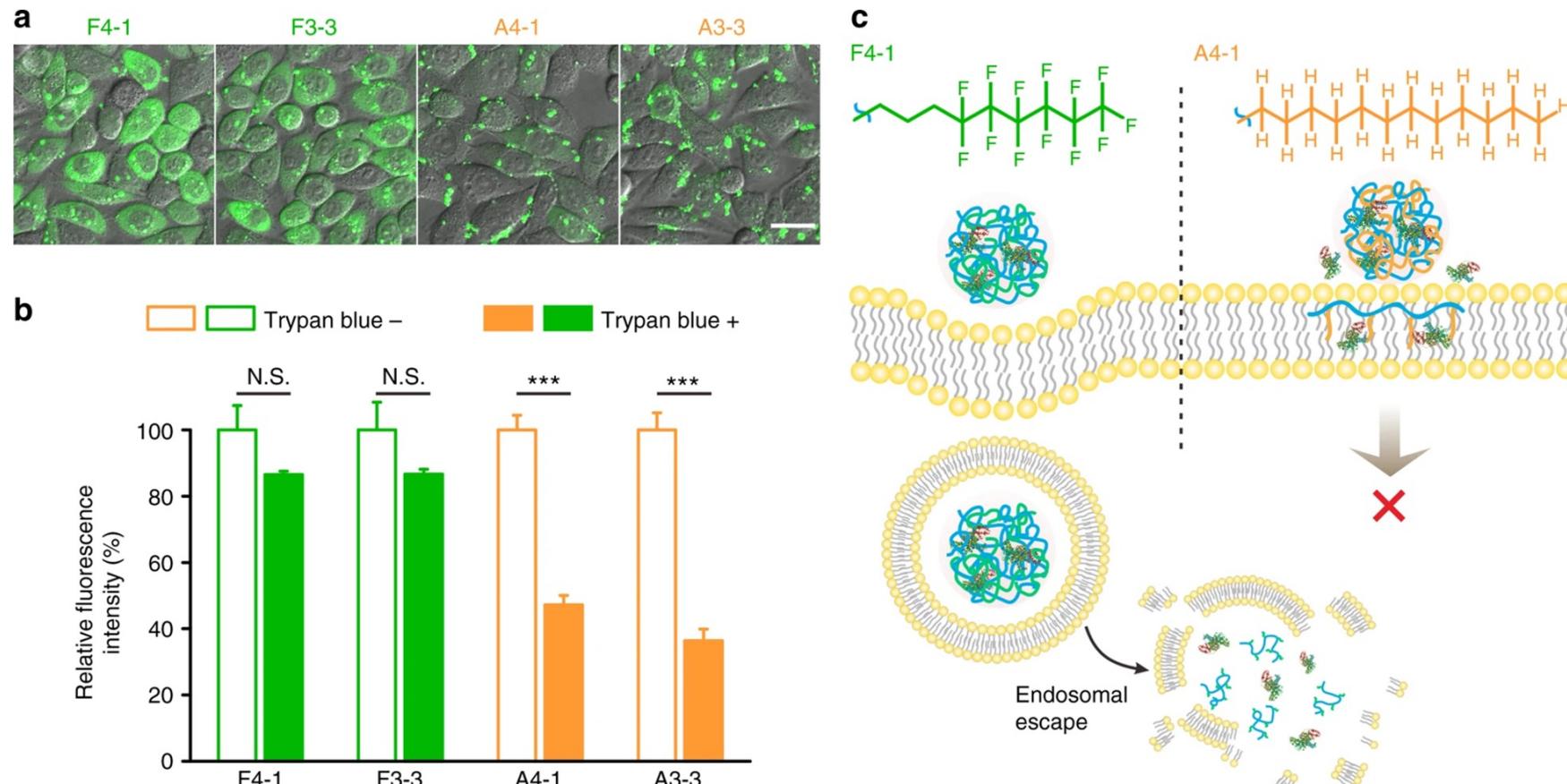
- **Mechanisms of endosomal escape:** four key strategies to facilitate endosomal escape for cytosolic cargo delivery: (1) the proton sponge effect, (2) pore formation, (3) membrane fusion, and (4) triggered response to external stimuli.



(a) Polymers can exploit the **proton sponge effect** through incorporation of chemical groups (e.g., amines and imidazoles) to trigger an influx of ions and water to burst the endosome. (b) Carriers can be equipped with peptides (e.g., CPPs) to **form pores in or disrupt the endosomal membrane**, facilitating cargo release into the cytosol. (c) While primarily employed by lipid-based carriers, **fusion with the cell membrane** can mediate cytosolic cargo delivery while bypassing the endosome. This can be achieved with fusogenic peptides or coatings. (d) **External stimuli**, such as light irradiation, can trigger endosomal disruption to release cargo into the cytosol.

