CH-413 Nanobiotechnology

Nucleic acid delivery

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May 8, 2025

A few words about proposal writing

The goal of writing a grant proposal is to **advance science and humanity.** You're asking for **money** to do that. To succeed, you must demonstrate that you've identified a **meaningful, unanswered question**, and that you have a **clear, feasible plan** to address it in a way that contributes to scientific knowledge or solves a real-world problem.

- **Structure** is super important, it should be easy to find information (think about evaluator's check list)
- Sell your project, especially in the abstract (first sentence!!), state of research and impact sections. **Why does it matter, why should we give money to you?**
- Structure your research plan with aims 1-3
- Make it clear what is known and where you propose something new, **what is the gap in the research?**

The evaluator's check list

Evaluator's Checklist – SNSF Spark Grant-Inspired Assessment

1. Novelty / Unconventionality of the Proposed Research Project (20%)

- ☐ Is the research idea distinct from established work in the field?
- ☐ Does the project address a topic with little prior literature or ongoing work?
- ☐ Is the methodological or conceptual approach clearly unconventional or original?

Score (0–20): ____

Comments:

_____

2. Scientific Quality of the Project (30%)

- ☐ Is the research plan logically structured and scientifically sound?
- ☐ Is the proposed methodology appropriate for the research goals?
- ☐ Is the project feasible within the proposed timeline and resources?
- ☐ Are the hypotheses or research questions clearly stated and testable?

Score (0–30): ____

Comments:

See online

Follow the structure from the guidelines

Title of the Project

1. Project Summary (1 page, DIN-A4 format)

- Provide a concise overview of the research question and objectives.
- Explain the significance of the proposed work.

2. Project Plan (3 pages total)

2.1 State of Research in the Field

- Describe the current state of research in the relevant area.
- Explain the novel and unconventional nature of the project.
- Justify how the proposed research is distinct from existing work and not a continuation of prior studies.

2.2 Detailed Description of Goals, Methods, Approach, Expected Results, and Potential Risks

- Clearly define the objectives and hypotheses.
- Describe the methodology and experimental design.
- Provide details on expected results and how they will be interpreted.
- Discuss potential challenges and risks, along with mitigation strategies.

2.3 Potential Impact of the Research

- Explain how the project could contribute to the field.
- Discuss broader implications and possible applications.

3. Bibliography (No page limit)

- Cite relevant literature appropriately using the reference format of the American Chemical Society (ACS).

1. Structure of project summary (1 page, DIN-A4 format)

To structure your project summary, you can follow this recipe:

1. Background/problem (1-2 sentences)
2. Goal/hypothesis (1 sentence)
3. Innovation/novelty (2-3 sentences)
4. Approach/methods (3-4 sentences)
5. Impact and outcomes (1-2 sentences)

1. Example project summary

Viral capsids offer a compelling blueprint for RNA delivery vehicles, but their application is hindered by poor solubility, low production yields, and limited tunability. A key barrier is the intrinsic aggregation of many capsid proteins when expressed recombinantly, making large-scale production and therapeutic translation challenging.

This project aims to engineer soluble, self-assembling capsid analogs fused to solubility-enhancing tags to enable efficient mRNA packaging and protection. Our hypothesis is that solubility-optimized capsid variants can retain RNA-binding and assembly functions while achieving higher expression and improved biochemical tractability.

The innovation lies in combining principles from structural virology, protein engineering, and synthetic delivery systems to produce non-viral, modular mRNA carriers. By fusing known capsid proteins to rationally chosen solubility tags, we aim to overcome expression bottlenecks while preserving or restoring the capacity for RNA encapsidation. Importantly, we will evaluate these constructs using quantitative in vitro assays for particle formation, mRNA protection, and structural integrity—providing both mechanistic insight and functional validation.

Our approach integrates recombinant protein design, biophysical characterization, and RNA encapsulation assays to establish a foundational platform for mRNA delivery. If successful, this work will produce a versatile, scalable alternative to viral and lipid-based RNA vectors, advancing the development of safer and more controllable gene therapies.

2.1 Structure of “State of research in the field”

To structure your project summary, you can follow this recipe:

1. Brief overview of the field
2. Summary of key studies **with citations**
3. Clear articulation of current limits
4. Then close with justification for this project

2.2 Structure of “Detailed description of goals, methods, approach, expected results, and potential risks"

Aim 1: *What is the aim?*

1) Rationale (why?)

2) Approach (how?)

3) Expected results

4) Risks and alternative approaches

Aim 2: *What is the aim?*

1) Rationale (why?)

2) Approach (how?)

3) Expected results

4) Risks and alternative approaches

(Aim 3..)

Example Aim 1

Aim 1: To engineer and express soluble capsid protein analogs fused to solubility-enhancing tags.

Rationale: Native viral capsid proteins often exhibit poor solubility when expressed recombinantly as monomers, limiting their utility as customizable platforms for mRNA delivery. Solubility tags such as maltose-binding protein (MBP), SUMO, or short, hydrophilic peptide domains have been shown to improve expression yields and folding efficiency while preserving protein function. By integrating such tags into capsid scaffolds, we aim to develop soluble, self-assembling protein carriers compatible with downstream mRNA encapsulation.

Approach: We will select one or more capsid proteins known to assemble into nanoscale containers, such as the bacteriophage MS2 coat protein or the hepatitis B virus core antigen. These proteins will be genetically fused to a panel of solubility-enhancing tags and cloned into expression vectors. Expression will be performed in *E. coli* or insect cells, followed by purification using affinity chromatography and size-exclusion chromatography. Solubility will be assessed via SDS-PAGE and SEC profiles, while folding and oligomeric state will be evaluated using dynamic light scattering (DLS) and negative-stain TEM.

Expected results: We anticipate that one or more fusion constructs will yield high levels of soluble, properly folded capsid protein capable of forming higher-order assemblies under physiological conditions. These constructs will provide the basis for RNA encapsulation studies in Aim 2.

Risks and alternative approaches: If solubility remains insufficient despite fusion tags, we will pursue alternative strategies such as truncation of aggregation-prone domains or mutation of exposed hydrophobic residues identified by in silico modeling. If assembly is compromised, we will test protease-cleavable solubility tags to decouple solubility enhancement from capsid self-assembly.

Example Aim 2

Aim 2: To evaluate the ability of engineered capsid analogs to package and protect mRNA in vitro.

Rationale: To serve as functional delivery vehicles, the engineered capsid analogs must retain the ability to bind and encapsulate mRNA. This encapsulation should result in particle formation and confer protection against nuclease degradation. Demonstrating this capability is essential for validating the platform as a non-viral alternative for RNA delivery.

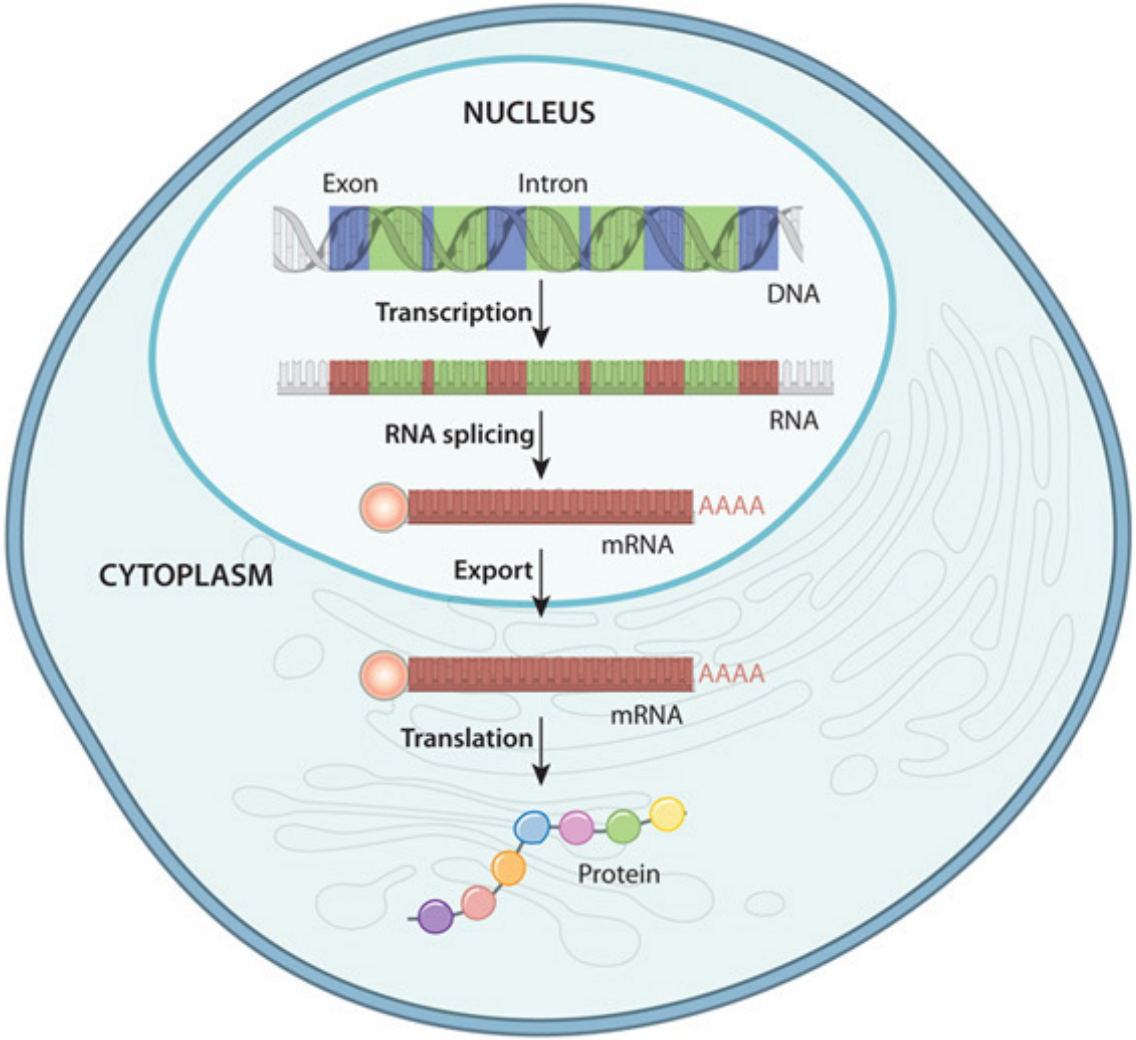
Approach: We will prepare model mRNA transcripts of defined sequence and length via in vitro transcription and label them with fluorescent dyes for quantitative detection. Capsid-mRNA mixtures will be incubated under conditions conducive to self-assembly, and the resulting complexes will be analyzed via native gel shift assays and RNase protection assays to confirm encapsulation. Particle formation will be confirmed by cryo-TEM. Quantification of protected mRNA will be performed by fluorescence intensity after enzymatic degradation of unencapsidated RNA.

Expected results: We expect to observe the formation of discrete protein-RNA complexes that are resistant to nuclease treatment and structurally well-defined. These results will indicate successful packaging and stabilization of mRNA by the capsid analogs.

Risks and alternative approaches: If encapsidation is inefficient, we will explore the inclusion of auxiliary RNA-binding peptides (e.g., arginine-rich motifs) fused to the capsid interior to enhance RNA affinity. Alternatively, electrostatic condensation of RNA using polycations or engineered scaffolds could be employed to promote encapsulation by capsid proteins with reduced RNA-binding domains.

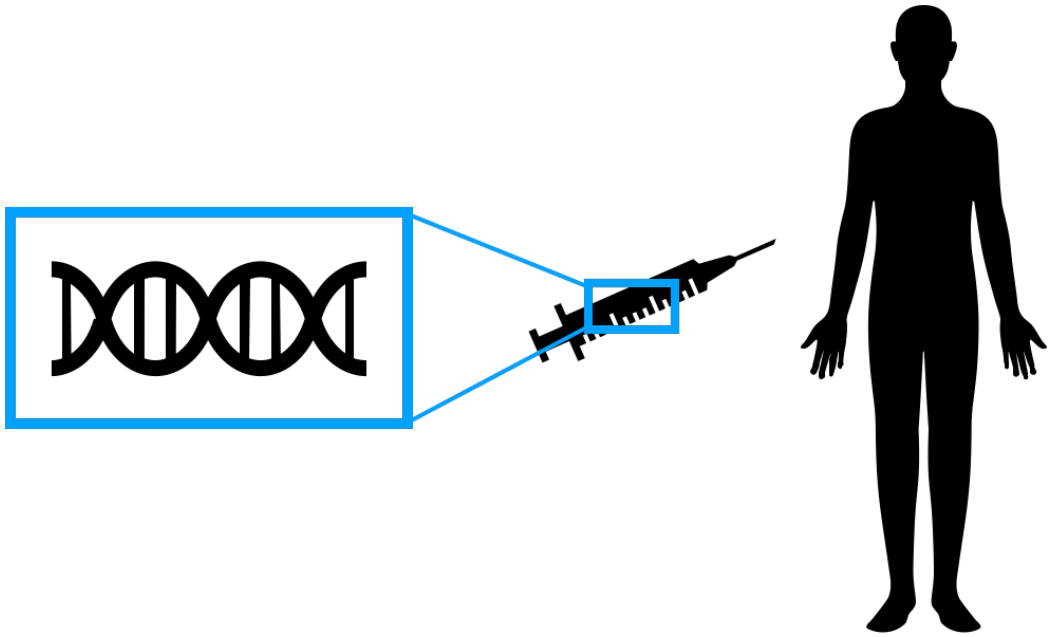
Netflix recommendation

Gene expression in eukaryotes



https://www.nature.com/scitable/ebooks/cell-biology-for-seminars-14760004/122995513/

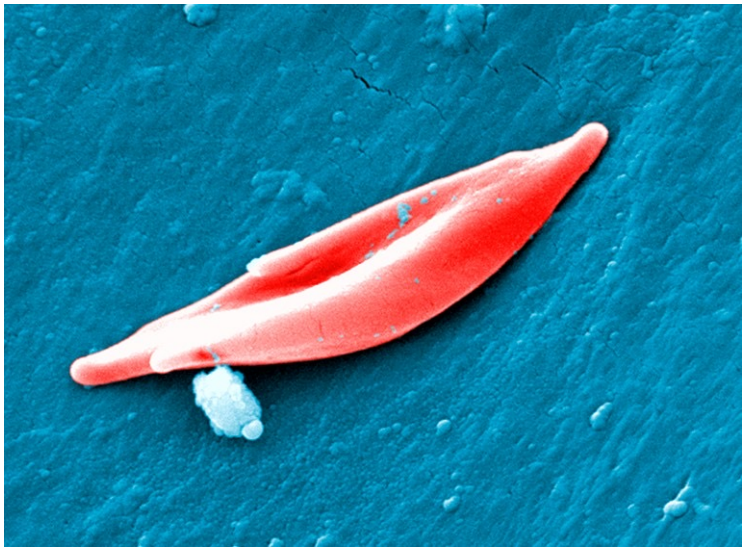
Gene therapy



- **Medical Intervention**: Modifies genetic material in cells to treat/prevent disease.
- **Goal**: Correct or compensate for defective genes causing disease.
- **Methods**:
  - Replace Mutated Gene: Introduce healthy gene copy.
  - Inactivate Mutated Gene: Silence improperly functioning gene.
  - Introduce New Gene: Add gene to combat disease.

