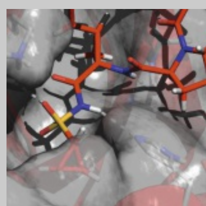
doi:10.2533/chimia.2024.885 Chimia 78 (2024) 885–888 © Swiss Chemical Society



Medicinal Chemistry and Chemical Biology Highlights

Division of Medicinal Chemistry and Chemical Biology

A Division of the Swiss Chemical Society

Protein Engineering and Directed Evolution for Nanocarrier Innovation

Angela Steinauer*

*Correspondence: Prof. A. Steinauer, E-mail: angela.steinauer@epfl.ch
Laboratory of Biomolecular Engineering and Nanomedicine, EPFL-SB-ISIC-LIBN, Station 6, CH-1015 Lausanne, Switzerland

Abstract: The design of nonviral protein-based delivery systems has gained significant attention as an alternative to viral vectors and lipid nanoparticles (LNPs) for gene therapy. While viral vectors offer efficient gene delivery, they present challenges such as immunogenicity and size limitations. LNPs, though pivotal in recent advancements like messenger ribonucleic acid (mRNA)-based vaccines, cause inflammation and exhibit low endosomal escape efficiency. This article explores the development of engineered nonviral protein cages through rational design and directed evolution as an additional nanocarrier option for RNA packaging and delivery. We highlight key advances, including the design and evolution of capsid-forming proteins capable of encapsulating and protecting their own encoding mRNA, a critical step in establishing a genotype-phenotype link for evolutionary optimization. Evolved protein cages, such as the I53-50-v4 and NC-4 variants, demonstrate enhanced stability and RNA protection, achieving structural transformations and packaging efficiencies akin to viral systems. The application of directed evolution has expanded the capacity of these nanocarriers enhancing their *in vivo* stability and biodistribution. This work underscores the potential of evolving nonviral capsids to become customizable platforms for therapeutic gene delivery, while also addressing current limitations in RNA cargo size and tissue targeting specificity. Future directions involve refining these systems to accommodate larger RNAs, and improving immunogenicity properties and dynamic control over cargo release.

Keywords: Capsid engineering · Directed evolution · Gene therapy · Protein design · RNA delivery

Introduction

Gene therapy has the potential to transform medicine by enabling the correction of genetic disorders, the treatment of chronic diseases, and the cure of conditions once considered untreatable.[1–3] Over the past few decades, significant advances, such as the genetic information provided by the Human Genome Project,[4] the development of CRISPR-Cas9 genome-editing technology,[5] and the successful development of mRNA vaccines,[6,7] have propelled the field forward. Despite advancements, significant challenges persist in creating systems for efficient, tissue-specific, and safe genetic delivery across a broader range of diseases. Issues like immunogenicity, stability, and the complexity of endosomal escape during intracellular delivery continue to hinder progress. The lack of a universal nanocarrier further emphasizes the need for diverse and adaptable delivery solutions.

One class of agents known—and feared—for their ability to deliver nucleic acid-based materials across cellular membranes are viruses. Viruses consist of a protein shell or capsid that protects and transports their encoding genome. Because viruses depend on the resources of the host they infect, they have perfected the delivery of their genetic information into host cells. They use many different strategies to circumnavigate membranes, for example by directly fusing with the plasma membrane or by hijacking the endosomal pathway to gain access to the cytosol.[8] Because of the efficiency with which viruses deliver their genomes into cells, many studies have focused on repurposing viral vectors for therapeutic gene delivery. As a result, viral transduction has become one of the most effective methods for gene delivery, both *in vivo* and *ex vivo*, and is used to treat diseases such as B-cell leukemia, β-thalassemia, and spinal muscular atrophy.[9] However, while some vectors, *e.g.* adeno-associated virus (AAV), are generally well tolerated by the immune system, they suffer from size limitations and cannot be employed repeatedly.[10] Others, such as adenovirus itself, efficiently deliver larger genes, but have caused such violent antiviral immune responses that patients have died in clinical trials.[11] While viral vectors have been critical in advancing gene therapy, their limitations highlight the ongoing need to explore alternative delivery systems that can provide the same level of transduction with improved safety and broader applicability.