Proton Sponge Effect

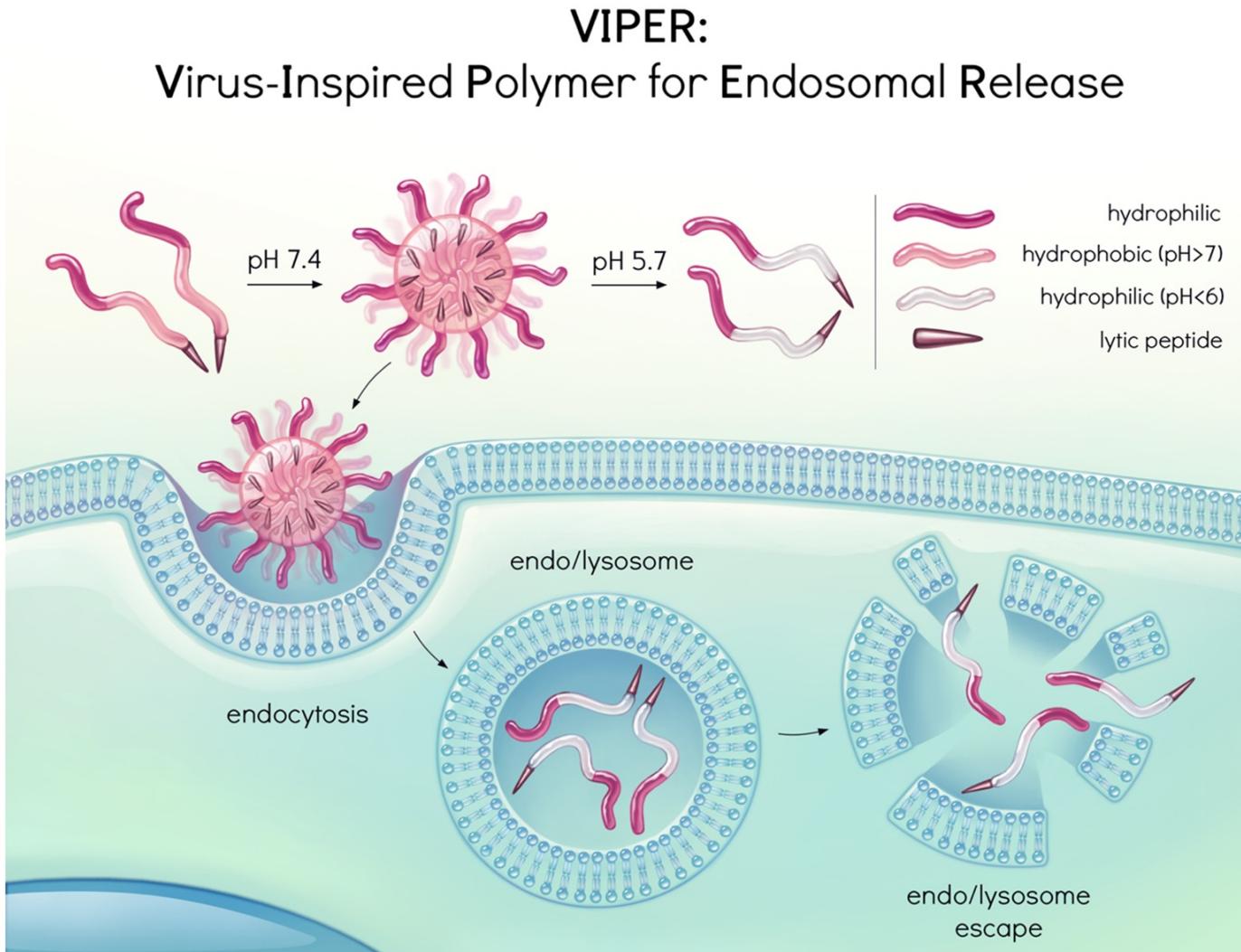
- The proton sponge effect hypothesizes that agents with high buffering capacity can induce endosomal rupture and subsequent release of entrapped components. Specifically, protonation of amine groups triggers an influx of protons and chloride ions, resulting in osmotic swelling and endosomal bursting.



Fluorination of polymers to mediate cytosolic protein delivery. PEI (with its high density of primary, secondary, and tertiary amines, demonstrating high buffering capacity and the proton sponge effect) was grafted with fluoroalkanes to facilitate protein/peptide encapsulation and endosomal escape. Longer fluoroalkyl chains and higher fluorination enhanced delivery.