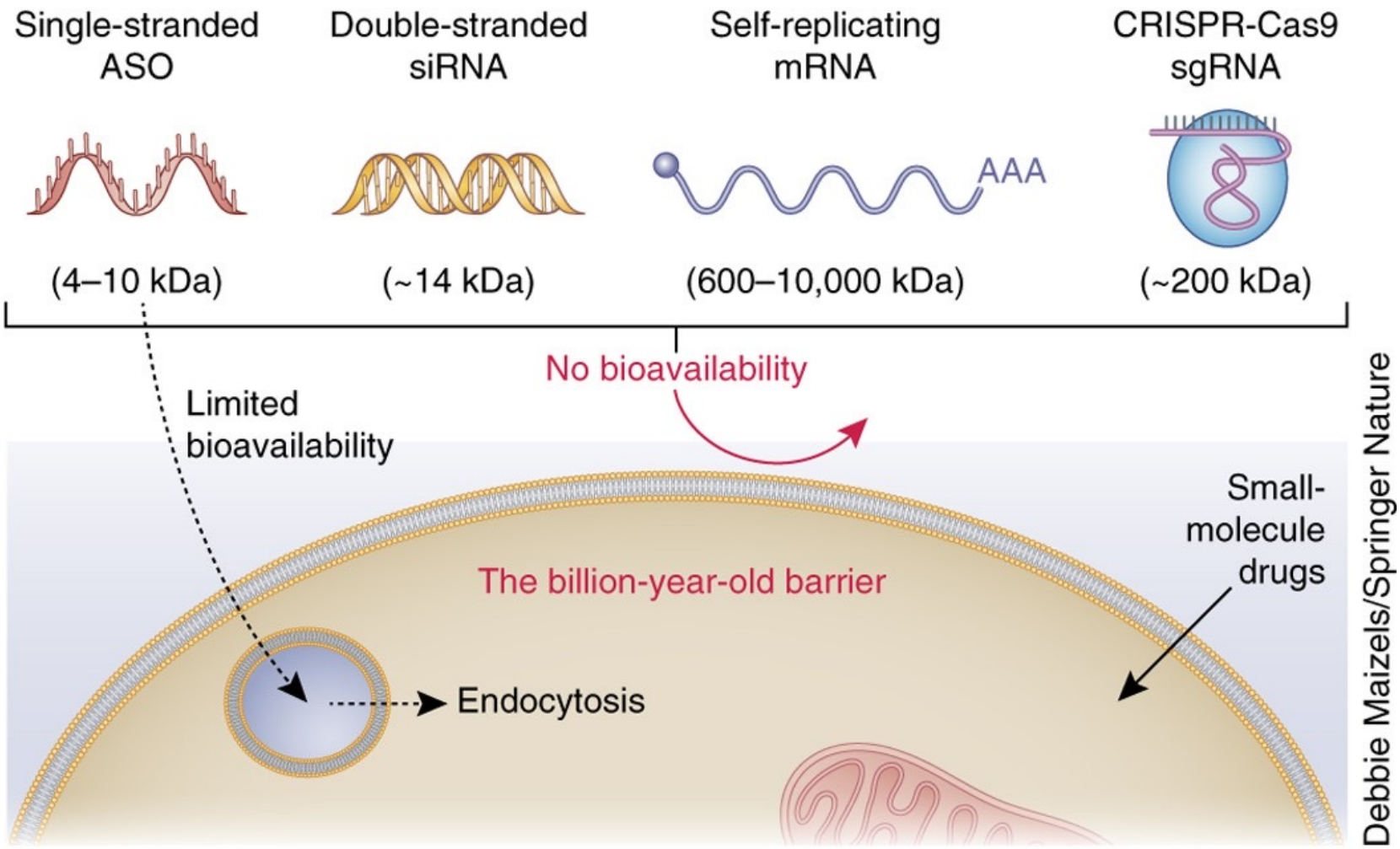
Gene therapy targets

- **Correction of single-gene defect**
  - X-SCID
  - Hemophilia
  - Cystic fibrosis
  - Muscular dystrophy
  - Sickle cell anemia
- **Insertion of therapeutic gene**
  - Tumor suppression gene
  - Expression of therapeutic enzyme
  - Cytokine, growth factor
  - DNA vaccination, mRNA vaccination
- **Silencing of pathologic gene expression**
- **Restoration of correct gene expression**



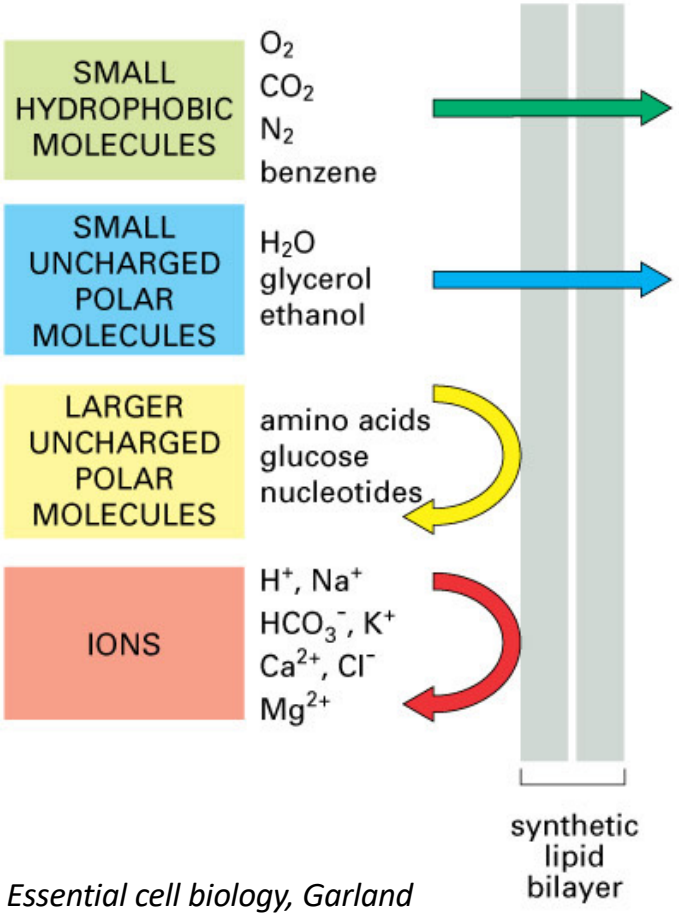
https://www.casgevy.com/sickle-cell-disease/how-casgevy-works

The billion-year-old barrier



Dowdy, S. Overcoming cellular barriers for RNA therapeutics. *Nat Biotechnol* **35**, 222–229 (2017).

Drug transport into cells



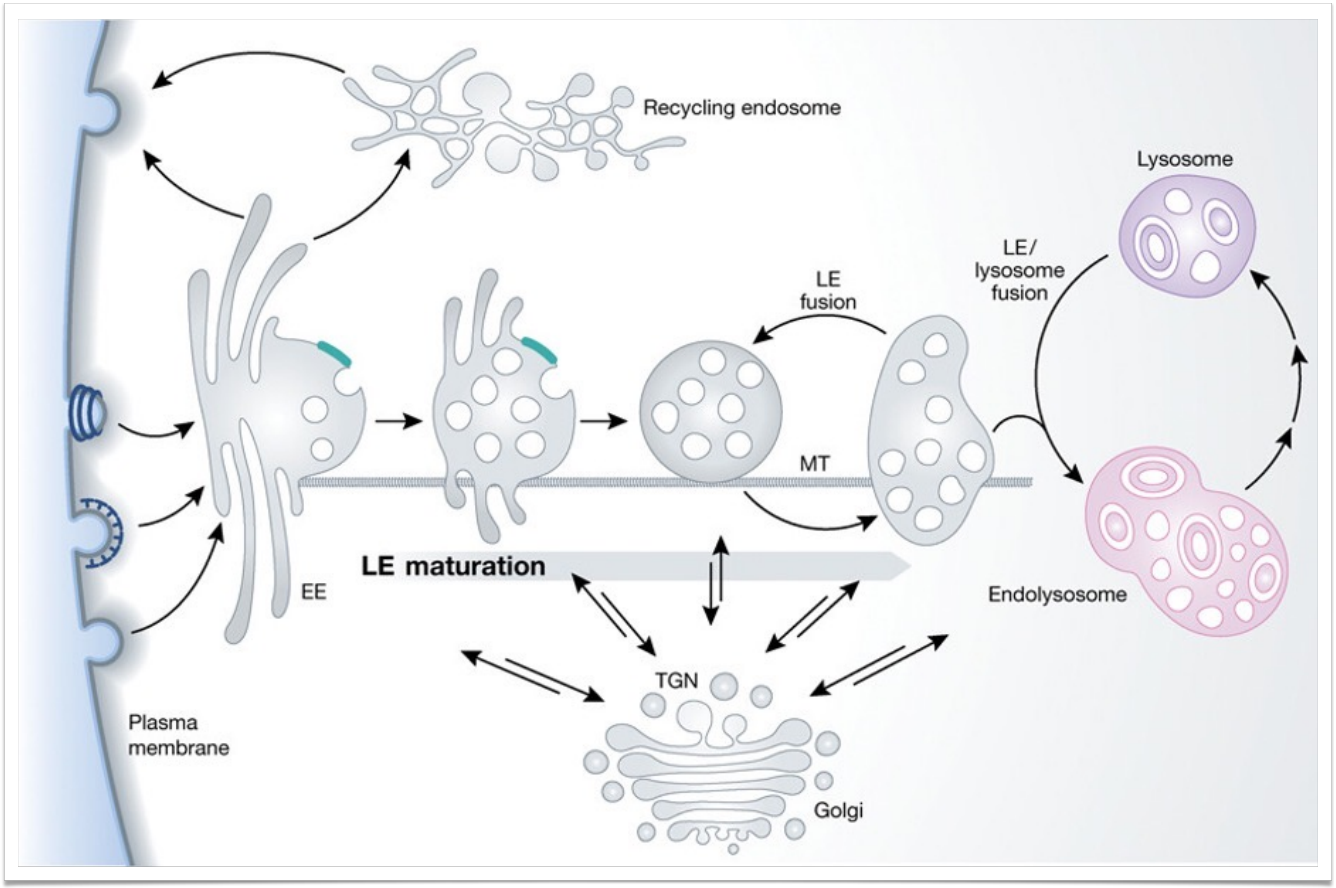
Essential cell biology, Garland

Classical small molecule drugs: Lipinski's rule of 5

- Not more than **5 hydrogen bond donors** (nitrogen or oxygen atoms with one or more hydrogen atoms)
- Not more than **10 hydrogen bond acceptors** (nitrogen or oxygen atoms)
- A molecular mass **less than 500 daltons**
- An octanol-water partition coefficient (log P) less than 5 (logP > 5: very hydrophobic and very lipophilic)

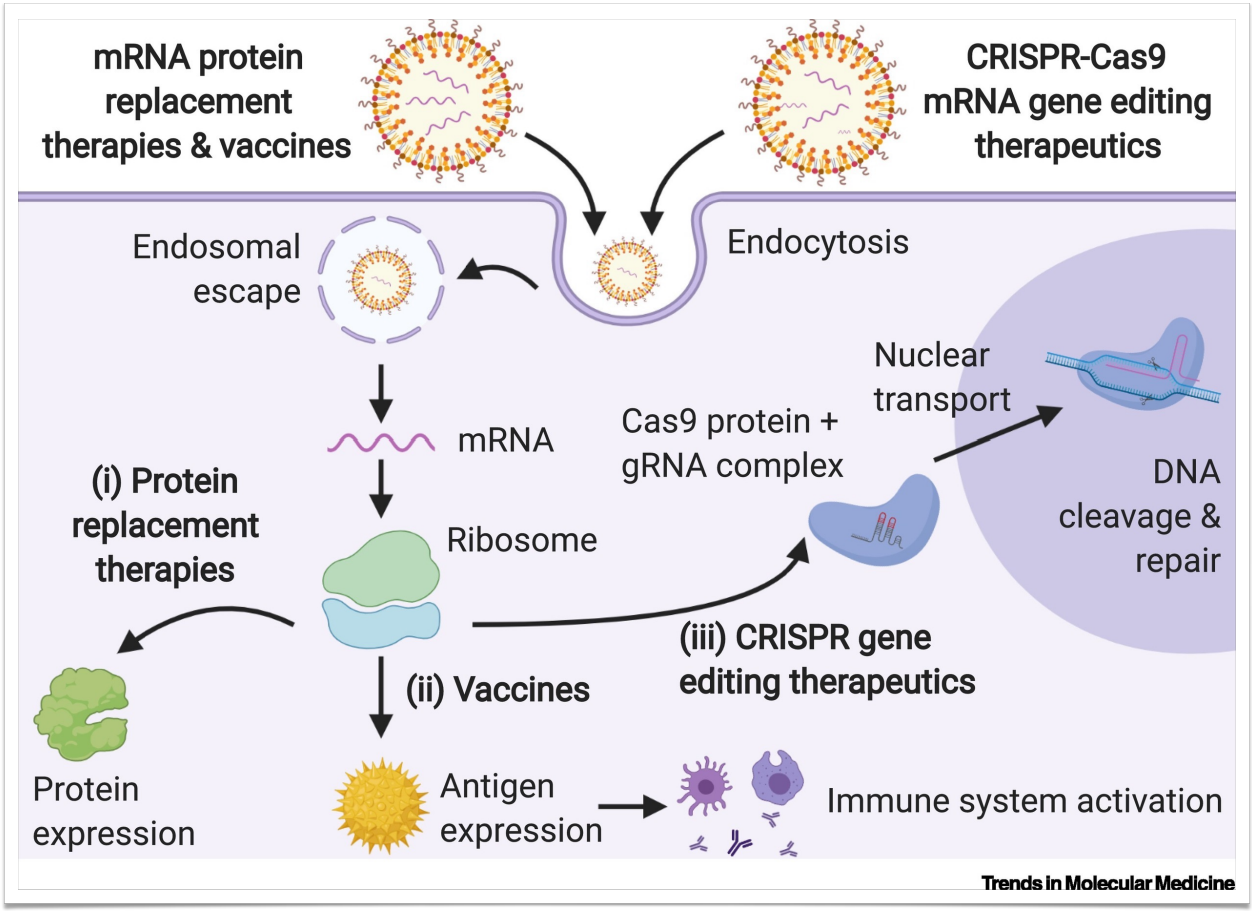
Many modern drugs (biologics) violate those rules!

Endosomal maturation



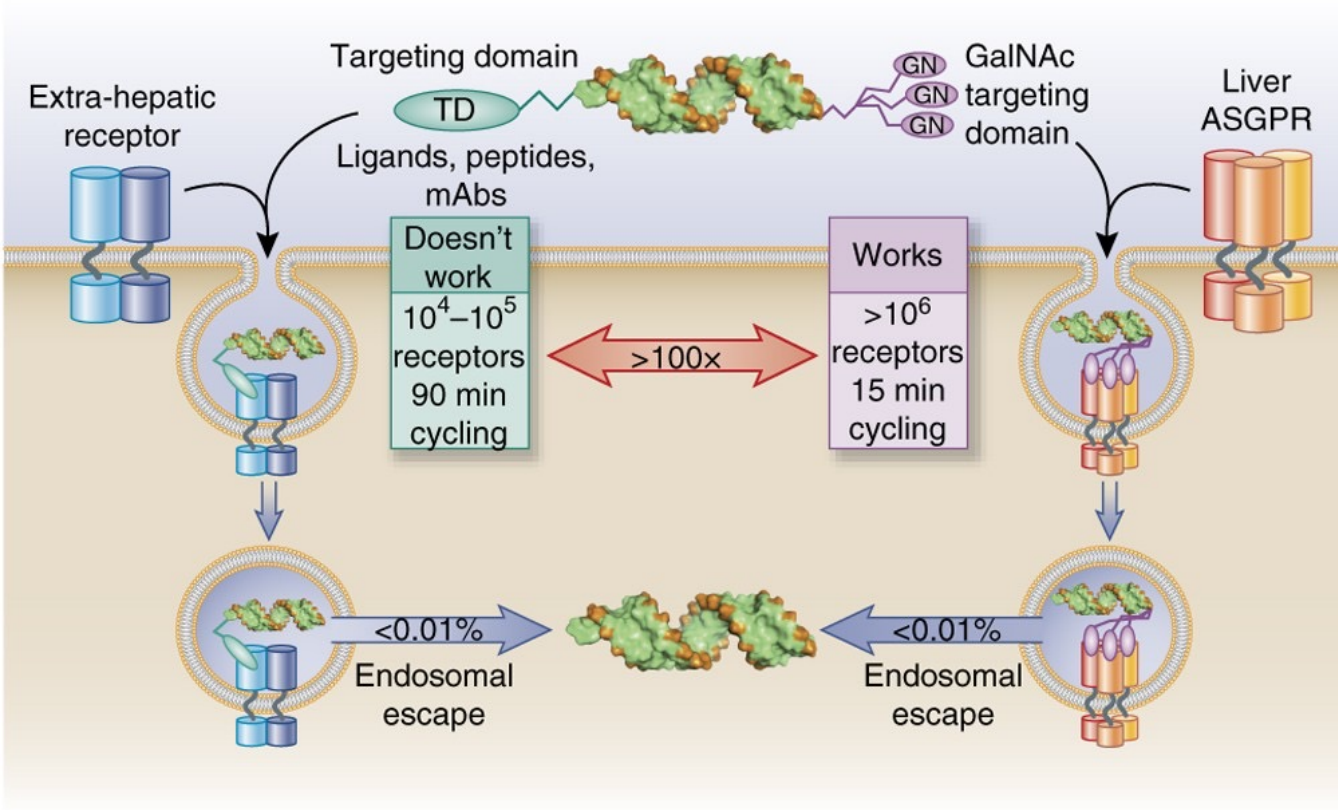
EMBO J. **2011**, *30*, 3481.

Endosomal escape



Trends Mol. Med. **2021**, *27*, 616.