The advent of mRNA vaccines has highlighted the potential of nonviral gene delivery platforms such as LNPs.[12] LNPs typically consist of ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-lipid conjugates, which self-assemble into nanostructures capable of encapsulating nucleic acids.[13] Their success has largely been ascribed to the inclusion of ionizable lipids, which are proposed to be protonated in the acidic environment of the endosome, potentially promoting membrane fusion and facilitating cargo release into the cytosol.[14,15] The LNP nanocarrier has emerged as a central platform for gene delivery, particularly in RNA-based therapeutics, and is employed in the treatment of diseases such as hereditary transthyretin-mediated amyloidosis and acute hepatic porphyria.[16] It is also used for prophylactic treatments, as demonstrated by the two LNP-based vaccines developed against SARS-CoV-2.[6,7] Despite these advantages, LNP formulations still face significant limitations. They can induce inflammation,[17] exhibit low efficiency in endosomal escape,[18] and rely heavily on lipid composition for tissue specificity,[19] which limits their versatility across different cell types. Additionally — from a protein engineer's perspective — their lipid-based nature makes them unsuitable for optimization through directed evolution, limiting their adaptability.

Evolved Non-viral Protein Cages as Alternative Nanocarriers

An emerging strategy for designing new nanocarriers involves the bottom-up design of nonviral protein cages, using building blocks from either natural or synthetic sources, to pack-

Can you show us your Medicinal Chemistry and Chemical Biology Highlight?
Please contact: Prof. Dr. Kathrin Lang, Dept. of Chemistry and Applied Bioscience, ETH Zurich, Vladimir-Prelog-Weg 1-5/10, CH-8093 Zurich, E-mail: kathrin.lang@org.chem.ethz.ch

age, protect, and deliver nucleic acids.[20–22] As protein engineers, we design sequences with precise control, allowing for tailored modifications at the molecular level. This approach gives us flexibility to understand and optimize each component individually and in combination before increasing complexity. Using protein engineering, nonviral nanocages have been rationally designed to encapsulate small molecules, lipids, proteins, and nucleic acids,[23] others were equipped with external moieties to guide them to specific cell types[24] or to mask them from the immune system,[25] while still others were engineered to self-assemble into bio-orthogonal chemical reactors within cells.[26]

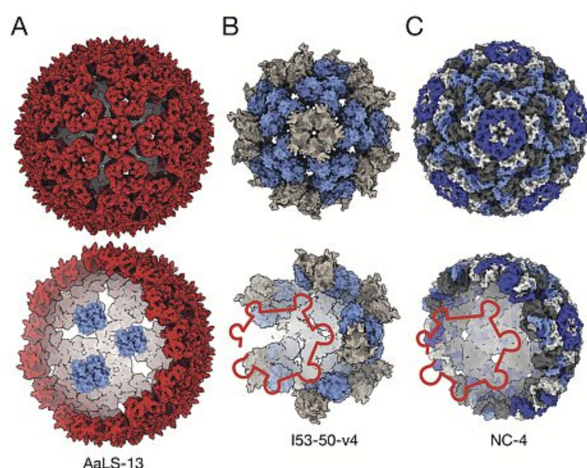


Fig. 1. Evolved non-viral capsids displayed as closed shells (top row) and partially open with schematic representations of cargo used in evolution (bottom row). Electrostatic interactions are represented by color: red indicates negative charge and blue indicates positive charge. (A) Structure of AaLS-13 (PDB: 5MQ7).[29] This capsid was evolved by selecting for sequestration of the toxic HIV protease (PDB: 1KJ7, schematic shown in the bottom row).[28] (B) Design model of I53-50-v4.[20] This capsid was evolved by selecting for capsids that bind their own encoding mRNA (represented by a red RNA cartoon in the bottom row). (C) Structure of NC-4 (PDB: 7A4J).[22] Evolved to bind its own mRNA (represented by a red RNA cartoon in the bottom row), this capsid is based on the bacterial microcompartment lumazine synthase from Aquifex aeolicus.