Pore Formation

➤ CPP-induced pore formation

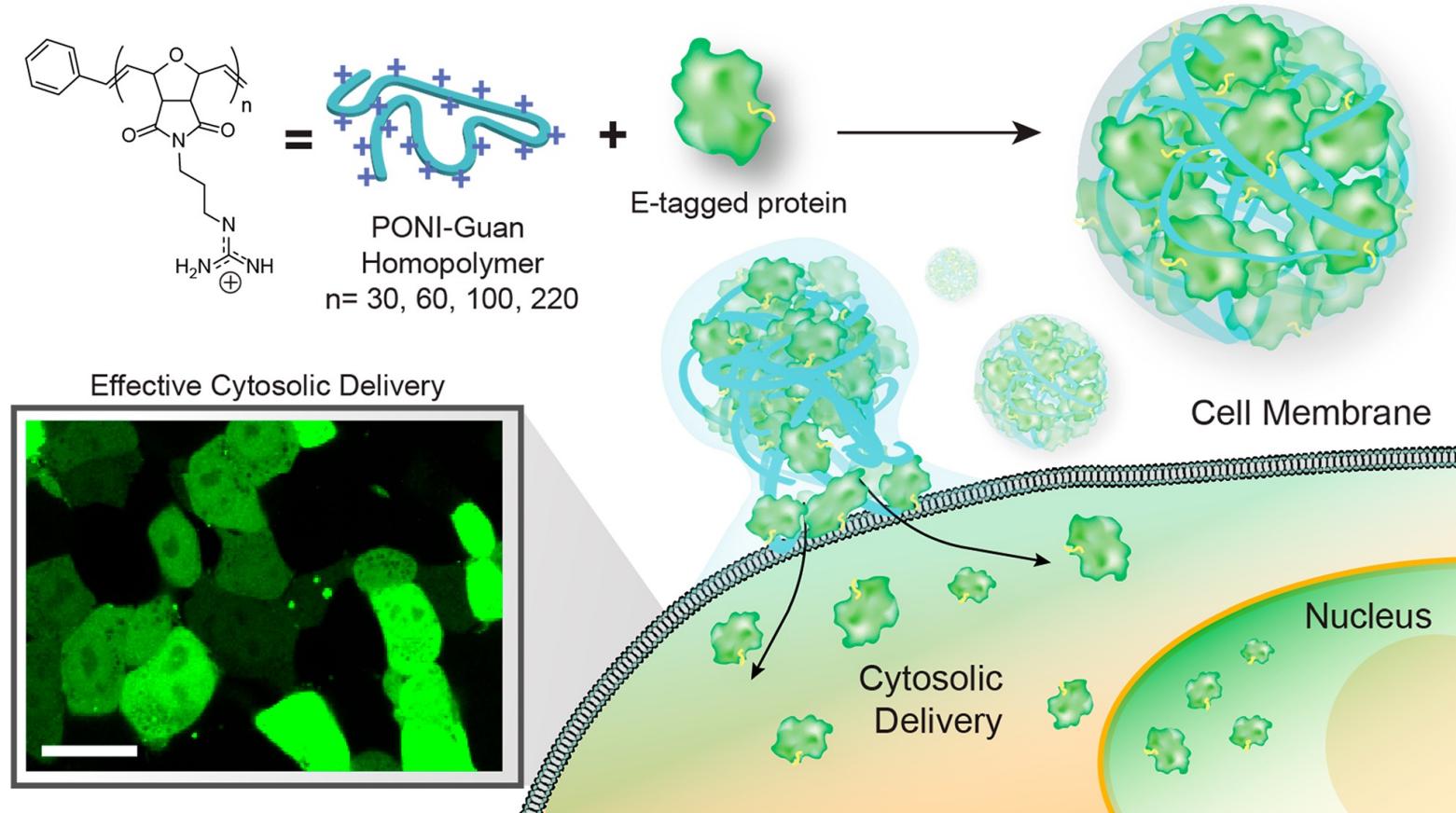


Pore formation occurs when the internal membrane tension that enlarges a pore is greater than the line tension that closes the pore.

Lytic peptides to mediate endosomal escape. **CPPs** (10–30 AA short peptides that are typically cationic, or amphipathic), such as **melittin**, are commonly employed to disrupt the endosomal membrane to facilitate cytosolic cargo delivery. In the VIPER platform, **melittin was conjugated to a pH-triggered polymer** which shielded the lytic peptide at physiological pH but exposed it at endosomal pH.