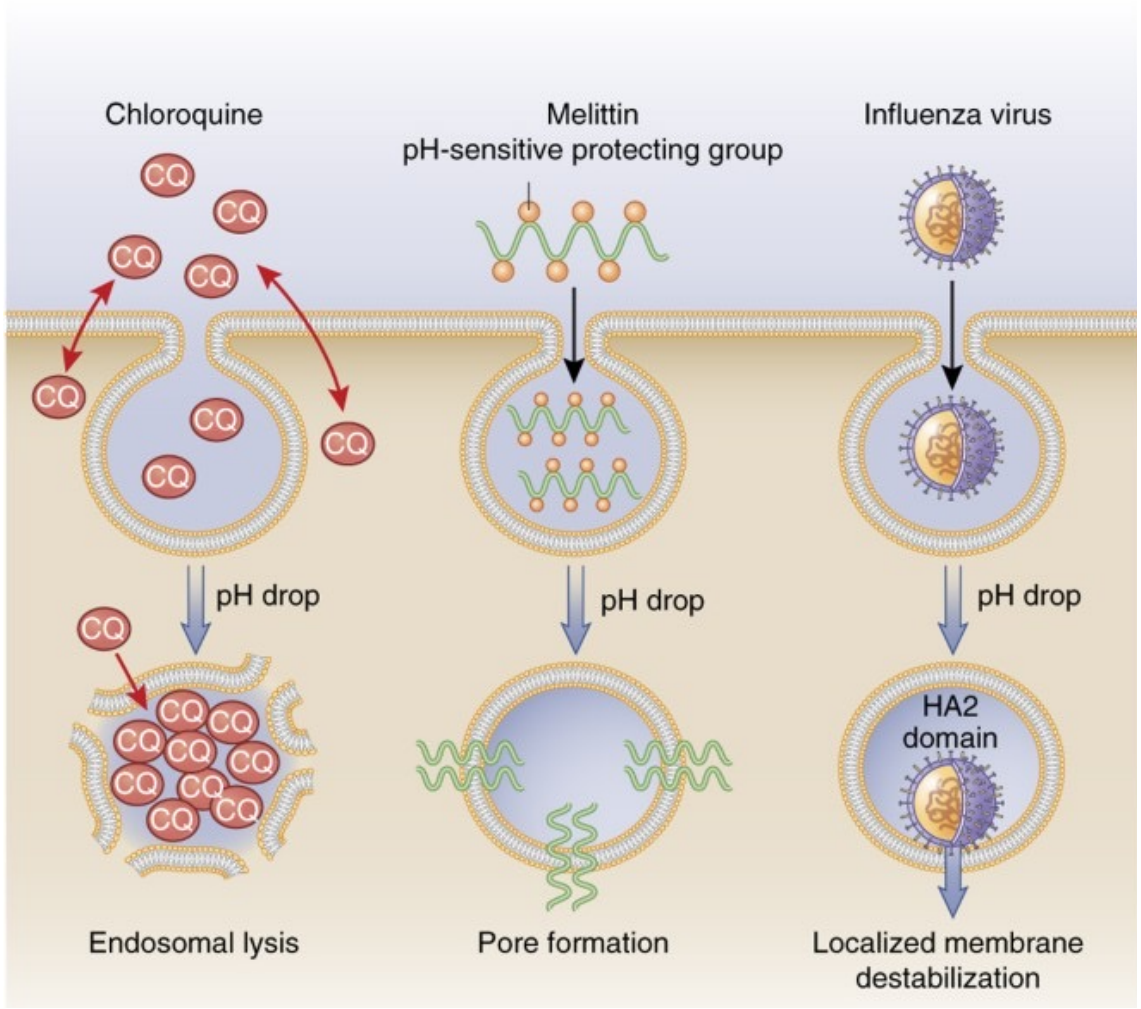
The numerology of endosomal escape



- Hepatocytes express **millions of copies of the ASGPR** on the cell surface
- Cycle of endocytosis-exocytosis: 10-15 min
- Need about 5000 siRNAs/cell
- Millions enter cells every 15 minutes
- **Localized destabilization of endosomal membrane at escape rate of <0.01% is enough to trigger knockdown**

Dowdy, S. Overcoming cellular barriers for RNA therapeutics. *Nat Biotechnol* **35**, 222–229 (2017).

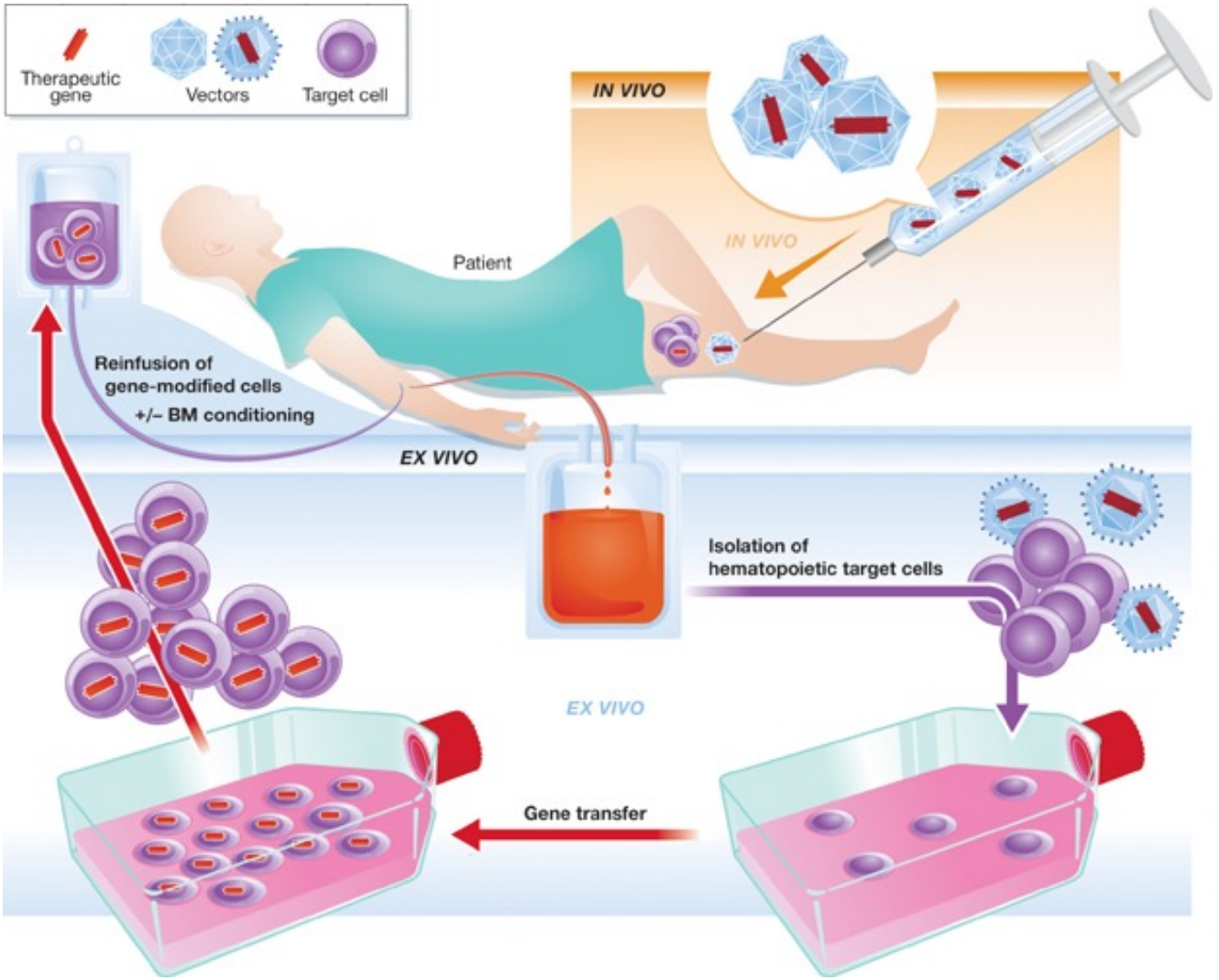
Endosomal escape agents



- **Chloroquine**: passive diffusion into cells, becomes protonated and trapped in endosomes, inserts a hydrophobic motif into endosomal membrane → lysis
- **Melittin**: pore-forming peptide from bee venom
- **HA2 domain**: from influenza virus contains a pH-sensitive **fusogenic** hemagglutinin-2 protein domain (HA2), inserts into endosomal membrane and facilitates virus entry

Dowdy, S. Overcoming cellular barriers for RNA therapeutics. *Nat Biotechnol* **35**, 222–229 (2017).

In vivo vs. ex vivo gene therapy



Kaufmann et al. *EMBO Mol. Med.* **5**, 1642–1661 (2013)

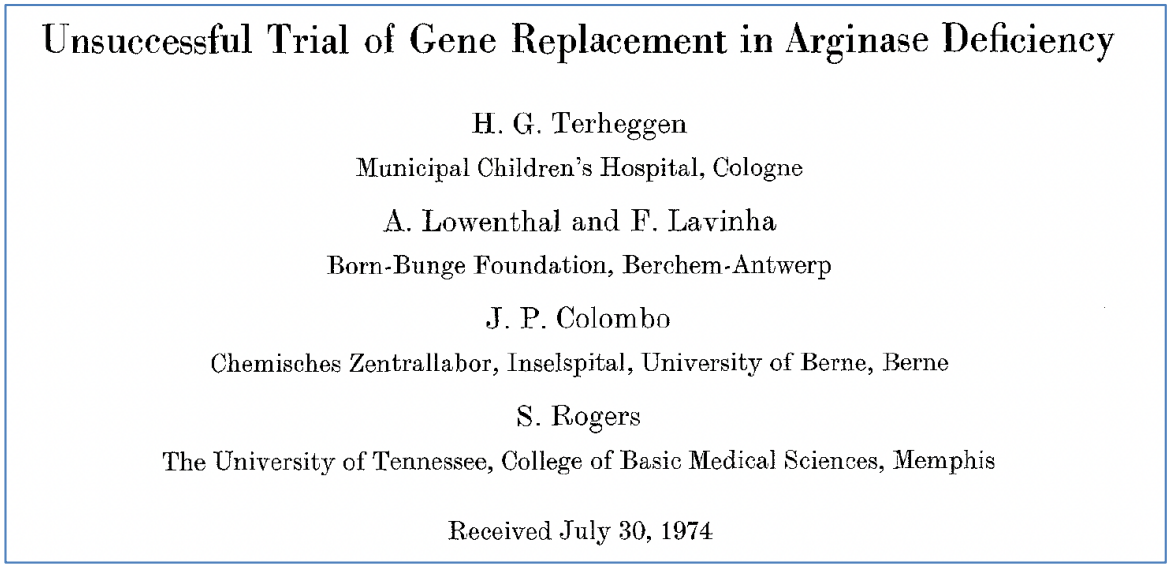
Important milestones in the history of gene therapy

1944: Avery-MacLeod-McCarty experiment: demonstrated that DNA is hereditary material

1953: Discovery of DNA double helix (Watson, Crick, Franklin)

1972: First proposal of gene therapy, Friedmann and Roblin in *Science* "Gene therapy for human genetic disease?"

1975: First gene therapy study (arginase deficiency)



Unsuccessful Trial of Gene Replacement in Arginase Deficiency

H. G. Terheggen
Municipal Children's Hospital, Cologne
A. Lowenthal and F. Lavinha
Born-Bunge Foundation, Berchem-Antwerp
J. P. Colombo
Chemisches Zentrallabor, Inselspital, University of Berne, Berne
S. Rogers
The University of Tennessee, College of Basic Medical Sciences, Memphis

Received July 30, 1974

Important milestones in the history of gene therapy

1980s: Development of recombinant DNA technology (restriction enzymes, plasmid cloning, viral vectors etc.)

1990: First approved gene therapy for severe combined immunodeficiency disease (SCID) patient.

1993: First clinical trial for gene therapy for cystic fibrosis

1999: Setback with death of Jesse Gelsinger (clinical trial for ornithine transcarbamylase deficiency)

2002: Successful gene therapy of SCID

2012: Approval of Glybera (lipoprotein lipase deficiency)

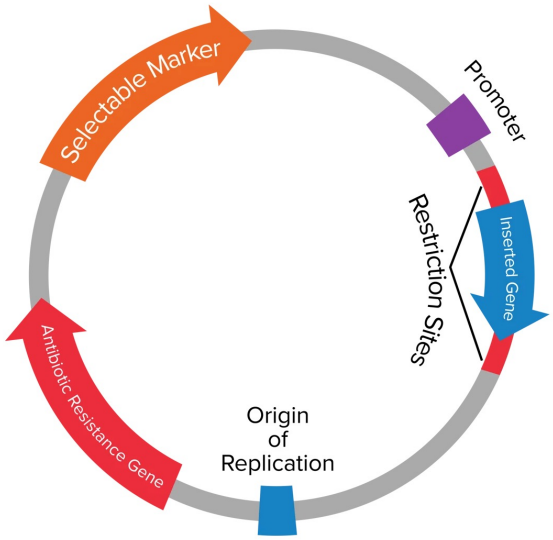
2017: Approval of CAR-T therapies (certain blood cancers) and Luxturna

2019: Approval of Zolgensma

2016: First clinical trial with CRISPR technology

2023: First CRISPR-Cas9 based gene therapy for sickle cell disease

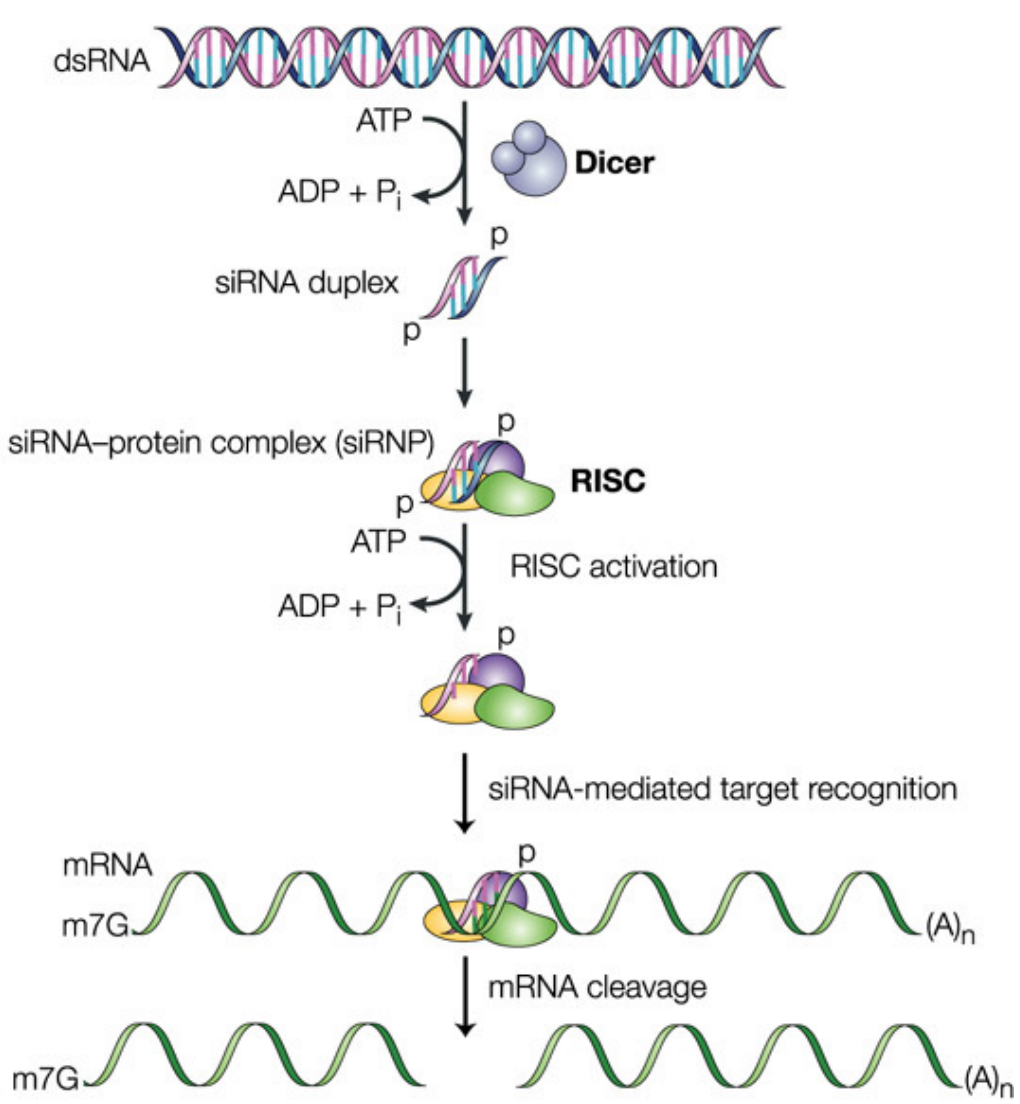
Plasmid DNA



Vector Element	Description
Origin of Replication (ORI)	DNA sequence which allows initiation of replication within a plasmid by recruiting replication machinery proteins
Antibiotic Resistance Gene	Allows for selection of plasmid-containing bacteria.
Multiple Cloning Site (MCS)	Short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Insert	Gene, promoter or other DNA fragment cloned into the MCS for further study.
Promoter Region	Drives transcription of the target gene. Vital component for expression vectors: determines which cell types the gene is expressed in and amount of recombinant protein obtained.
Selectable Marker	The antibiotic resistance gene allows for selection in bacteria. However, many plasmids also have selectable markers for use in other cell types.
Primer Binding Site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Primers can be exploited for sequence verification of plasmids.

https://blog.addgene.org/plasmids-101-what-is-a-plasmid

Switching off genes: RNA interference



Innate virus defense

Viral RNA is digested by an enzyme (dicer)

RNA duplex is bound by a protein complex (RISC)