When rational engineering falls short or yields only partial success, directed evolution becomes a secret weapon in protein engineering capable of optimizing desired traits by harnessing the process of natural selection to refine protein function.[27] In a directed evolution experiment, iterative rounds of mutagenesis, screening or selection, and amplification are performed to identify mutants with the desired properties. The first example of using directed evolution to optimize a self-assembling protein nanoparticle was published by Wörsdörfer, Hilvert and coworkers. They developed the AaLS-13 capsid, a protein container capable of encapsulating the toxic HIV protease (Fig. 1A).[28] The evolution was driven using an electrostatic tagging strategy: the HIV protease was tagged with a poly-arginine peptide, while the interior lumen of the starting capsid, the bacterial microcompartment *Aquifex aeolicus* lumazine synthase (AaLS), was modified to carry numerous glutamate residues, resulting in the capsid AaLS-neg. The electrostatic interaction between the positively charged HIV protease and the negatively charged interior of the AaLS-neg capsid, combined with the toxicity of the protease, was leveraged to drive the selection process. Only cells that successfully segregated the HIV protease within the evolving capsid survived the selection. During the course of the evolution process, the capsid exhibited an increase in size, expanding from an initial diameter of approximately 30 nm in AaLS-neg to around 35 nm in the evolved AaLS-13 variant. This pioneering work demonstrated the potential of selections and directed evolution to create new, robust protein assemblies capable of encapsulating and protecting toxic cargo within biological systems.

When screening large mutant libraries in directed evolution experiments, it is crucial to trace back the activity of a protein to its genetic sequence as we are currently still unable to efficiently sequence entire proteins. This relationship is referred to as 'the genotype-phenotype link'. Without this link, the identified protein cannot be amplified, studied, or characterized. The link can be established in different ways: some methods employ a physical, covalent bond,[30] while others utilize compartmentalization into cells or droplets.[31] Viruses are highly amenable to this requirement, as they inherently exhibit a genotype-phenotype link by packaging their own genome (genotype) within their capsid (phenotype). This property has been harnessed by various directed evolution strategies, including phage display,[32] phage-assisted continuous (PACE) and non-continuous evolution (PANCE),[33] as well as adenovirus-[34] and RNA alphavirus sindbis-assisted[35] continuous evolution. In these approaches, viral replication is directly tied to the activity of the evolving gene, ensuring that only variants with the desired traits are amplified and beneficial mutations are efficiently propagated. In contrast to virus-assisted evolution methods, where the genotype-phenotype link is inherent and leveraged as a feature, directed evolution experiments in nonviral protein cages have recently focused on establishing this link by evolving capsids that encapsulate and protect their own encoding mRNA.[20–22] Even minimal initial binding of mRNA can serve as a starting point for directed evolution to improve packaging efficiency, potentially reaching RNA packaging levels comparable to viral systems. To establish the genotype-phenotype link in nonviral proteins, a plasmid encoding a capsid-forming protein is introduced into bacteria, where the DNA is transcribed into mRNA and translated into many copies of the capsid protein. By applying a selection pressure that enforces the association between the capsid protein and its encoding mRNA, self-mRNA packaging can be evolved (Fig. 2A). This process establishes a genotype-phenotype link, enabling efficient identification and amplification of functional variants.

The experimental evolution of capsids that package their own mRNA has been demonstrated in two separate cases: the Baker group utilized a computationally designed protein cage as a starting point and evolved capsid **I53-v4** (Fig. 1B) [20,36] and the Hilvert group evolved the bacterial protein cage lumazine synthase from *Aquifex aeolicus* (AaLS) to package and protect its own encoding mRNA yielding the capsid **NC-4** (Fig. 1C).[21,22] Both directed evolution campaigns (Fig. 2B) began with rational protein design. Baker and colleagues initially compared two strategies to promote RNA encapsulation: they introduced the Tat RNA-binding peptide from bovine immunodeficiency virus to the lumenal surface and modified the interior capsid surface with positive charges to enhance interactions with negatively charged RNA.[20] The lumenal surface charge strategy yielded superior results. In the case of the Hilvert group, an arginine-rich RNA-binding peptide termed 'λN+' was added to the *N*-terminus of the capsid-forming protein, with its RNA stem-loop binding partner 'BoxB' encoded within the untranslated regions of the RNA.[21] After the protein design stage, capsids in both campaigns were able to package RNA, but inefficiently packaged their full-length mRNA, providing a starting point for evolution. Following these design-based modifications, mutagenesis was employed – either through targeted mutations or error-prone PCR (epPCR) – to introduce additional diversity into the capsid-forming proteins. After mutagenesis, capsids were purified by size exclusion chromatography (Selection A), enriching for assembled structures. To further refine stability, the capsids were subjected to various challenges (Selection B), such as exposure to nucleases (*e.g.* RNase A or benzonase), heat, or blood circulation in mice, se-