Cell Membrane Fusion

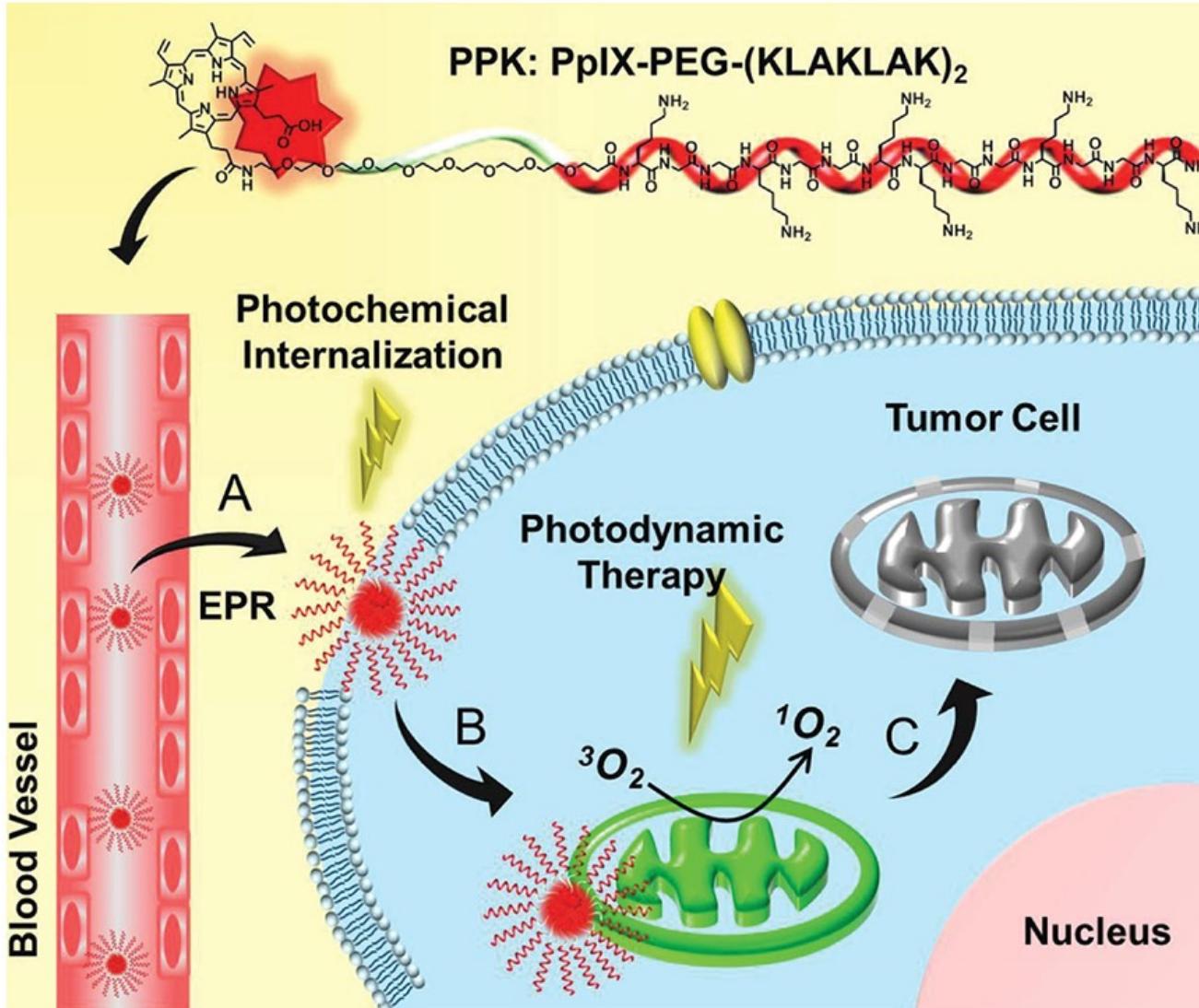
- Fusogenic peptides facilitate cytosolic delivery



A library of **guanidium**-functionalized poly-(oxanorborneneimide) (PONI) homopolymers to determine optimal molecular weight (8k - 60k) for protein delivery. The highest molecular weight PONI facilitated the highest (~90%) cytosolic and nuclear delivery efficiency. **Cargo delivery occurred within ~40 s of PPNC incubation with cells, indicating that delivery undergoes membrane fusion rather than an endocytosis-dependent pathway.** Cellular treatment with inhibitors of endocytosis had negligible effect on cargo delivery; only a cholesterol-depletion agent interfered with delivery.