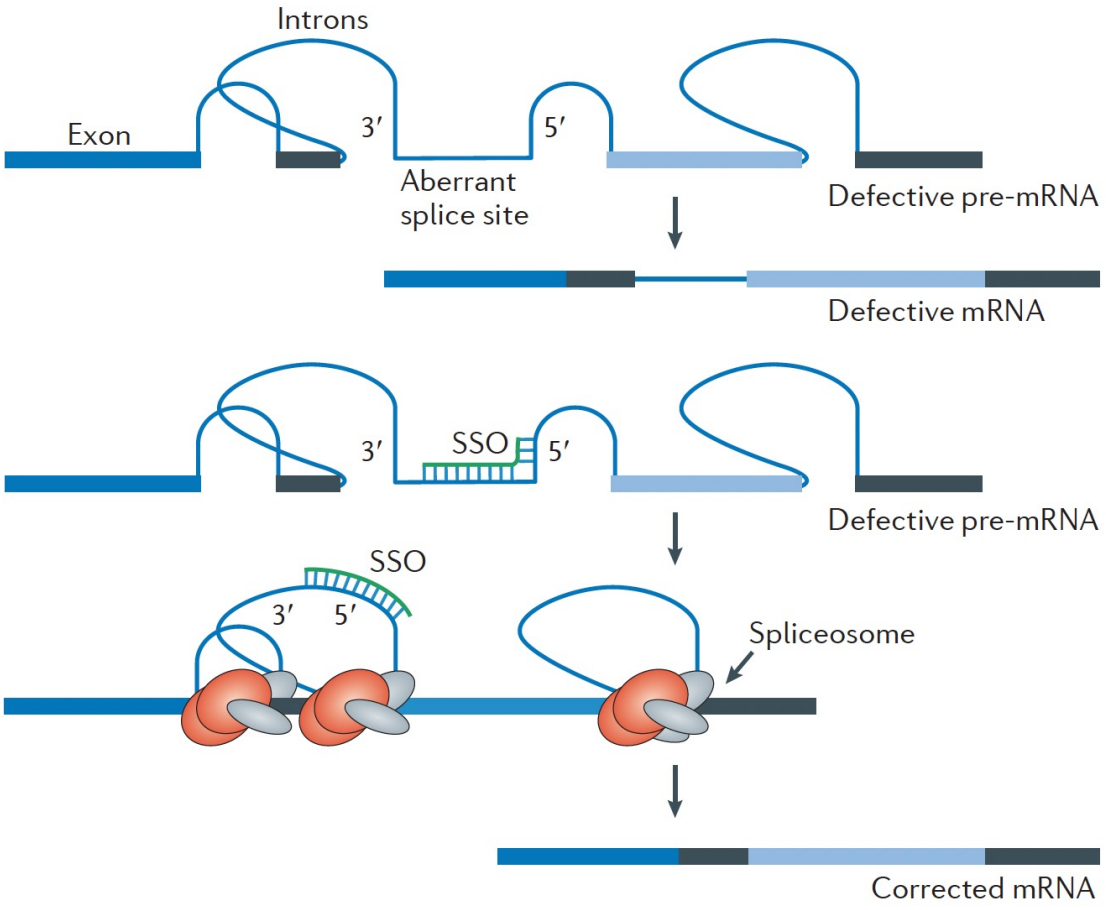
RISC activation: ssRNA is produced

Basepairing to mRNA -> specific gene recognition

mRNA cleavage

Dykxhoorn, et al., *Nature Rev. Mol. Cell Biol.* **4,** 457–467 (2003)

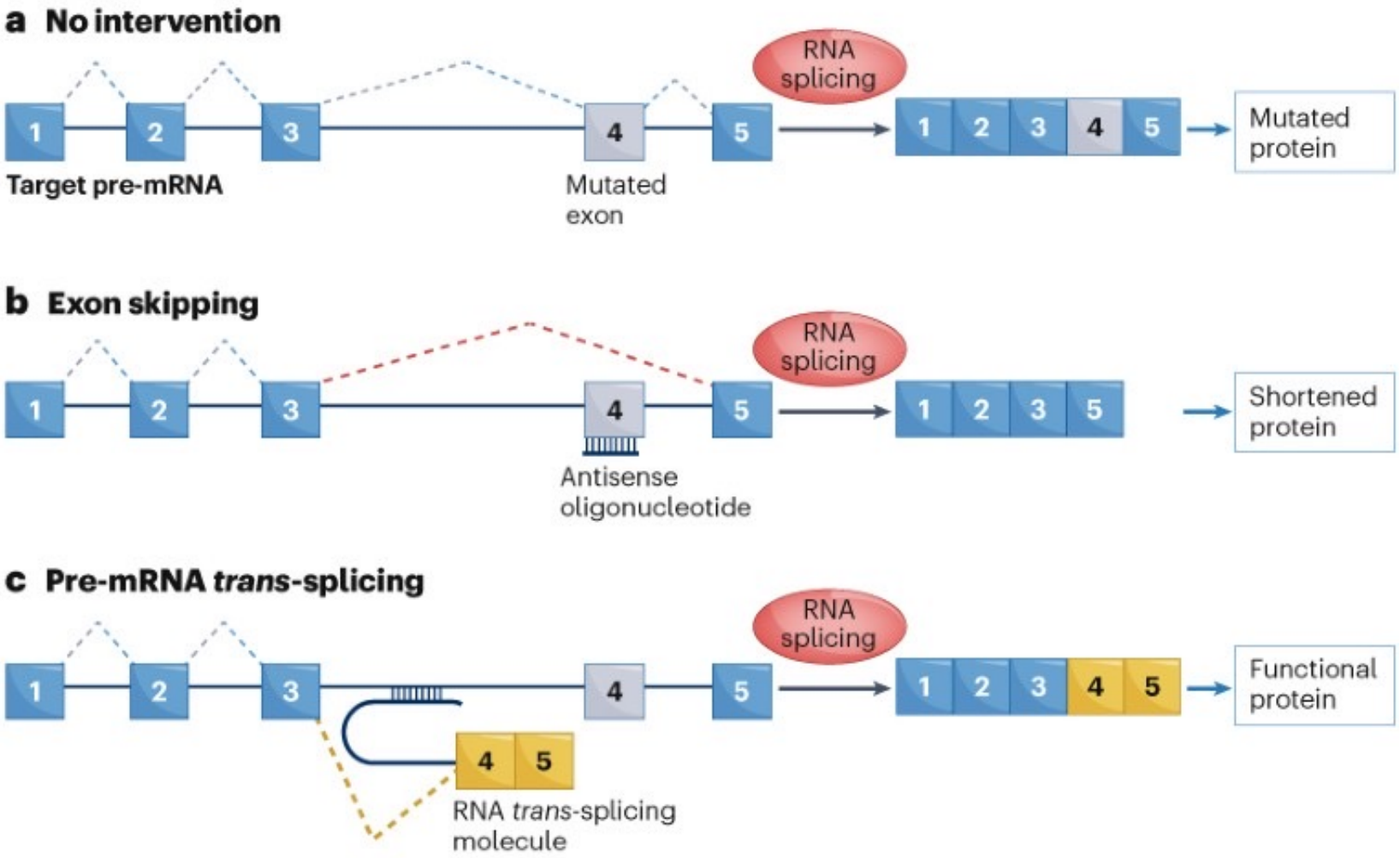
Strand-switching oligonucleotides (SSOs)



Exon retention by SSOs. Some exons are poorly spliced into mRNA because they contain exonic splicing silencer (ESS) elements. An SSO designed to block an ESS interferes with this element's role in splicing and promotes exon inclusion, as has been demonstrated in the case of **spinal muscular atrophy**, a genetic disorder.

Kole, R. et al., *Nature Rev. Drug Discov.* **11**, 125 (2012).

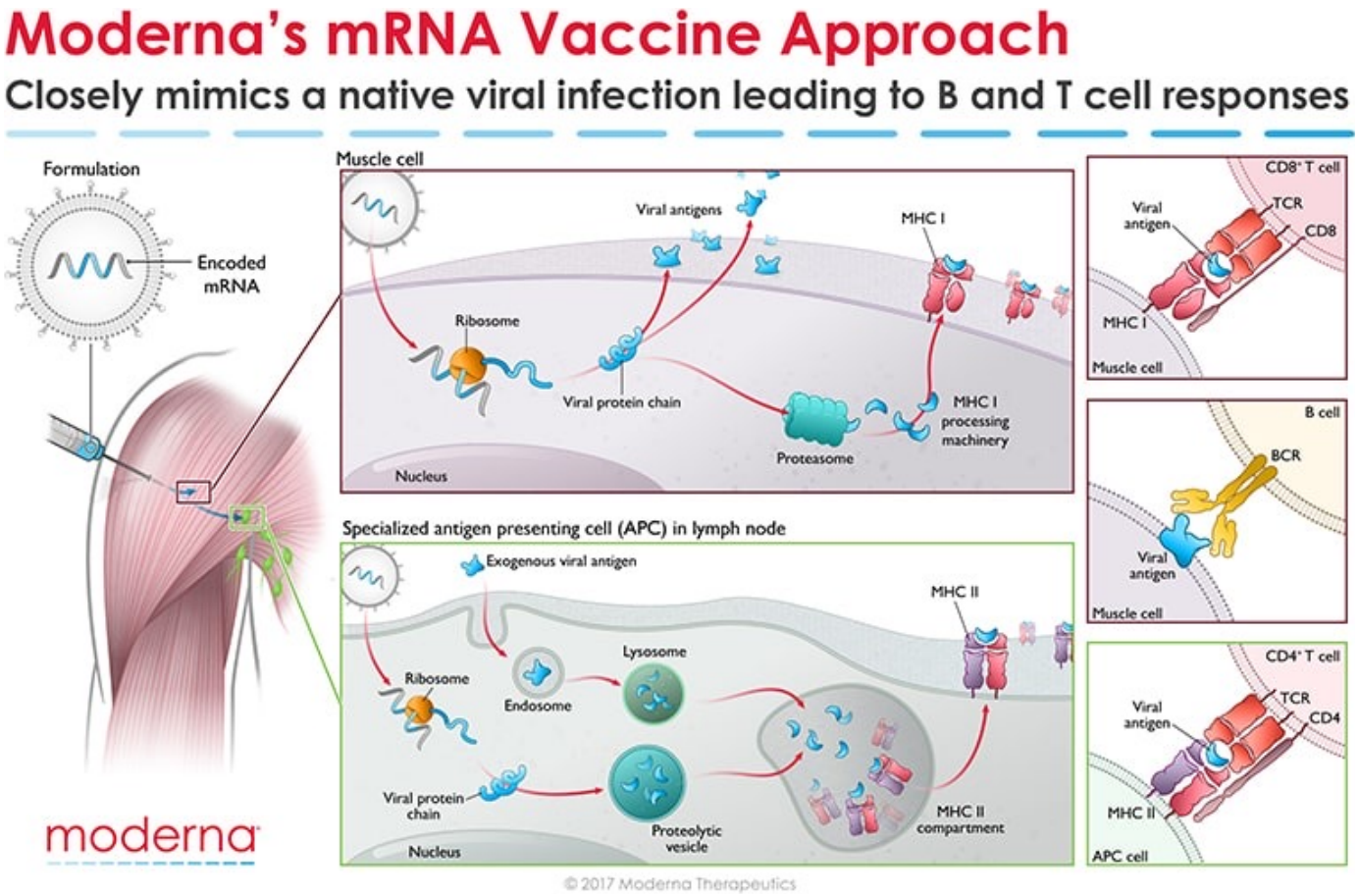
Pre-mRNA *trans*-splicing' drugs



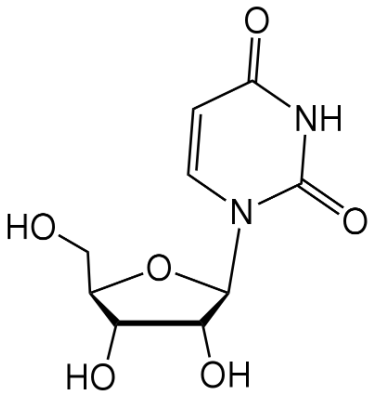
https://doi.org/10.1038/d41573-024-00086-4

Current application of RNA nanoparticles: mRNA vaccines

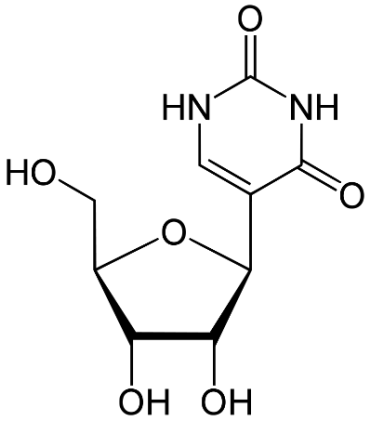
- Intramuscular administration: local inflammation
- Attracts neutrophils and antigen presenting cells (APGs)
- APCs internalize mRNA LNP & express the protein
- APCs migrate to the local draining lymph node and prime T-Cells



mRNA engineering – low immunogenicity, high stability, high translatability



uridine



pseudouridine ψ

Immunogenicity, stability & translatability

nucleoside-modified mRNA

e.g., Ψ,m1Ψ, 5meC

sequence-engineered mRNA

e.g., GC motifs, structural UTR elements

Translatability

Cap analogs
e.g., ARCA, β-S-ARCA

Stability and translatability

5' Cap

open reading frame

AAAAA

5' UTR

3' UTR

self-amplifying mRNA

\+

nonstructural (replicase)

subgenomic promotor

end structures
e.g., Polyadenylation, circular mRNA

Stability & translatability

Schoenmaker et al. Intl J of Pharmaceutics 2021

Comparison of different gene modalities

Modality	Needs Nuclear Entry	Expression Type	Integration Risk	Duration	Ideal Use Case
pDNA	Yes	Transcriptional	Low	Weeks–months	Protein replacement in dividing cells
mRNA	No	Translational	None	Hours–days	Vaccines, rapid protein expression
siRNA/ASO	No	RNA silencing	None	Days–weeks	Knockdown of disease-causing genes
CRISPR/editors	Yes or No (depending on system)	Genomic	Possible	Permanent	Mutation correction or functional knockouts

LNPs as a mRNA delivery vehicle

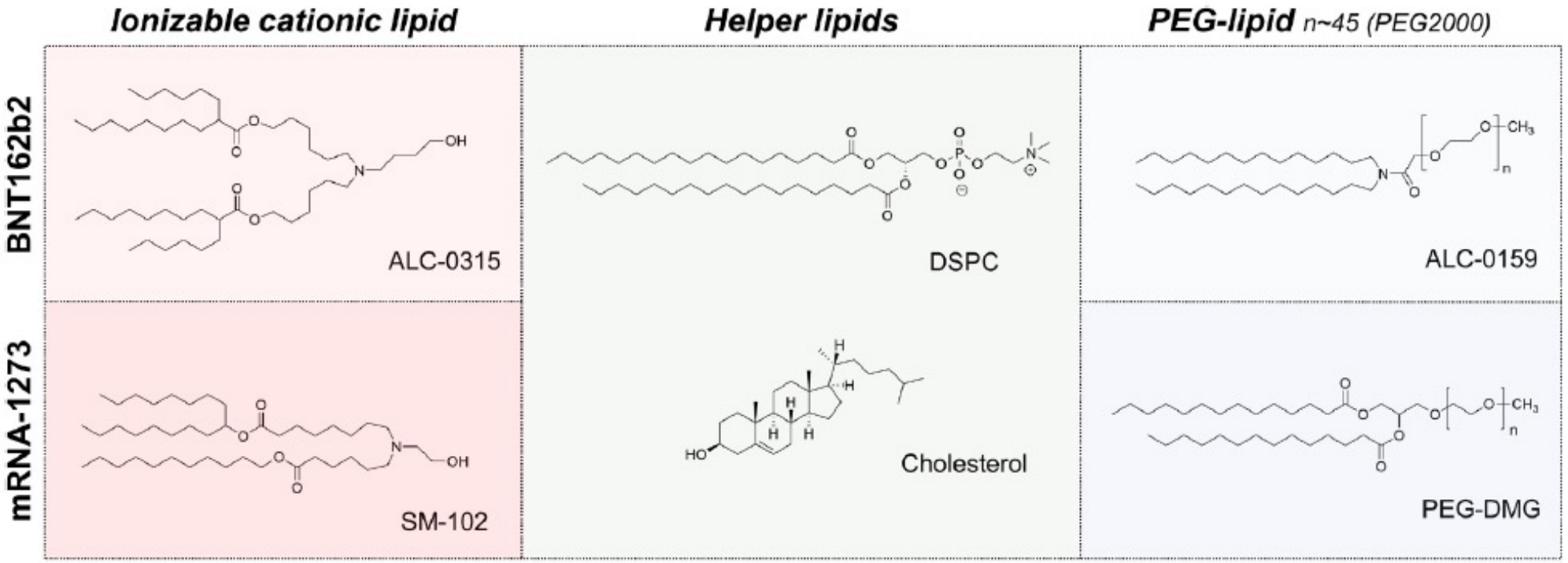


Fig. 6. Lipids used in the mRNA-LNP COVID-19 vaccines BNT162b2 (Comirnaty) and mRNA-1273.