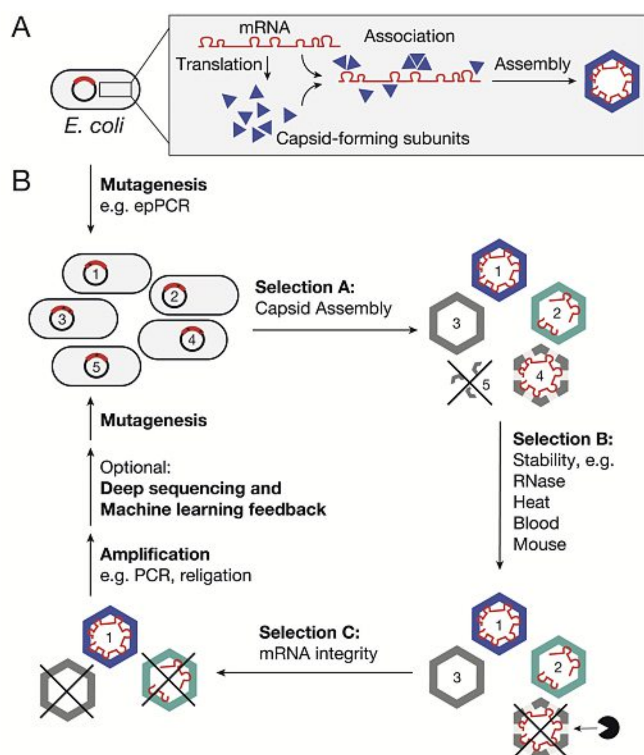


Fig. 2. Directed evolution strategy for evolving non-viral capsids. (A) Capsid-encoding plasmids are transformed into *Escherichia coli*, where they are transcribed into capsid-encoding mRNAs and translated into capsid-forming subunits. The interaction between mRNA and capsid-forming subunits, followed by assembly, yields nucleocapsids. In the absence of evolution, self-mRNA binding and packaging efficiency has been low in published examples.[20,21] (B) Directed evolution approach to enhance self-mRNA binding and packaging. The process begins by introducing diversity into the capsid gene pool through methods like targeted mutations[20] or error-prone PCR.[21,22] Capsids then undergo selection steps: (Selection A) assembly is verified *via* size exclusion chromatography; (Selection B) stability is tested by exposure to nucleases, heat, blood, or injection into mice; and (Selection C) mRNA integrity is checked *via* reverse transcription using gene-specific primers. Surviving mRNAs are amplified, re-ligated into plasmids, and subjected to further rounds of mutagenesis and selection. Optionally, final surviving mRNAs can be sequenced and incorporated into machine learning models to refine RNA-capsid interactions.

lecting for variants that protect their encapsulated RNA. A final selection (Selection C) focused on mRNA integrity by reverse transcribing the remaining RNA with capsid gene-specific primers after Selection B, ensuring only those capsids that effectively preserve their mRNA are retained. The remaining cDNA was amplified and sequenced to identify winning capsid variants. Deep sequencing data could also optionally be fed into machine learning algorithms to identify enriched variants, which could then be reintroduced into the next round of mutagenesis and selection. Although not employed in these two pioneering studies, machine learning has become a transformative tool in directed evolution[37–40] and capsid evolution studies[41–43] helping predict beneficial mutations by analyzing large datasets and enabling the exploration of vast sequence spaces that are otherwise challenging to sample experimentally.