External Stimuli

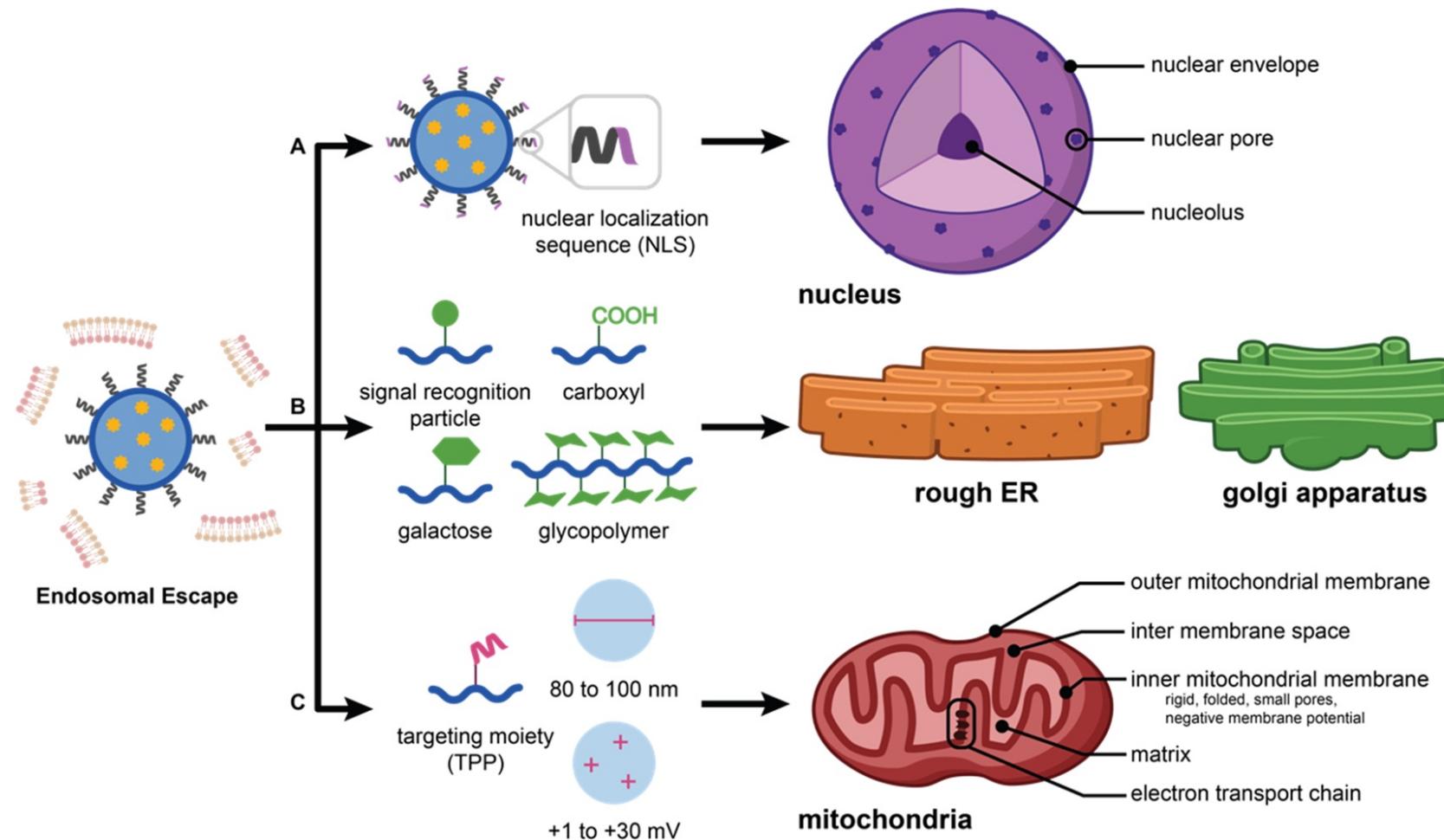
- External stimuli, particularly, light-activation of photo-sensitizers (to generate reactive oxygen species, ROS) to burst endosomes through oxidation



A delivery system to target **cellular mitochondria** for photosensitizer and peptide delivery. This system (PPK) comprised the photosensitizer protoporphyrin IX (PpIX) and the pro-apoptotic peptide (KLAKLAK)₂, linked via a short PEG linker. The authors confirmed ROS generation under light irradiation, which increased cellular internalization of PPK, facilitating mitochondrial targeting and disruption. The combinatorial ROS generation and cytotoxic peptide delivery translated to robust tumor inhibition in an H22 hepatic carcinoma tumor model.