4 main components:
phospholipid, cholesterol, ionizable cationic lipid and PEG lipid

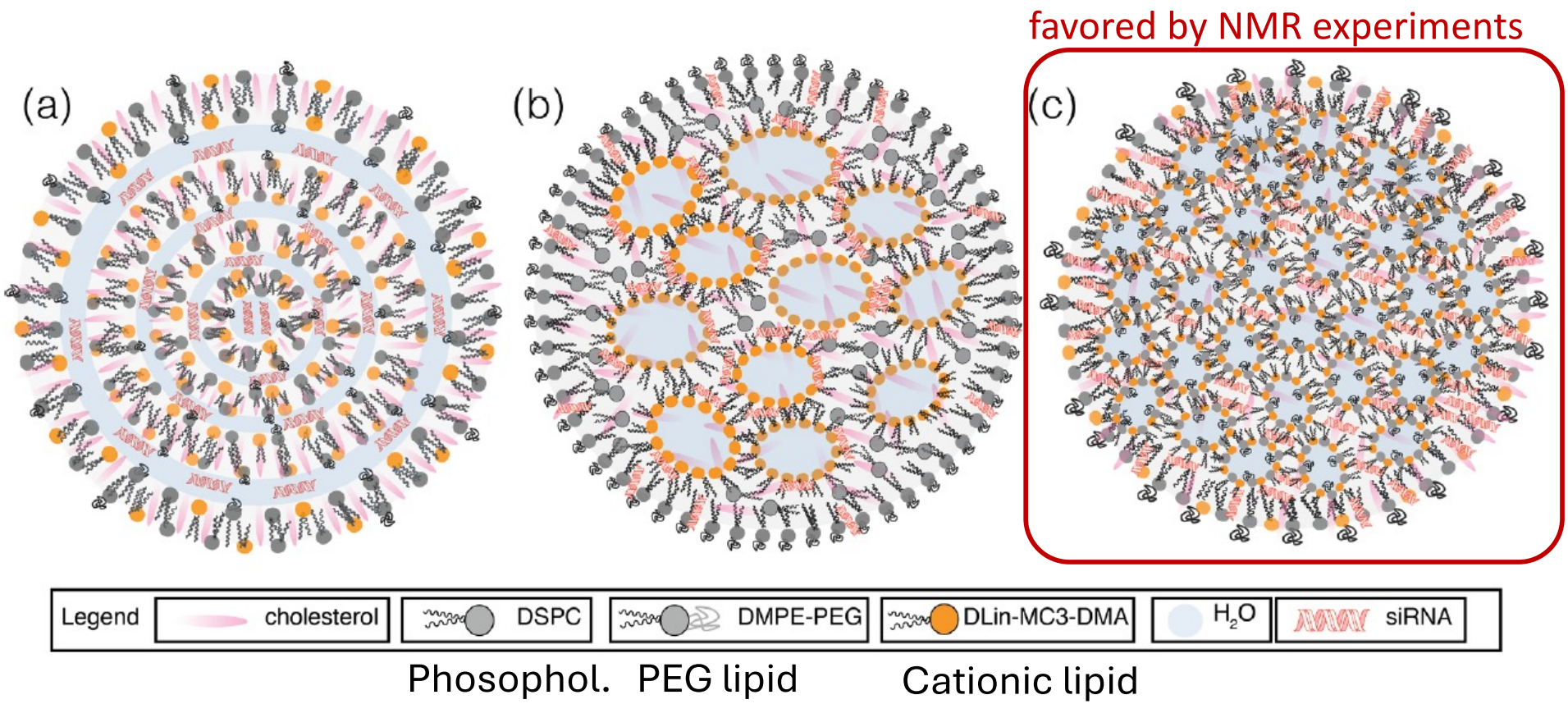
particle size

preparation, membrane fusion

Schoenmaker et al. Intl J of Pharmaceutics 2021

Models for mRNA LNP structure

Multilamellar vesicles (onion), (b) nanostructure core, and (c) homogeneous core shell.



J. Phys. Chem. B 2018, 122, 2073−2081

Alternative models of mRNA LNP structures

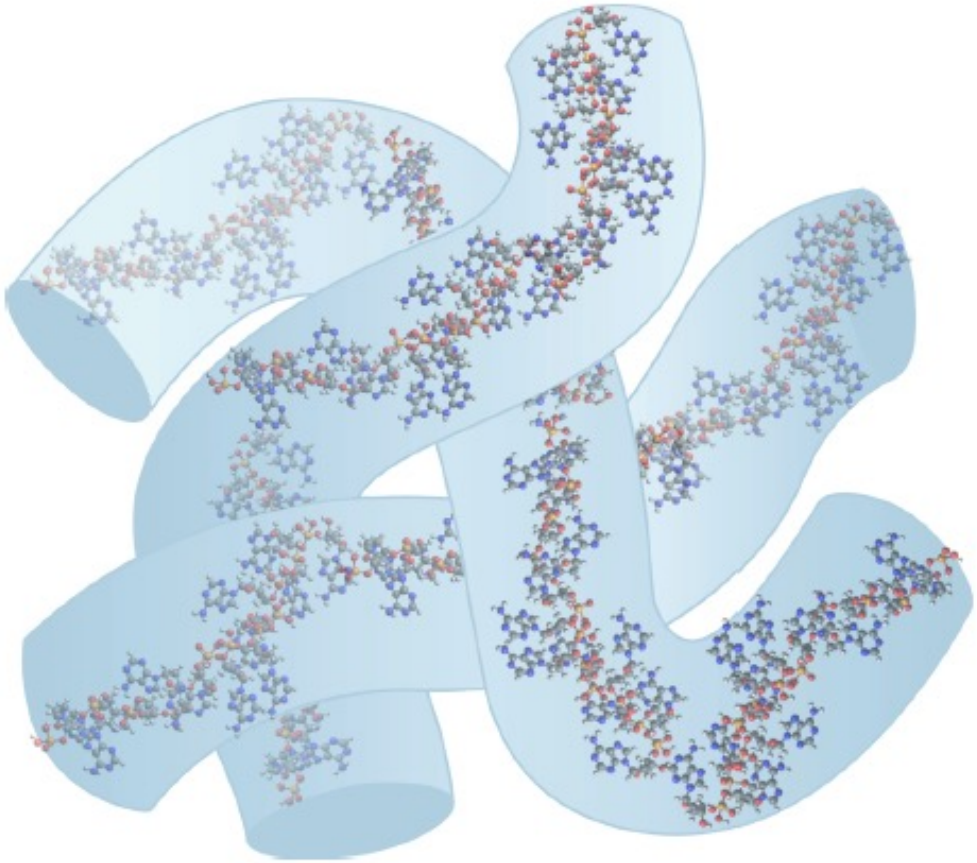
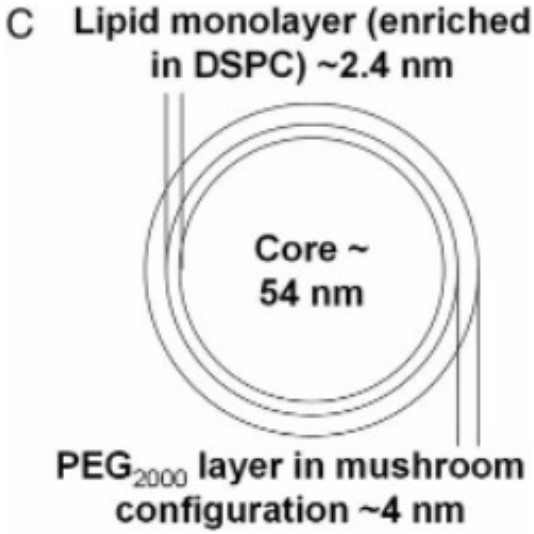


Fig. 4. Schematic representation of the mRNA-water cylinders in the core of mRNA- LNPs (Arteta et al., 2018). Courtesy of the authors.



- mRNA water cylinders in the center of nanoparticles
- mRNA is exposed to water in the interior

mRNA lipid nanoparticles form complex dense bleb-like structures

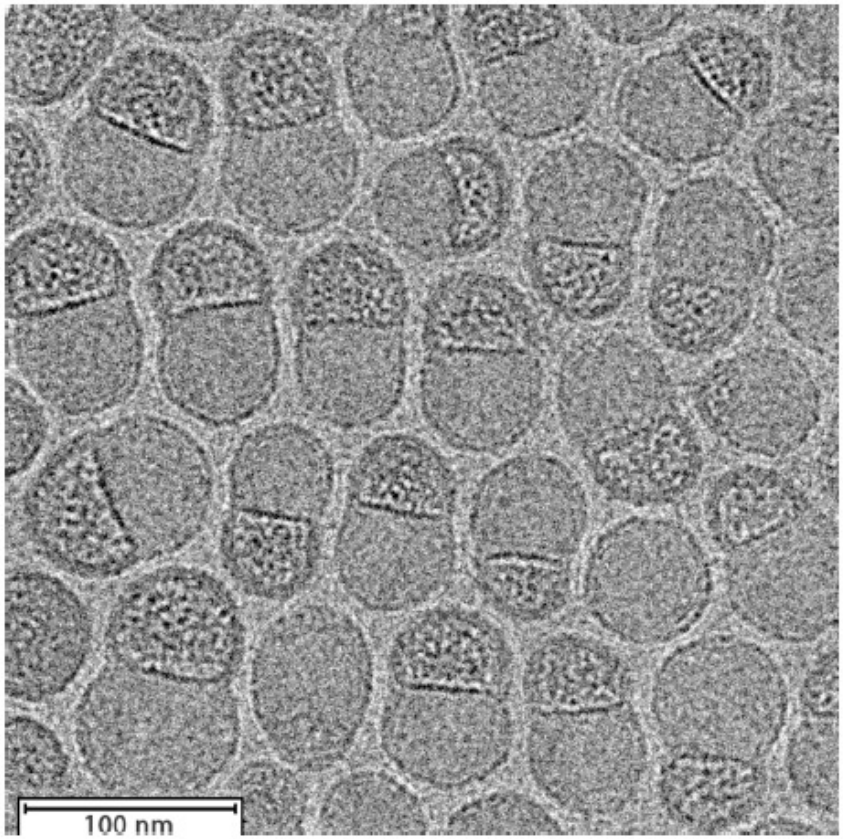
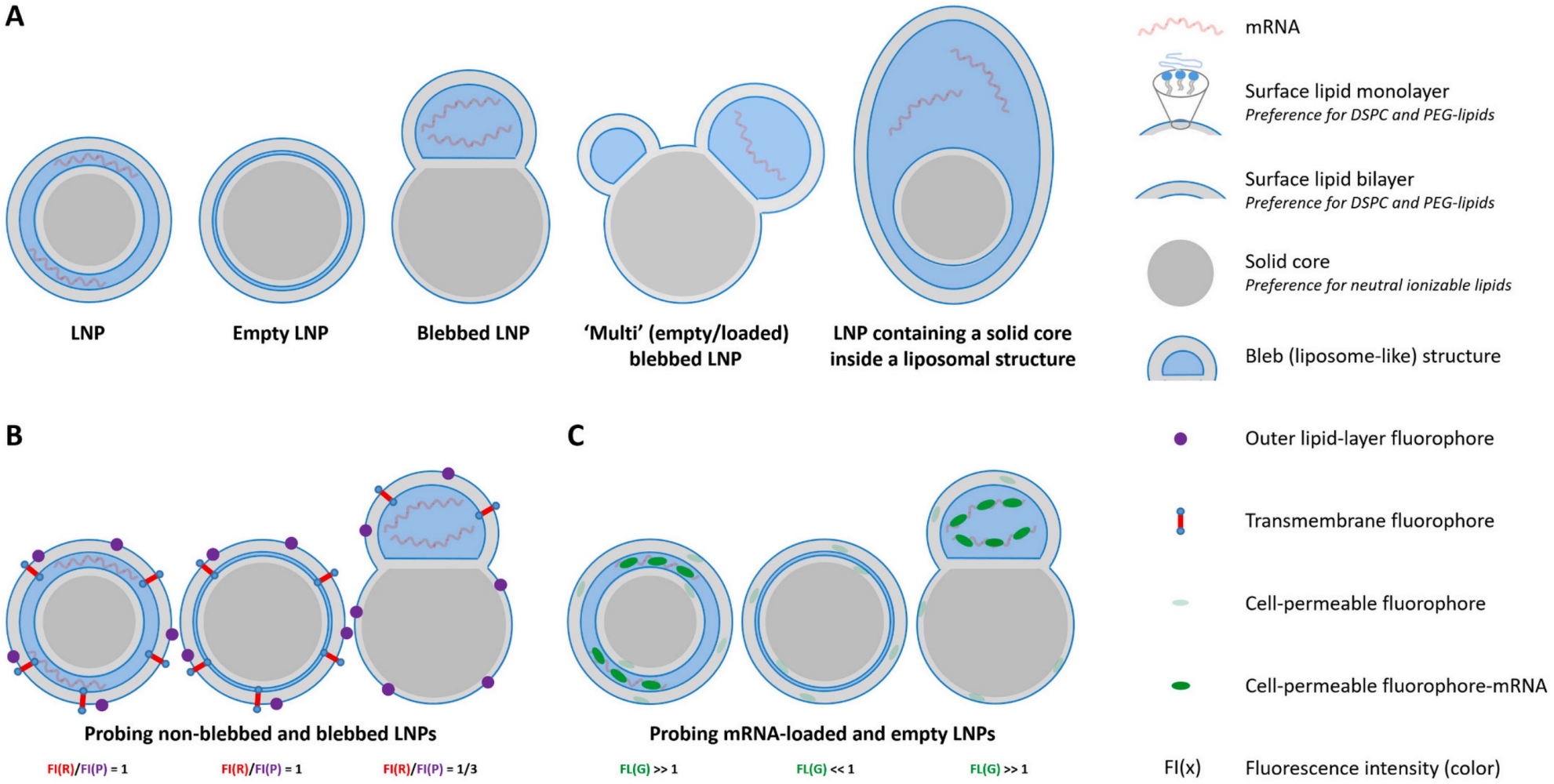


Fig. 2. Cryo-TEM image of mRNA-LNP showing 'bleb' structures with distinctly different electron density. Adapted from Brader et al. (2021) with permission.

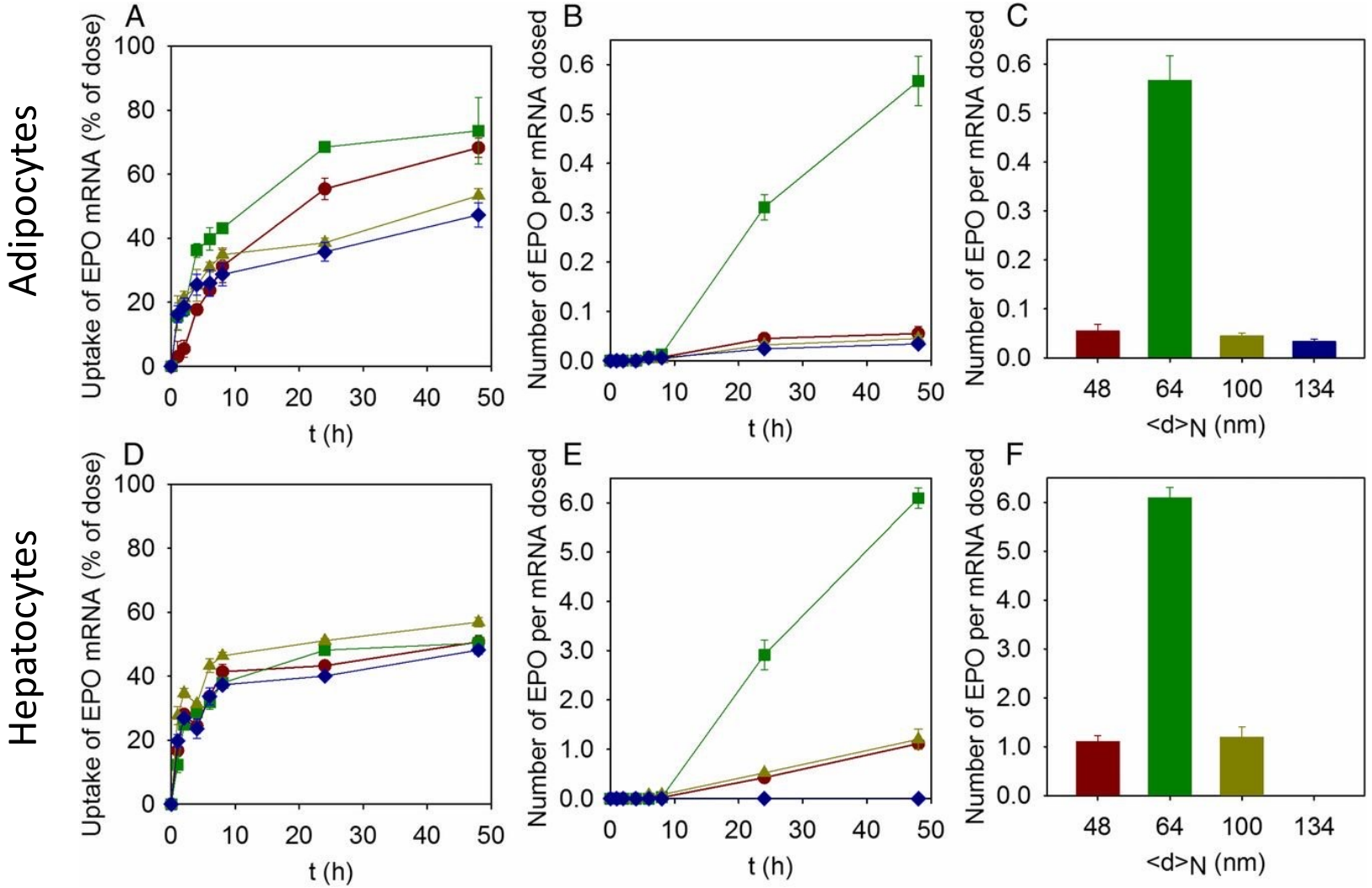
- Blebs: protruding liposome-like structures found in lipid nanoparticles (LNPs)
- Observed through cryo-TEM
- Contain an **aqueous core** surrounded by a lipid bilayer, likely **phase-separated from a solid core** that presumably consists of **ionizable lipids**
- Mixed reviews on relevance of blebs for mRNA vaccine activity
- More studies needed

mRNA lipid nanoparticles come in many different forms



J. Contr. Rel. 2024, 952-961.

Cellular uptake and cellular protein production for LNPs of different sizes



Formulation is critically important for successful mRNA delivery!