Both capsid evolution strategies achieved significant advances. The Baker group, building on their computationally designed protein cages, evolved variants with enhanced stability and mRNA protection through challenges such as RNase and *in vivo* blood circulation in mice. Their final variant, I53-50-v4, achieved a remarkable 54-fold increase in serum half-life, reaching approximately 4.5 hours. The Hilvert group's directed evolution of the AaLS bacterial protein cage expanded it from a $T = 1$, 16-nm, 60-subunit structure to a $T = 4$, 30-nm, 240-subunit icosahedral capsid capable of encapsulating multiple copies of its own mRNA. The increase in size was facilitated by destabilizing amino acid substitutions causing a domain swap that altered the oligomerization interfaces of the capsid-forming subunits. In addition to modifying the protein, directed evolution led to alterations in the encoding RNA structure, enabling efficient RNA packaging mimicking an RNA packaging signal-templated assembly process similar to what is observed in certain single-stranded RNA viruses. Both examples illustrate how directed evolution, through iterative selection for capsid assembly, stability, and efficient RNA packaging, can overcome the limitations of rational design. This approach generated particles with virus-like properties, characterized by serum stability and packaging efficiency similar to those observed in viruses, and a structure that begins to resemble a virus.

More recently, further development of *in vivo* library selection using synthetic nucleocapsids has been explored. Olshefsky *et al.* showed that nucleocapsids displaying mini-proteins could be evolved in mice to selectively accumulate in tissues such as lungs and muscles within just two rounds of selection.[44] These advances highlight the potential of *in vivo* evolution to optimize biodistribution and identify tissue-specific targeting ligands, contributing to more precise therapeutic delivery. Similar methods have been applied to AAV engineering, particularly to enhance tissue tropism and overcome biological barriers like the blood-brain barrier (BBB).[45] AAV variants have been evolved to cross the BBB in rodent models, and recent developments, such as the discovery of the capsid variant AAV.CAP-Mac,[46] have shown promising results in overcoming this barrier in non-human primates, enabling more effective gene delivery to the brain and expanding therapeutic possibilities.

Limitations Present Opportunities

While these examples demonstrate significant progress, each approach faces its own set of challenges. One key challenge is the engineering of dynamic capsids, particularly those that open and close in response to environmental cues, which remains difficult to engineer in bottom-up nonviral designs. The NC-4 system, although effective in certain contexts, originates from the bacterial protein lumazine synthase, which increases the risk of inducing an immune response in humans. Additionally, both the NC-4-based[21,22] and *de novo*[20,44] scaffolds, while successful in packaging RNA, are limited by their inability to encapsulate larger RNA molecules, such as those needed to encode the Cas9 protein for gene editing. Even AAV vectors, which are widely studied due to their efficiency, are not without drawbacks — their packaging limit is at around 5 kb and they are unsuitable for repeated administrations. Despite these limitations, the methodologies developed for protein engineering and directed evolution stand and offer a robust framework that can be applied to other proteins or to engineer existing nonviral proteins further. Recent innovations offer promising new directions for overcoming these limitations. For example, the Zhang group has explored the human retrovirus-like proteome to identify proteins that, due to their retained ancestral viral functions, naturally bind their own mRNA. Among these, PEG10 was re-engineered into a novel gene delivery system known as selective endogenous encapsidation for cellular delivery (SEND).[47] This system involves grafting untranslated regions (UTRs) from the PEG10 gene to flank the mRNA of interest and employs an endogenous retroviral fusogen to modify the capsid, allowing packaging and delivery of different mRNAs into cells. Their work not only highlights the potential of utilizing human-derived proteins for gene delivery but also illustrates how leveraging our understanding of viral evolution can lead to the development of new, potentially less immunogenic nanocarriers.

Conclusions

As directed evolution continues to drive innovation in capsid protein design, the field is poised for further breakthroughs. Future research may focus on expanding these systems to accommodate larger RNA cargo, such as genome editing tools like Cas9, and improving specificity for targeted tissue delivery. Additionally, the engineering of dynamic capsid behaviors — such as the ability to open and close in response to biological triggers — could enable more precise control over cargo release. Bringing machine learning into directed evolution opens up new possibilities for predicting useful mutations and improving nanocarriers more efficiently, helping to create the next generation of versatile, effective, and safe protein-based delivery systems. In summary, the integration of protein engineering with lessons from nature and viruses could lead to important breakthroughs in gene therapy and other areas of medicine.

Received: October 14, 2024

[1] T. Friedmann, R. Roblin, *Science* **1972**, *175*, 949, https://doi.org/10.1126/science.175.4025.949.

[2] L. Naldini, *Nature* **2015**, *526*, 351, https://doi.org/10.1038/nature15818.