Organelle-Specific Targeting

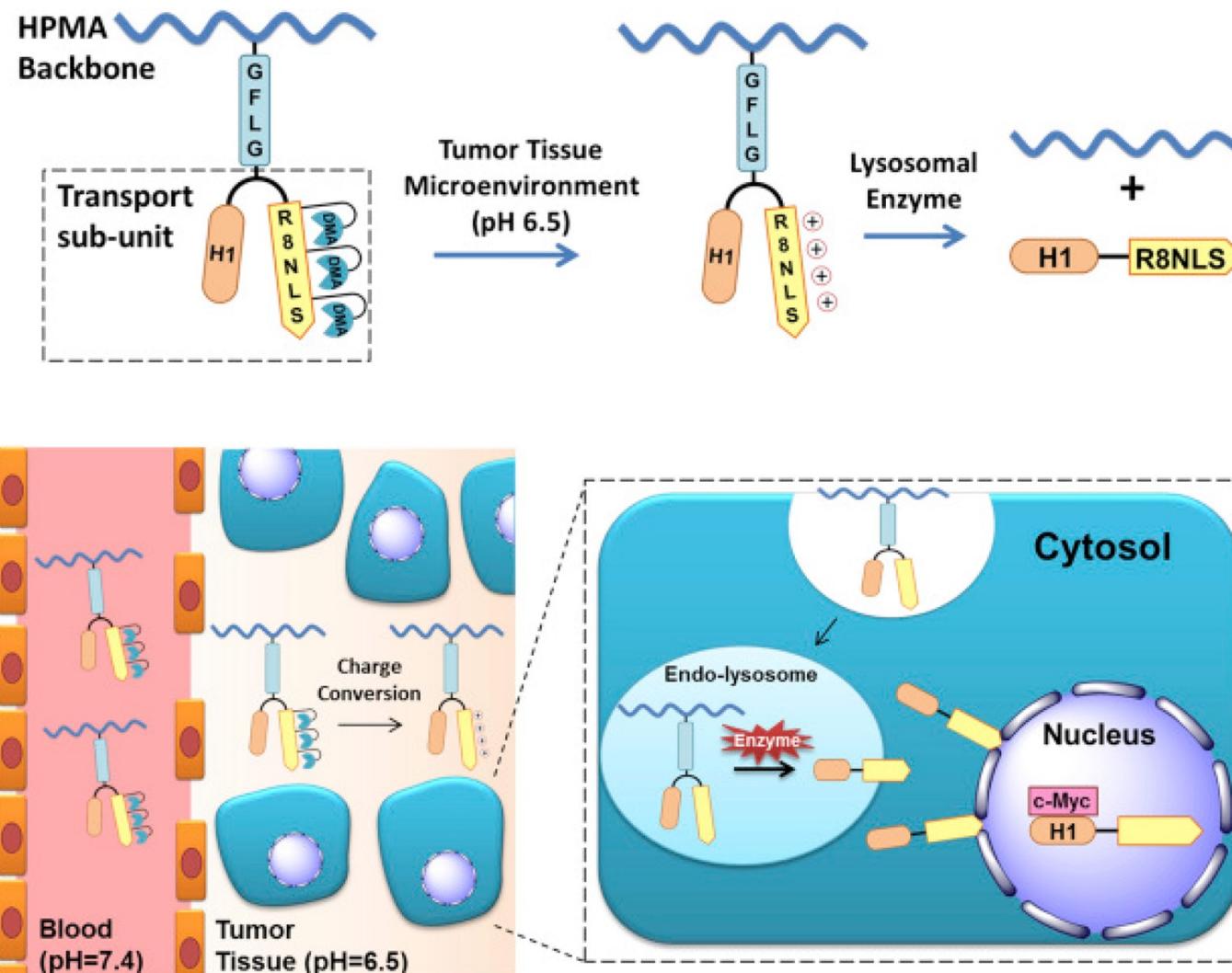
- Intracellular organelle targeting is often the ultimate goal to exert therapeutic efficacy, following cell entry and endosomal escape.



(A) **NLS peptides** can guide NPs to the nucleus for cargo delivery. (B) Incorporation of different chemical moieties i.e. **signal recognition particles**, can facilitate Golgi apparatus or ER localization. (C) **Varying nanoparticle size or charge** can permit passive targeting of mitochondria. Another strategy is to incorporate targeting moieties, such as **TPP or KLA peptides**.