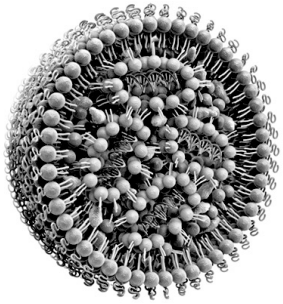
The most popular delivery approaches

Viral vectors

Protein-based

+ Delivery efficiency high
– Immunogenic
– Limited in gene size

Top-down

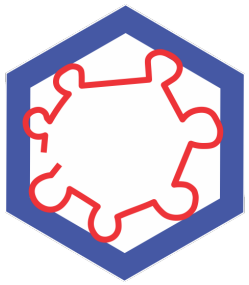


Lipid nanoparticles

Lipid-based

+ Easy to produce
+ Can package larger genes
+ Less immunogenic
– Delivery efficiency low
– Cannot be optimized by evolution

Bottom-up



Engineered & evolved protein cages

Protein-based

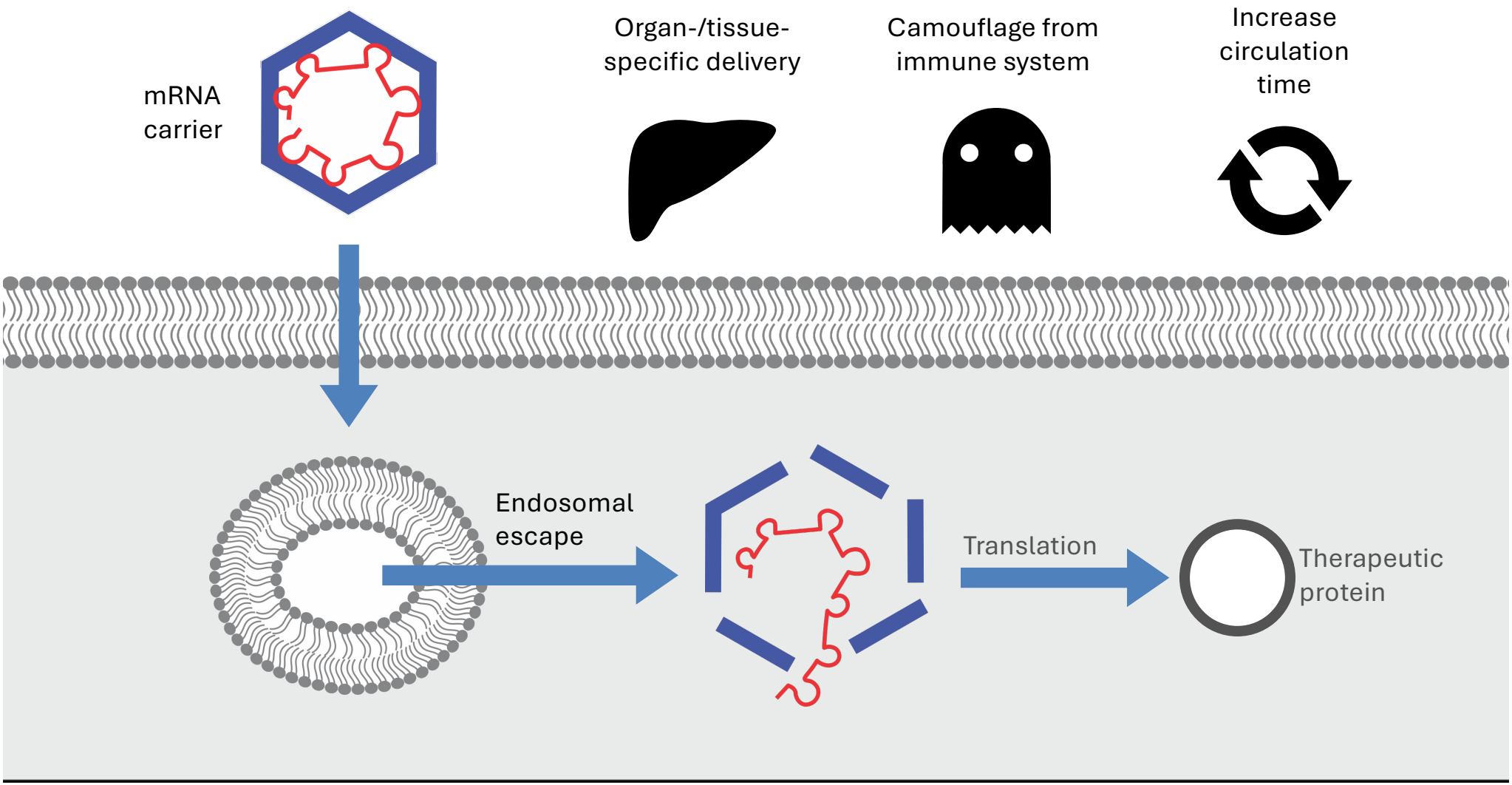
+ Designed
+ Genetically encoded
+ Engineerable & evolvable

Bottom-up

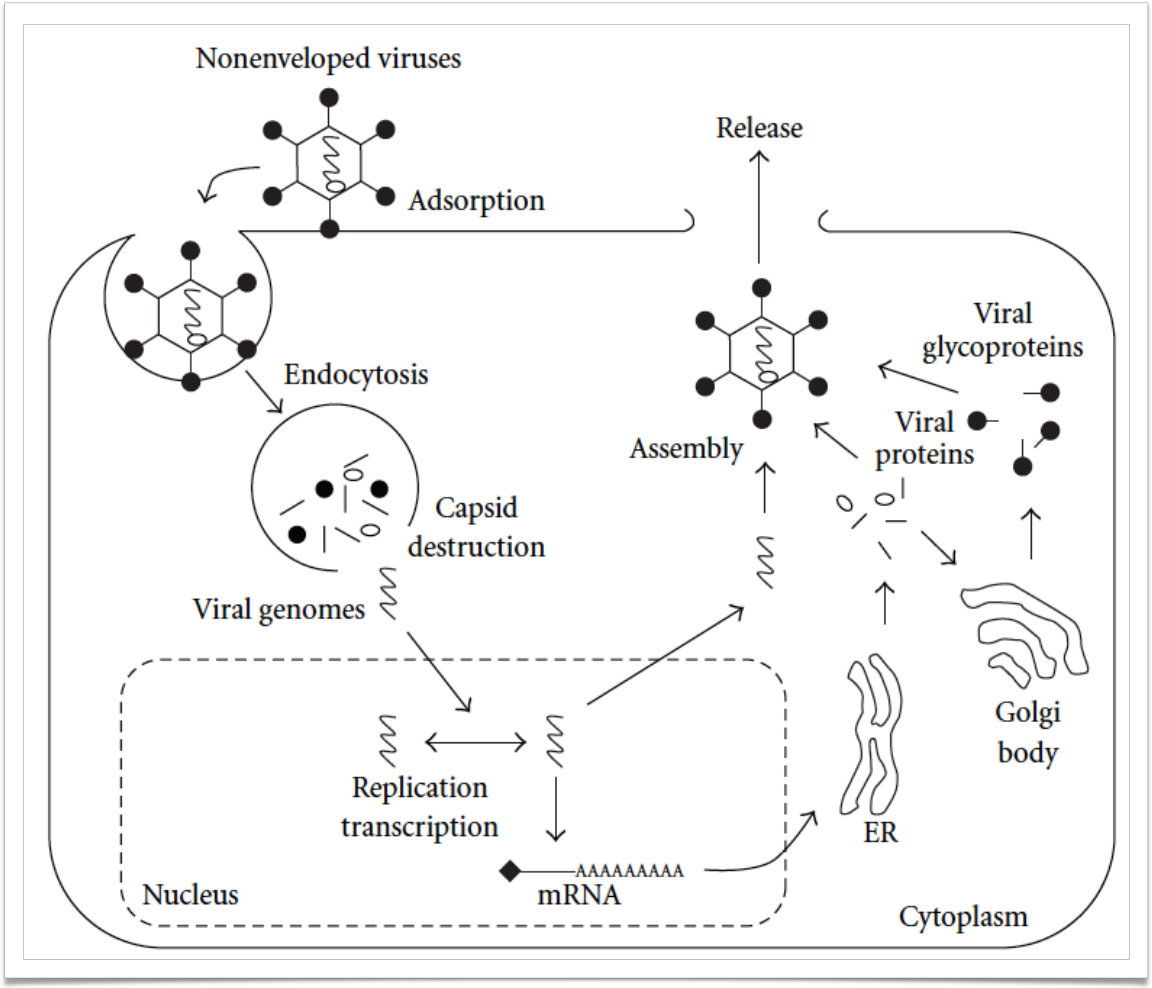
Many more delivery approaches are being studied

- Viral vectors
  - Adeno-associated viruses (AAVs) for certain genetic retinal diseases and spinal muscular atrophy
  - Lentivirus for certain blood disorders
  - Adenovirus for cancer therapy
- Non-viral vectors
  - LNPs for mRNA COVID-19 vaccines
- Physical methods
  - Electroporation in electrochemotherapy for cancer treatment
  - Microinjection for intracytoplasmic sperm injection (ICSI) during in vitro fertilization (IVF)
- Cell-based delivery
  - Stem cell therapies for blood disorders like beta-thalassemia

Gene delivery challenges



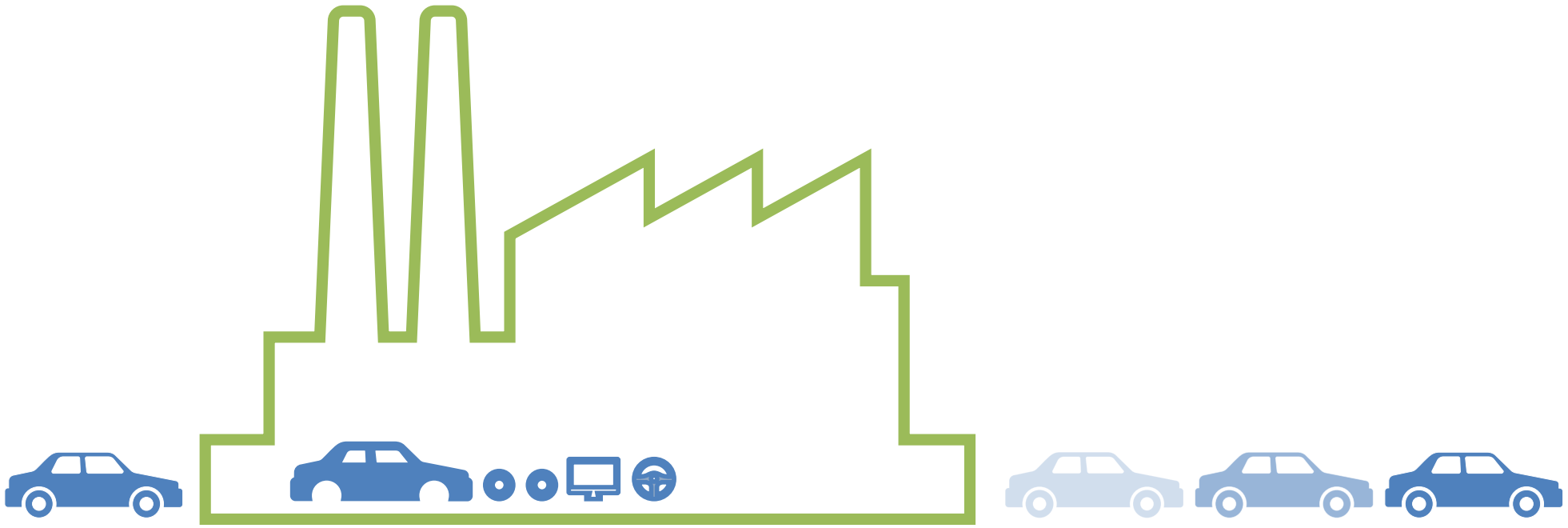
Viruses are excellent gene delivery vehicles



- Viral protein capsid protects genome from degradation
- Packages its genome with high selectivity
- The external face of the protein shell is equipped with targeting domains
- Protein shell acts as a Trojan horse, it is masking it from the immune system

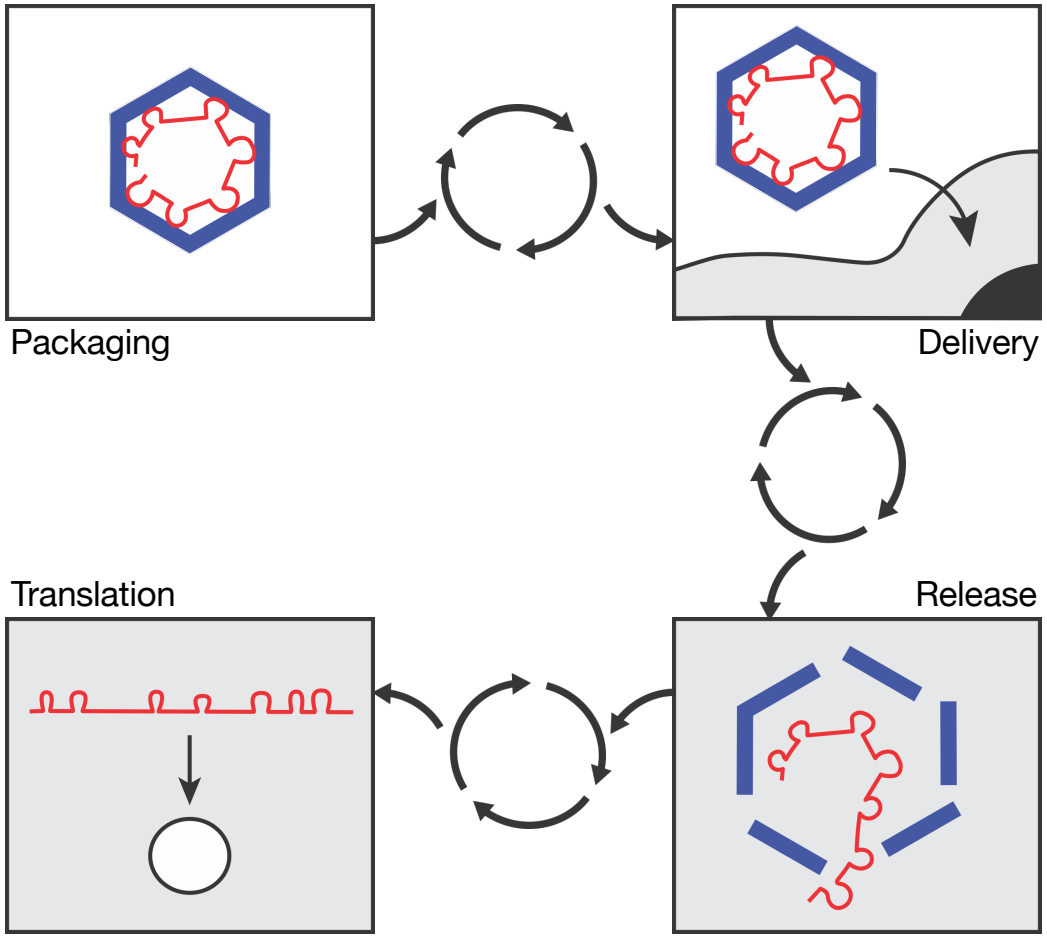
Takahashi, T. *Biochem. Res. Int.* **2011**, *245090*.

Viruses are just (bio)machines



What I cannot create, I do not understand.