[3] X. M. Anguela, K. A. High, *Annu. Rev. Med.* **2019**, *70*, 273, https://doi.org/10.1146/annurev-med-012017-043332.

[4] M. D. Adams, J. M. Kelley, J. D. Gocayne, M. Dubnick, M. H. Polymeropoulos, H. Xiao, C. R. Merril, A. Wu, B. Olde, R. F. Moreno, A. R. Kerlavage, W. R. McCombie, J. C. Venter, *Science* **1991**, *252*, 1651, https://doi.org/10.1126/science.2047873.

[5] J. A. Doudna, E. Charpentier, *Science* **2014**, *346*, 1258096, https://doi.org/10.1126/science.1258096.

[6] F. P. Polack, S. J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J. L. Perez, G. Pérez Marc, E. D. Moreira, C. Zerbini, R. Bailey, K. A. Swanson, S. Roychoudhury, K. Koury, P. Li, W. V. Kalina, D. Cooper, R. W. Frenck, L. L. Hammitt, Ö. Türeci, H. Nell, A. Schaefer, S. Ünal, D. B. Tresnan, S. Mather, P. R. Dormitzer, U. Şahin, K. U. Jansen, W. C. Gruber, *N. Engl. J. Med*. **2020**, *383*, 2603, https://doi.org/10.1056/NEJMoa2034577.

[7] L. R. Baden, H. M. El Sahly, B. Essink, K. Kotloff, S. Frey, R. Novak, D. Diemert, S. A. Spector, N. Rouphael, C. B. Creech, J. McGettigan, S. Khetan, N. Segall, J. Solis, A. Brosz, C. Fierro, H. Schwartz, K. Neuzil, L. Corey, P. Gilbert, H. Janes, D. Follmann, M. Marovich, J. Mascola, L. Polakowski, J. Ledgerwood, B. S. Graham, H. Bennett, R. Pajon, C. Knightly, B. Leav, W. Deng, H. Zhou, S. Han, M. Ivarsson, J. Miller, T. Zaks, *N. Engl. J. Med.* **2021**, *384*, 403, https://doi.org/10.1056/NEJMoa2035389.

[8] Y. Yamauchi, A. Helenius, *J. Cell Sci*. **2013**, *126*, 1289, https://doi.org/10.1242/jcs.119685.

[9] J. T. Bulcha, Y. Wang, H. Ma, P. W. L. Tai, G. Gao, *Signal Transduct. Target. Ther*. **2021**, *6*, 1, https://doi.org/10.1038/s41392-021-00487-6.

[10] D. Wang, P. W. L. Tai, G. Gao, *Nat. Rev. Drug Discov*. **2019**, *18*, 358, https://doi.org/10.1038/s41573-019-0012-9.

[11] S. Atasheva, J. Yao, D. M. Shayakhmetov, *FEBS Lett*. **2019**, *593*, 3461, https://doi.org/10.1002/1873-3468.13696.

[12] E. Samaridou, J. Heyes, P. Lutwyche, *Adv. Drug Deliv. Rev.* **2020**, *37*, 154, https://doi.org/10.1016/J.ADDR.2020.06.002.

[13] X. Hou, T. Zaks, R. Langer, Y. Dong, *Nat. Rev. Mater*. **2021**, *6*, 1078, https://doi.org/10.1038/s41578-021-00358-0.

[14] J. A. Kulkarni, M. M. Darjuan, J. E. Mercer, S. Chen, R. Van Der Meel, J. L. Thewalt, Y. Y. C. Tam, P. R. Cullis, *ACS Nano* **2018**, *12*, 4787, https://doi.org/10.1021/ACSNANO.8B01516.

[15] S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Y. Sah, D. Stebbing, E. J. Crosley, E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope, *Nat. Biotechnol*. **2010**, *28*, 172, https://doi.org/10.1038/nbt.1602.

[16] J. A. Kulkarni, D. Witzigmann, S. B. Thomson, S. Chen, B. R. Leavitt, P. R. Cullis, R. Van Der Meel, *Nat. Nanotechnol*. **2021**, *16*, 630, https://doi.org/10.1038/s41565-021-00898-0.