Nuclear targeting

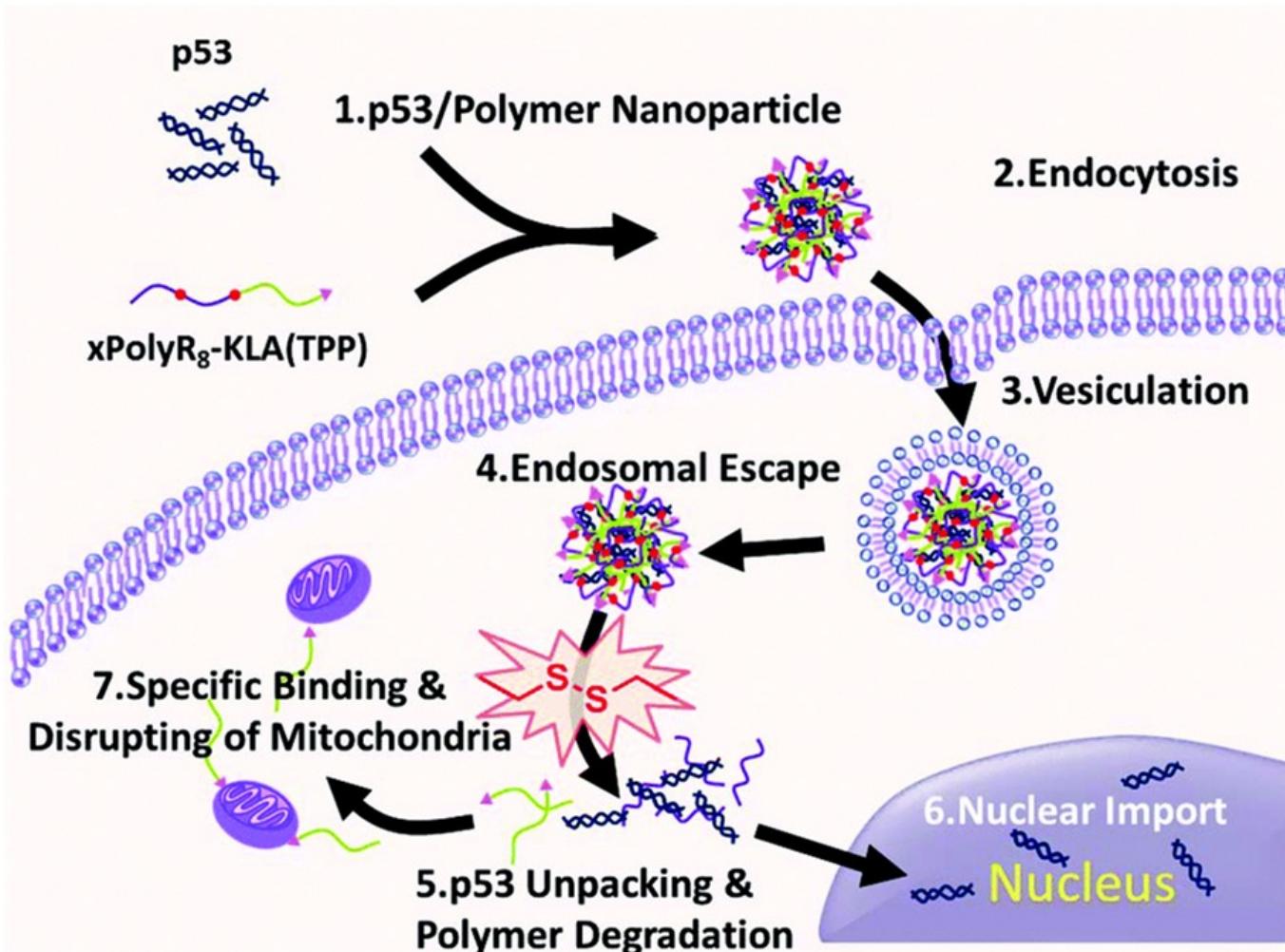
- Targeting the nucleus is challenging. NPC has pores that allow passive transport of molecules smaller than 30–50 kDa, while larger molecules require energy-dependent transport.



- ✓ Nuclear targeting of pH-responsive carriers via NLS and CPP, R8. The targeting CPP R8 and drug peptide H1 were conjugated to an HPMA backbone via a degradable linker.
- ✓ R8 was concealed with DMA at pH 7.4 but was exposed at mildly acidic tumor pH to enhance tumor penetration. Following cellular internalization, R8 mediated endosomal escape and nuclear targeting.
- ✓ pH-sensitive nuclear transport was confirmed in cells and tumor spheroids, and significant tumor inhibition was demonstrated in murine HeLa tumors.

Mitochondrial Targeting

- Mitochondrial targeting is complicated by the outer and inner mitochondrial membranes (OMM and IMM), intermembrane space, and matrix.