- Richard Feynman



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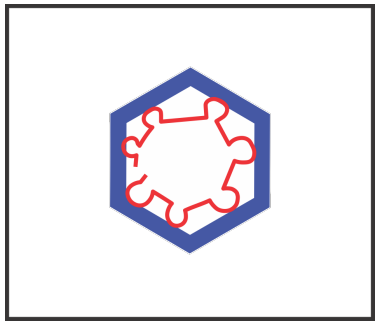
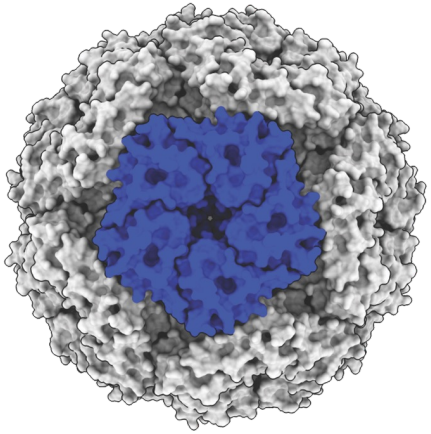


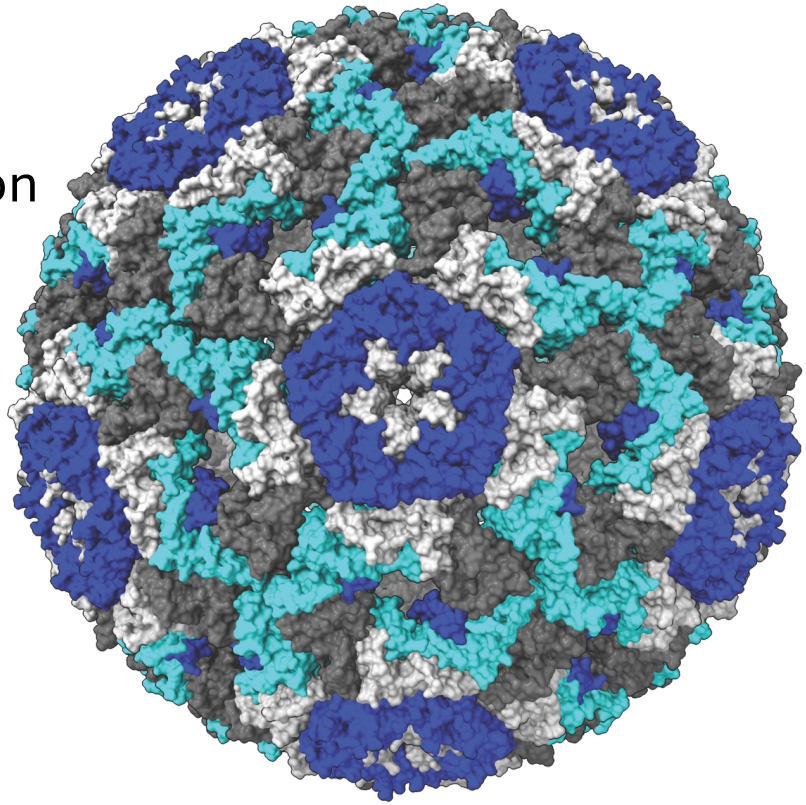
Packaging

Evolution of a capsid that binds its own mRNA



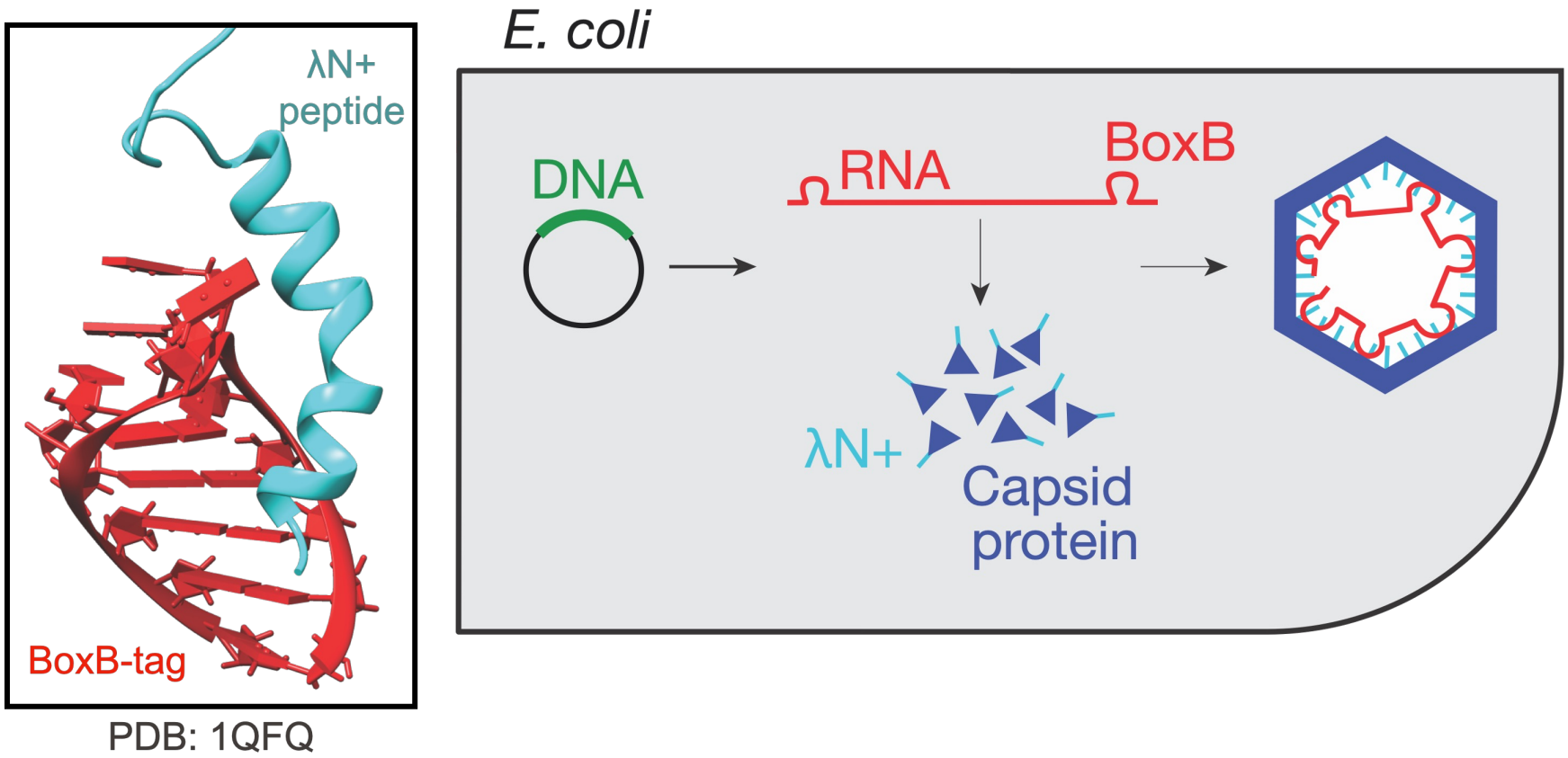
Design:
1. Circular permutation
2. Addition of RNA-binding peptide

Evolution:
Selection for
1. Capsid assembly
2. Stability towards nucleases
3. mRNA integrity



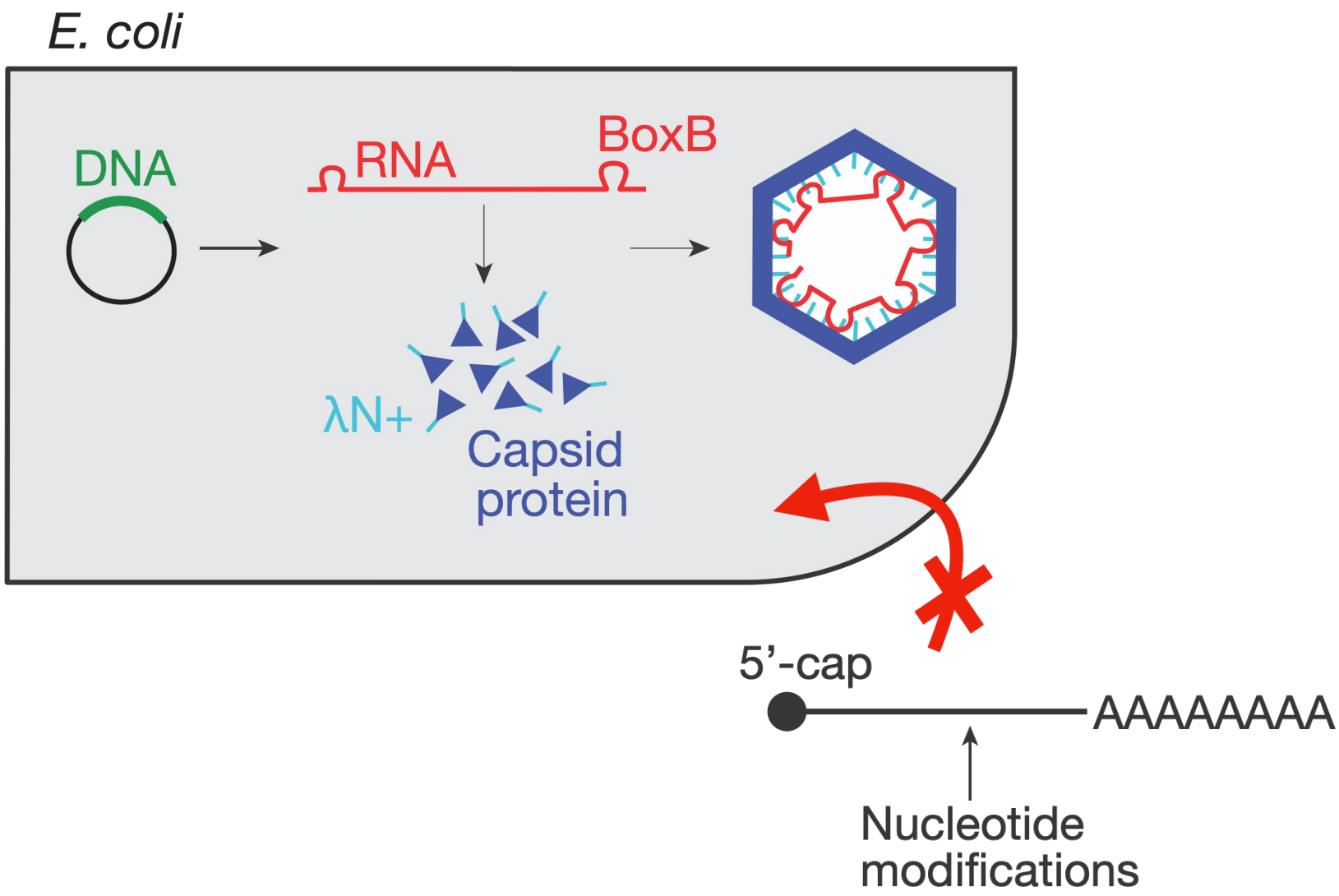
Science **2021**, *372*, 1220.

Capsids assemble in bacteria

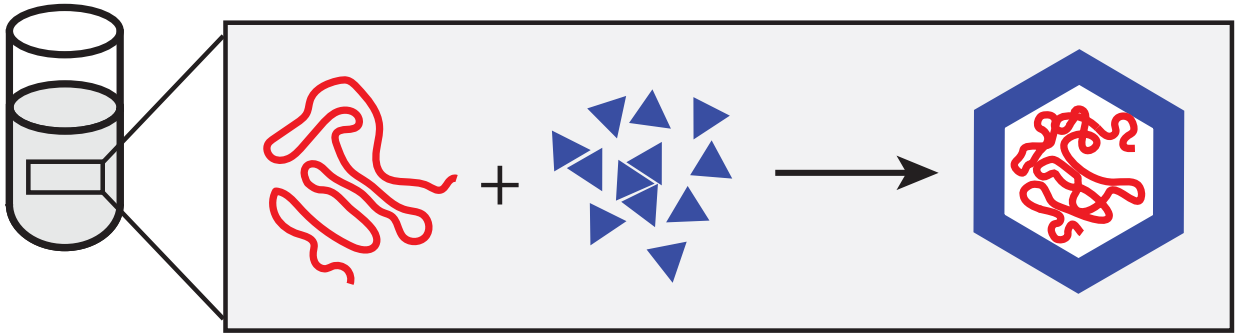


Science **2021**, *372*, 1220.

Capsid loading is limited to bacterial RNA



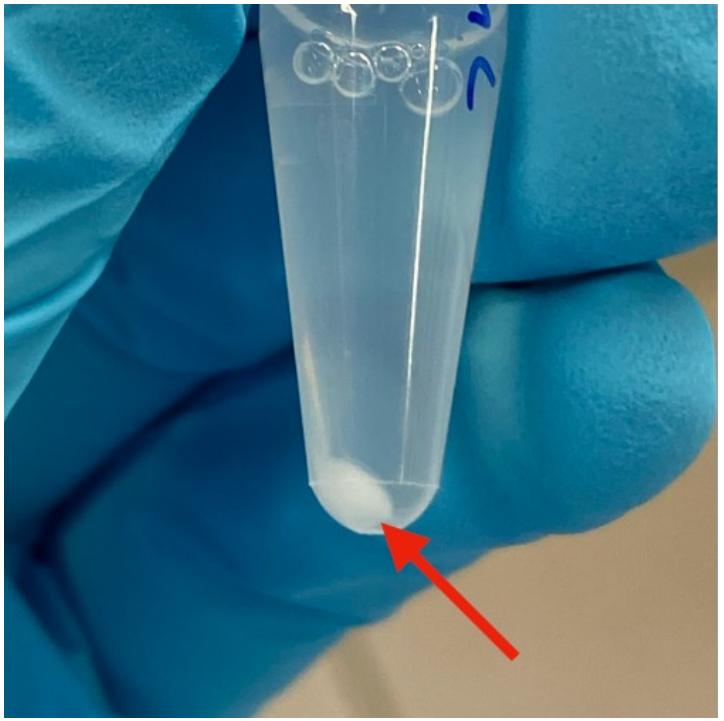
In vitro assembly: a story from my own work



Required components for in vitro assembly

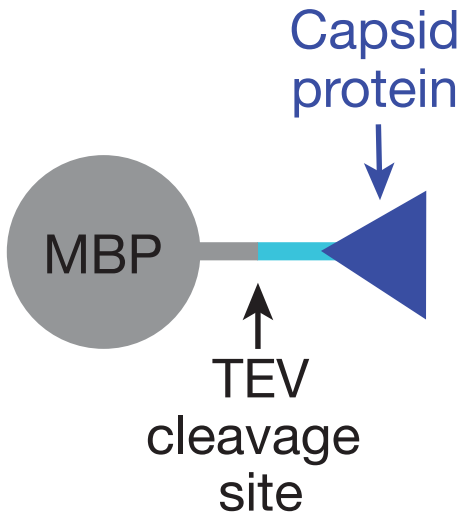
- Purified RNA (in vitro-transcribed)
- Purified protein subunits
  - RNA-free
  - Soluble
  - Monomeric
- Trigger to assemble

- Tested many approaches to produces soluble protein monomers:
  - Two-component assembly
  - Divalent cations to remove RNA
  - Varying ionic strength, pH, etc.
  - Detergents



Always observed precipitation

Solution: steric block solubility tag

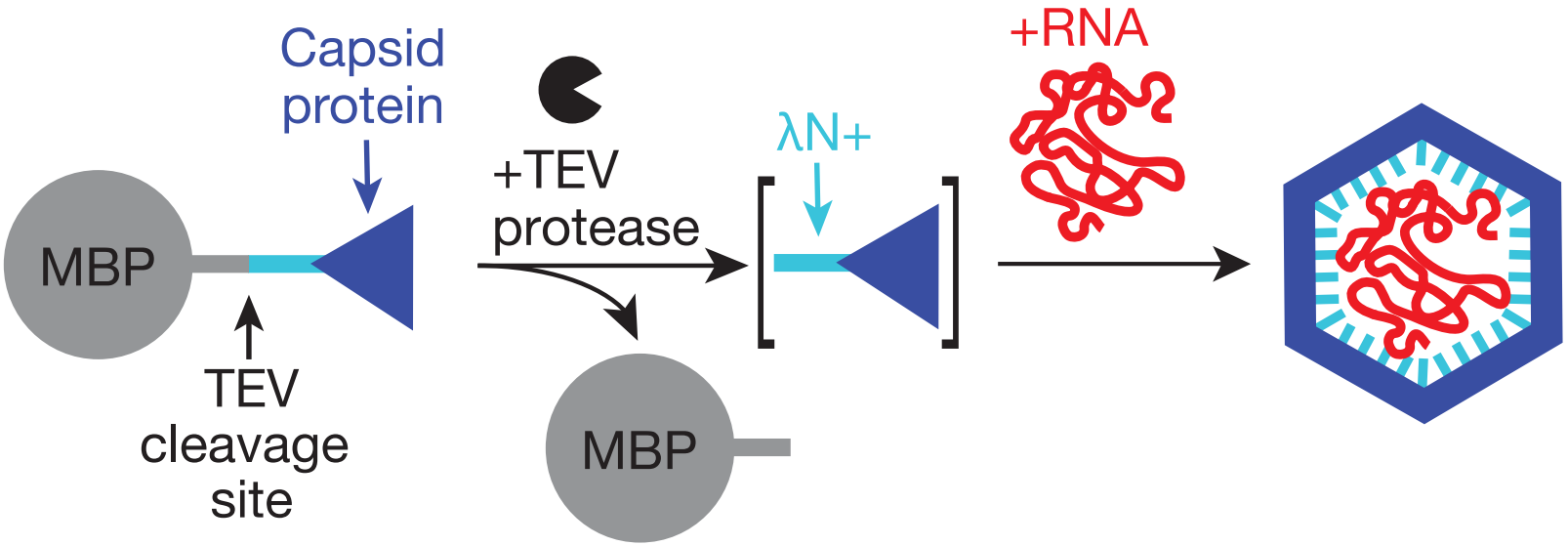


Advantages:

- Increased solubility
- Steric block prohibits assembly
- RNA peptide is masked
- Cleavage can be induced by the addition of protease

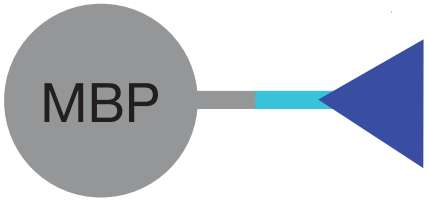
Nat. Commun **2024**, *15*, 3576.