[17] S. Omo-Lamai, Y. Wang, M. N. Patel, E.-O. Essien, M. Shen, A. Majumdar, C. Espy, J. Wu, B. Channer, M. Tobin, S. Murali, T. E. Papp, R. Maheshwari, L. Wang, L. S. Chase, M. E. Zamora, M. L. Arral, O. A. Marcos-Contreras, J. W. Myerson, C. A. Hunter, A. Tsourkas, V. Muzykantov, I. Brodsky, S. Shin, K. A. Whitehead, P. Gaskill, D. Discher, H. Parhiz, J. S. Brenner, *bioRxiv* **2024**, https://doi.org/10.1101/2024.04.16.589801.

[18] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, *Nat. Biotechnol*. **2013**, *31*, 638, https://doi.org/10.1038/nbt.2612.

[19] S. A. Dilliard, Q. Cheng, D. J. Siegwart, *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2109256118, https://doi.org/10.1073/pnas.2109256118.

[20] G. L. Butterfield, M. J. Lajoie, H. H. Gustafson, D. L. Sellers, U. Nattermann, D. Ellis, J. B. Bale, S. Ke, G. H. Lenz, A. Yehdego, R. Ravichandran, S. H. Pun, N. P. King, D. Baker, *Nature* **2017**, *552*, 415, https://doi.org/10.1038/nature25157.

[21] N. Terasaka, Y. Azuma, D. Hilvert, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 5432, https://doi.org/10.1073/pnas.1800527115.

[22] S. Tetter, N. Terasaka, A. Steinauer, R. J. Bingham, S. Clark, A. J. P. Scott, N. Patel, M. Leibundgut, E. Wroblewski, N. Ban, P. G. Stockley, R. Twarock, D. Hilvert, *Science* **2021**, *372*, 1220, https://doi.org/10.1126/science.abg2822.

[23] S. Bhaskar, S. Lim, *NPG Asia Mater*. **2017**, *9*, e371, https://doi.org/10.1038/am.2016.128.

[24] Z. Zhao, A. Ukidve, J. Kim, S. Mitragotri, *Cell* **2020**, *181*, 151, https://doi.org/10.1016/j.cell.2020.02.001.

[25] M. A. Kotterman, D. V. Schaffer, *Nat. Rev. Genet*. **2014**, *15*, 445, https://doi.org/10.1038/nrg3742.

[26] S. A. Bode, I. J. Minten, R. J. M. Nolte, J. J. L. M. Cornelissen, *Nanoscale* **2011**, *3*, 2376, https://doi.org/10.1039/c0nr01013h.

[27] C. Zeymer, D. Hilvert, *Annu. Rev. Biochem*. **2018**, *87*, 131, https://doi.org/10.1146/annurev-biochem-062917-012034.

[28] B. Wörsdörfer, K. J. Woycechowsky, D. Hilvert, *Science* **2011**, *331*, 589, https://doi.org/10.1126/science.1199081.

[29] E. Sasaki, D. Böhringer, M. Van De Waterbeemd, M. Leibundgut, R. Zschoche, A. J. R. R. Heck, N. Ban, D. Hilvert, *Nat. Commun.* **2017**, *8*, 14663, https://doi.org/10.1038/ncomms14663.

[30] M. S. Newton, Y. Cabezas-Perusse, C. L. Tong, B. Seelig, *ACS Synth. Biol.* **2020**, *9*, 181, https://doi.org/10.1021/acssynbio.9b00419.

[31] P. Y. Colin, A. Zinchenko, F. Hollfelder, *Curr. Opin. Struct. Biol*. **2015**, *33*, 42, https://doi.org/10.1016/j.sbi.2015.06.001.

[32] J. C. Frei, J. R. Lai, *Methods Enzymol*. **2016**, *580*, 45, https://doi.org/10.1016/bs.mie.2016.05.005.

[33] S. M. Miller, T. Wang, D. R. Liu, *Nat. Protoc*. **2020**, *15*, 4101, https://doi.org/10.1038/s41596-020-00410-3.

[34] C. M. Berman, L. J. Papa, S. J. Hendel, C. L. Moore, P. H. Suen, A. F. Weickhardt, N. D. Doan, C. M. Kumar, T. G. Uil, V. L. Butty, R. C. Hoeben, M. D. Shoulders, *J. Am. Chem. Soc*. **2018**, *140*, 18093, https://doi.org/10.1021/jacs.8b10937.