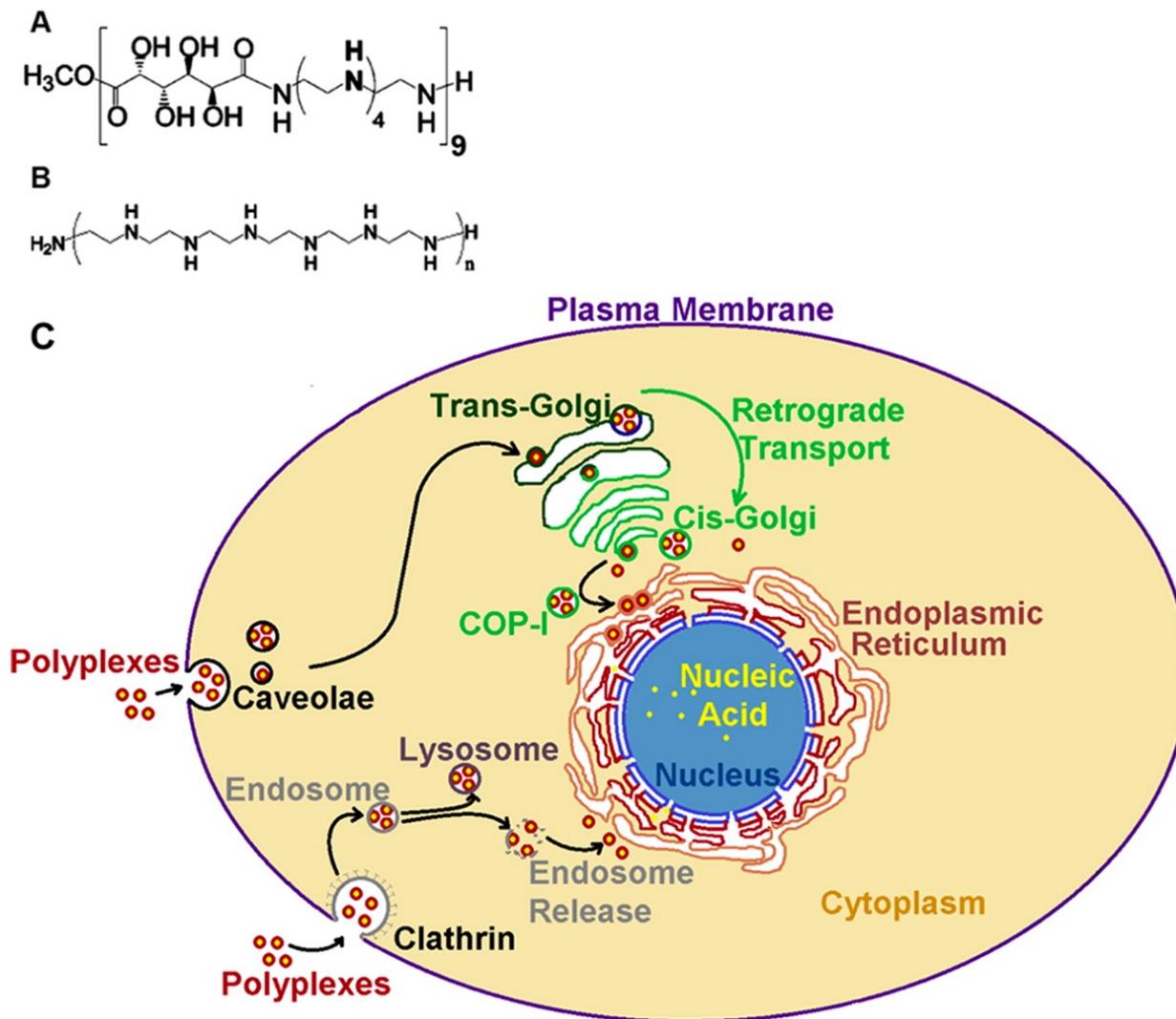


The most widely utilized mitochondrial targeting moiety is **triphenylphosphonium (TPP)**, a mitochondrion-penetrating peptide, as its hydrophobic and cationic properties.

Delivery of (KLAKLAK)₂ peptide and p53 plasmid to cancer cells utilizing **charge-switchable polypeptide complexes**. At physiological pH, this system shielded the positively charged polypeptide–plasmid complexes. At acidic tumor pH, the triggered charge switch shed the outer layer in order to expose folate, facilitating folate-mediated uptake. Upon cellular uptake, intracellular glutathione liberated the p53 plasmid and TPP-pro-apoptotic peptide for subsequent mitochondrial targeting and disruption.

Golgi Apparatus and ER Targeting

- Targeting to the ER can be facilitated via a signal peptide sequence (11–27 amino acids), combining with a signal recognition particle (SRP).



- ✓ Polyplexes formed with Glycofect exploited an **active transport pathway** via the GA and ER, resulting in more sustained gene delivery than its PEI counterpart.
- ✓ This was attributed to Glycofect's lower capacity to disrupt acidic vesicles, which **enabled routing through native active intracellular routes, slower gene delivery, and lower toxicity**.

Intracellular trafficking for nucleic acid delivery. (A) Glycofect and (B) linear PEI are commonly used polymeric, nonviral nucleic acid delivery vehicles. (C) While linear PEI appears to localize in acidic endocytic vesicles, Glycofect is trafficked through the Golgi and ER.