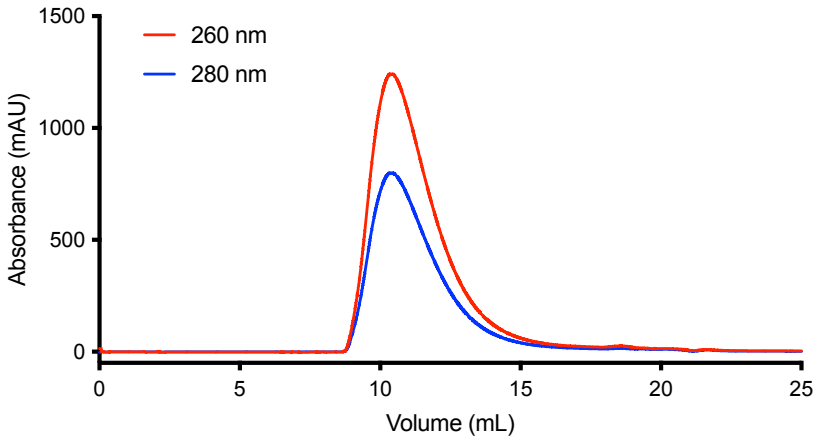
Stimulus-responsive capsid assembly

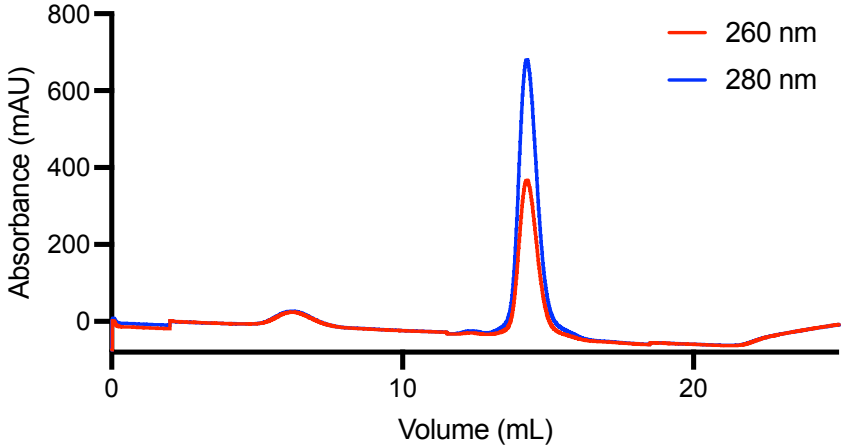


Nat. Commun **2024**, *15*, 3576.

Soluble monomeric capsid protein





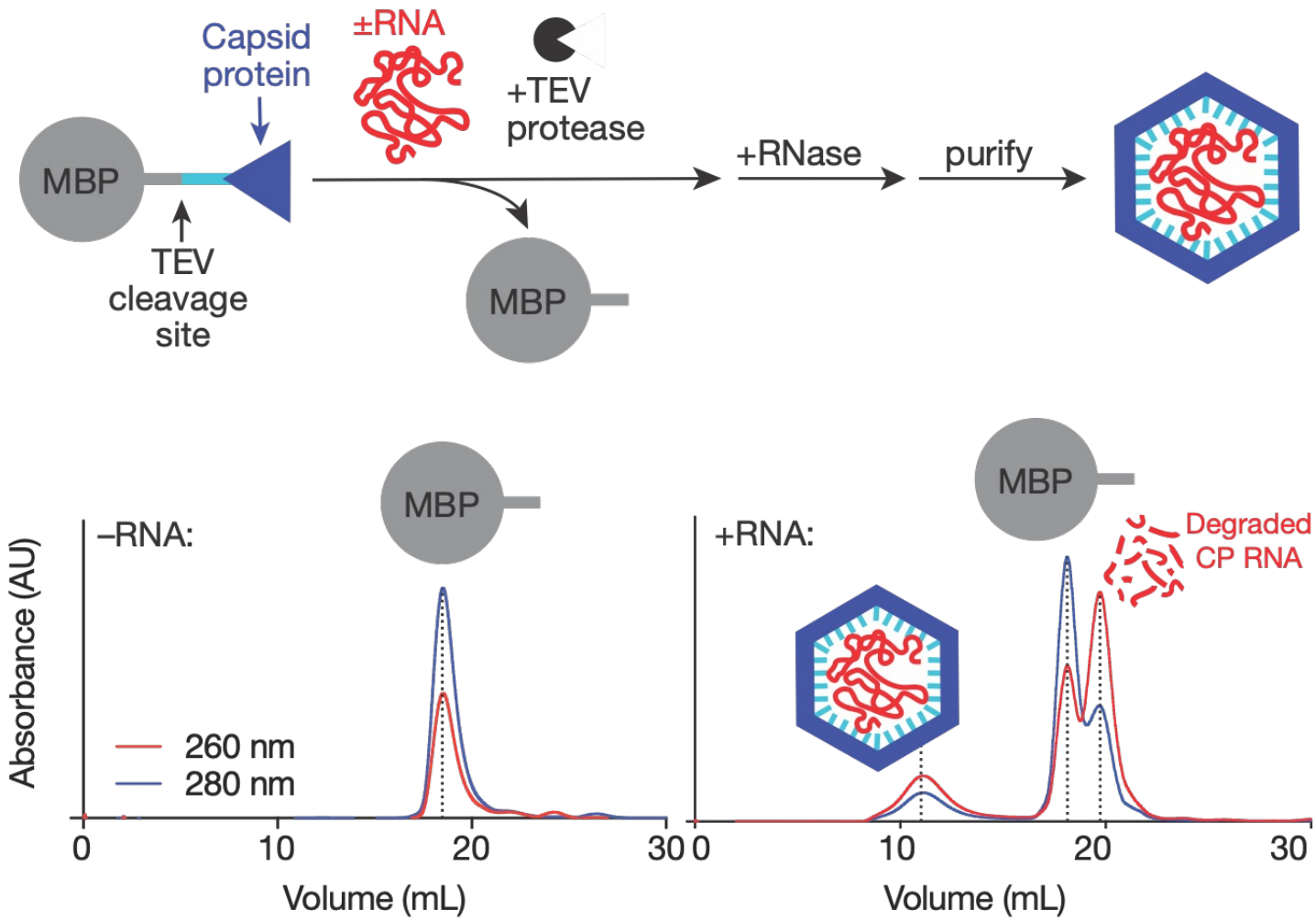


A260/A280 = 1.56

A260/A280 = 0.55

Nat. Commun **2024**, *15*, 3576.

Assembly occurs in the presence of RNA



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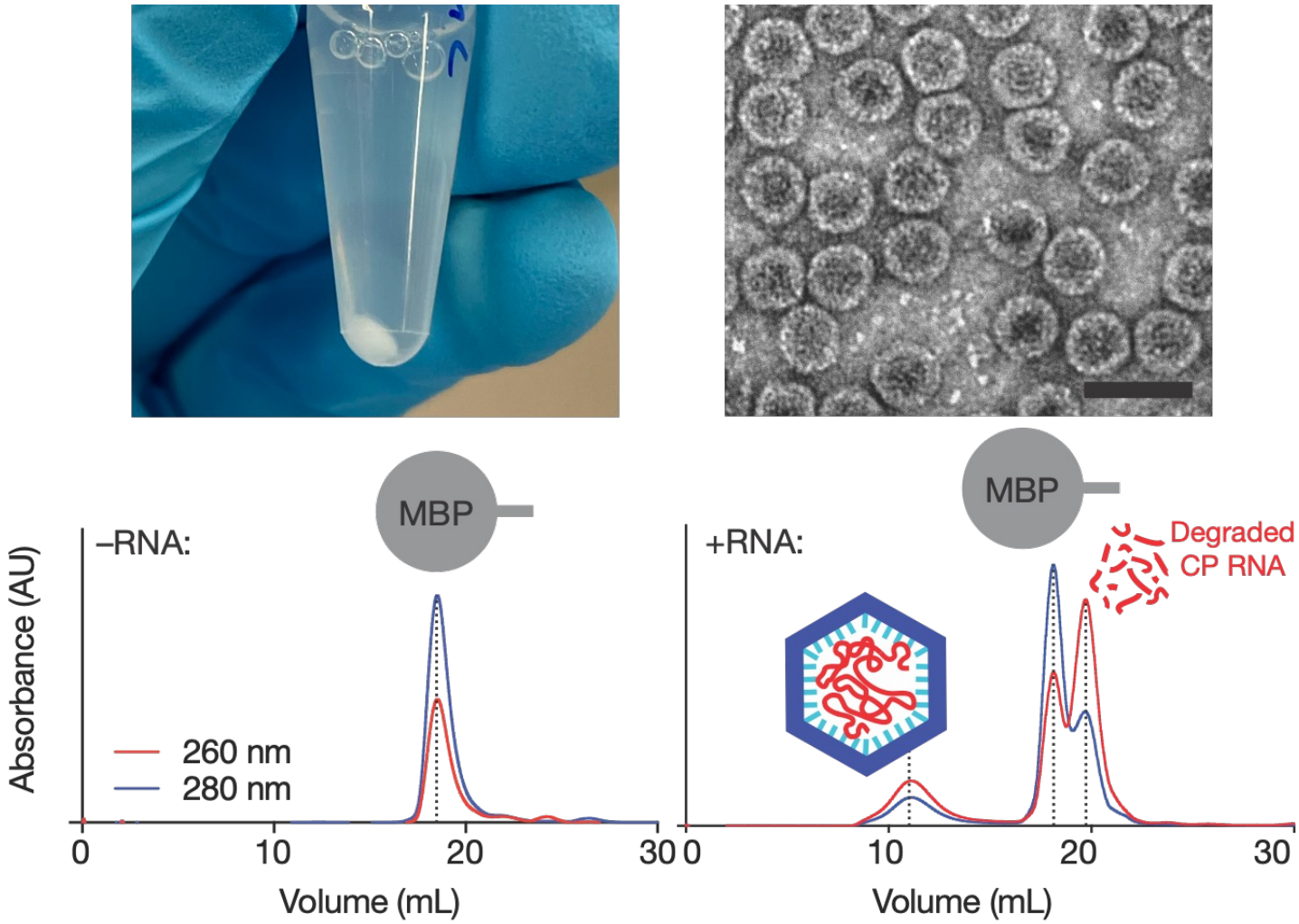
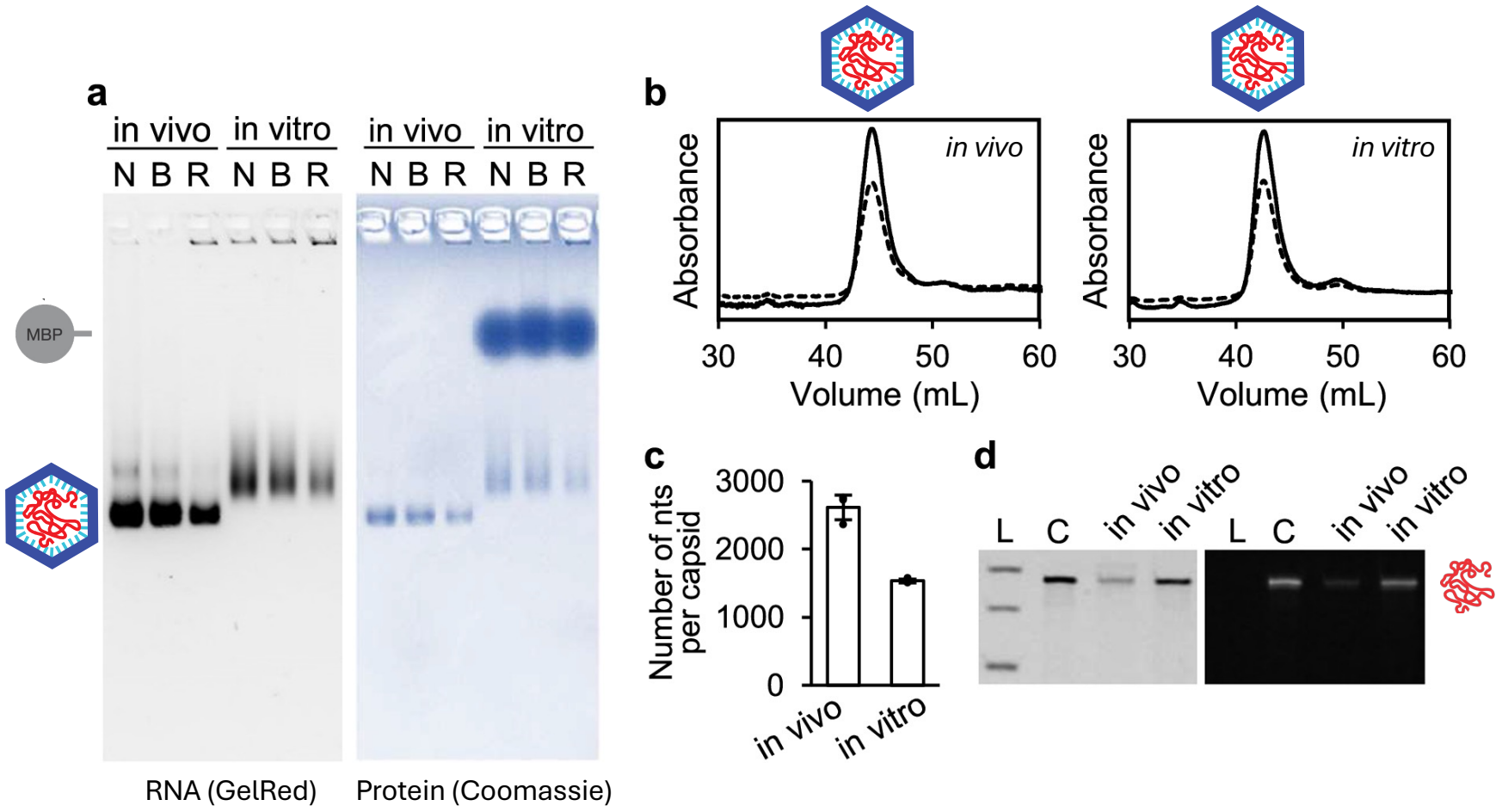


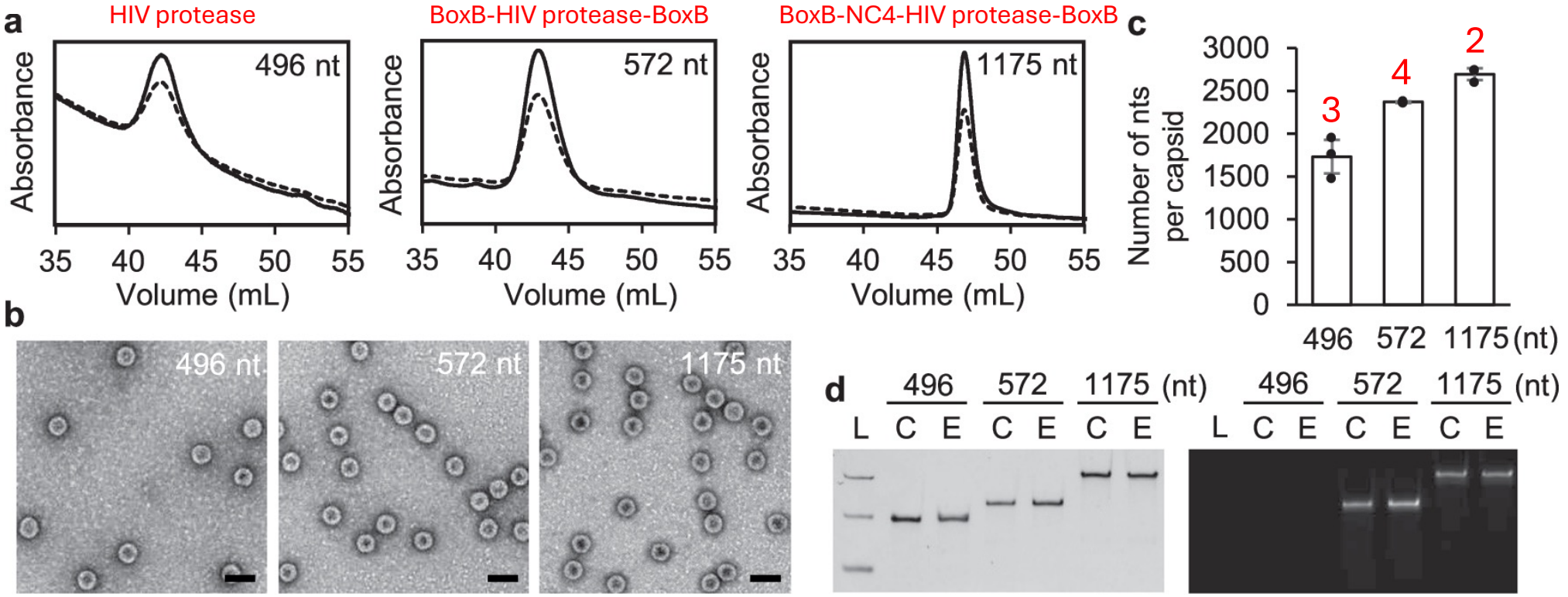
Nat. Commun **2024**, *15*, 3576.

In vivo vs. in vitro-assembled capsids



Nat. Commun **2024**, *15*, 3576.

In vitro assembly with different RNA cargos



Nat. Commun **2024**, *15*, 3576.

Conclusions

Evolved nucleocapsids can be assembled in vitro.

- Approach will be used to
  - decouple RNA and protein sequence
  - study assembly mechanism
  - assemble capsids with longer or synthetically modified RNAs