[35] J. G. English, R. H. J. Olsen, K. Lansu, M. Patel, K. White, A. S. Cockrell, D. Singh, R. T. Strachan, D. Wacker, B. L. Roth, *Cell* **2019**, *178*, 1, https://doi.org/10.1016/j.cell.2019.05.051.

[36] J. B. Bale, S. Gonen, Y. Liu, W. Sheffler, D. Ellis, C. Thomas, D. Cascio, T. O. Yeates, T. Gonen, N. P. King, D. Baker, *Science* **2016**, *353*, 389, https://doi.org/10.1126/science.aaf8818.

[37] K. K. Yang, Z. Wu, F. H. Arnold, *Nat. Methods* **2019**, *16*, 687, https://doi.org/10.1038/s41592-019-0496-6.

[38] A. L. Ferguson, R. Ranganathan, *ACS Macro. Lett*. **2021**, *10*, 327, https://doi.org/10.1021/acsmacrolett.0c00885.

[39] B. J. Wittmann, K. E. Johnston, Z. Wu, F. H. Arnold, *Curr. Opin. Struct. Biol*. **2021**, *69*, 11, https://doi.org/10.1016/j.sbi.2021.01.008.

[40] S. Biswas, G. Khimulya, E. C. Alley, K. M. Esvelt, G. M. Church, *Nat. Methods* **2021**, *18*, 389, https://doi.org/10.1038/s41592-021-01100-y.

[41] A. Z. Wec, K. S. Lin, J. C. Kwasnieski, S. Sinai, J. Gerold, E. D. Kelsic, *Front. Immunol*. **2021**, *12*, 674021, https://doi.org/10.3389/fimmu.2021.674021.

[42] J. Guo, L. F. Lin, S. V. Oraskovich, J. A. Rivera de Jesús, J. Listgarten, D. V. Schaffer, *Trends Biochem. Sci*. **2024**, *49*, 457, https://doi.org/10.1016/j.tibs.2024.03.002.

[43] J. Becker, J. Fakhiri, D. Grimm, *Pathogens* **2022**, *11*, 756, https://doi.org/10.3390/pathogens11070756.

[44] A. Olshefsky, H. Benasutti, M. Sylvestre, G. L. Butterfield, G. J. Rocklin, C. Richardson, D. R. Hicks, M. J. Lajoie, K. Song, E. Leaf, C. Treichel, J. Decarreau, S. Ke, G. Kher, L. Carter, J. S. Chamberlain, D. Baker, N. P. King, S. H. Pun, *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2306129120, https://doi.org/10.1073/pnas.2306129120.

[45] M. Tabebordbar, K. A. Lagerborg, A. Stanton, E. M. King, S. Ye, L. Tellez, A. Krunnfusz, S. Tavakoli, J. J. Widrick, K. A. Messemer, E. C. Troiano, B. Moghadaszadeh, B. L. Peacker, K. A. Leacock, N. Horwitz, A. H. Beggs, A. J. Wagers, P. C. Sabeti, *Cell* **2021**, *184*, 4919, https://doi.org/10.1016/j.cell.2021.08.028.

[46] M. R. Chuapoco, N. C. Flytzanis, N. Goeden, J. Christopher Octeau, K. M. Roxas, K. Y. Chan, J. Scherrer, J. Winchester, R. J. Blackburn, L. J. Campos, K. N. M. Man, J. Sun, X. Chen, A. Lefevre, V. P. Singh, C. M. Arokiaraj, T. F. Shay, J. Vendemiatti, M. J. Jang, J. K. Mich, Y. Bishaw, B. B. Gore, V. Omstead, N. Taskin, N. Weed, B. P. Levi, J. T. Ting, C. T. Miller, B. E. Deverman, J. Pickel, L. Tian, A. S. Fox, V. Gradinaru, *Nat. Nanotechnol*. **2023**, *18*, 1241, https://doi.org/10.1038/s41565-023-01419-x.

[47] M. Segel, B. Lash, J. Song, A. Ladha, C. C. Liu, X. Jin, S. L. Mekhedov, R. K. Macrae, E. V. Koonin, F. Zhang, *Science* **2021**, *373*, 882, https://doi.org/10.1126/science.abg6155.