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Current Opinion in Structural Biology

Artificial protein cages – inspiration, construction, and observation

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Protein cages are hollow, often spherical, protein structures. They are scientifically interesting for reasons including their capability to serve as protective containers for delivering medically useful cargoes to cells. Design and construction of artificial protein cages is a powerful strategy enabling them to be endowed with bespoke properties not seen in natural forms. To this end, structural studies are a vital tool: Structural analyses of naturally existing protein cages can provide an inspiration for artificial designs while determining structures of artificial proteins can confirm that they match expected designs and cryo-EM is now the tool of choice to achieve this. In this review we describe how natural protein cage structures can inform the design of artificial versions and how, in turn, these can exceed the limitations of their natural counterparts.

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Current Opinion in Structural Biology 2020, **64**:66–73

This review comes from a themed issue on **Cryo electron microscopy**

Edited by **Sara Sandin** and **Daniela Rhodes**

For a complete overview see the Issue and the Editorial

Available online 30th June 2020

https://doi.org/10.1016/j.sbi.2020.05.014

Introduction

The fundamental utility of nanometric protein cages is identical to that of macroscale containers: They can act as receptacles for objects placed inside them ('guests'). As a consequence, these guests can be protected from the outside environment (or *vice versa*, a useful capability if the guests are toxic). The container walls constrain the interior volume meaning that multiple objects within the same container are kept in close proximity to each other. This may be beneficial if it is desirable for the objects to interact with each other but would otherwise drift apart.

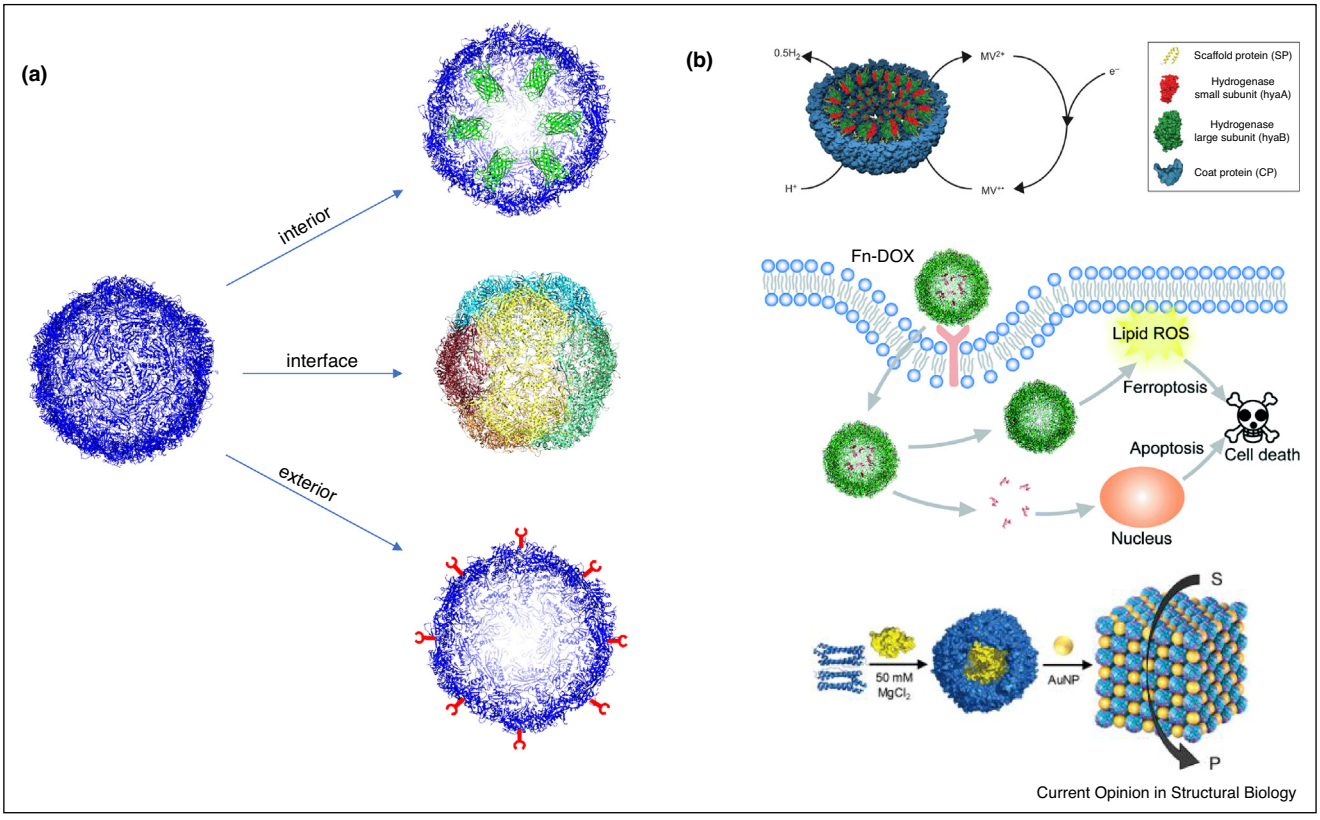
In the biological context, at the nanoscale, there are numerous self-assembled protein cages. Well-known examples include the iron storage protein ferritin [1], bacterial nano compartments such as encapsulins [2] and lumazine synthase [3] as well as the capsids of numerous species of viruses [4]. Recombinant and chemical techniques allow cages to be modified (Figure 1a). Target areas for modifications include the interior surface (for filling with guests); the interface between protein subunits (for modifying assembly/disassembly characteristics) and external surface decoration (for immune modulation/cell targeting). Indeed, natural protein cages have been engineered to provide useful nanocontainers functioning as chambers for chemical reactions [5], delivery vehicles for therapeutics [6], and building blocks to construct macroscale structures [7•] (Figure 1b).

The size of guests in natural protein cages ranges from macromolecules such as nucleic acids or proteins to metal ions [8]. Where protein cages require a large internal volume – as is the case for typical viruses whose lumens are in the several thousands of cubic nanometres range – a number of consequences arise: For example the diameter of such a cage would have to be around 20 nm or larger; were it to be made from a single polypeptide then its total molecular weight would number in the megadalton range. Clearly this is not possible to produce as a single chain. Furthermore, in the case of viruses, the gene encoding such a protein would potentially have a volume larger than the volume of the cage itself. The well-known solution is for protein cages to be constructed of multiple copies of a single or a few kinds of proteins that fit together to tile the cage wall. This leads to certain rules and constraints as to what structures can theoretically be formed. Structural studies on both natural and artificial protein cages have helped us to understand in more detail these rules which in turn will help in the design of new structures.

Natural cages as a guide to artificial cage design

Understanding the structure and function of natural cages can guide the design and construction of artificial cages. Thanks to early structural work, the geometrical principles of natural protein cage assemblies is now well defined, stemming from research on viral capsids, the protein shells encapsulating viral genetic materials. These were some of the earliest protein structures solved by X-ray diffraction analysis and helped Caspar and Klug to develop their quasi-equivalence theory, referred to as CK theory hereafter [9]. In it, virus cages can be viewed as having icosahedral symmetry: The smallest viral capsid having the triangulation number 1 ($T = 1$) indicating that

Figure 1

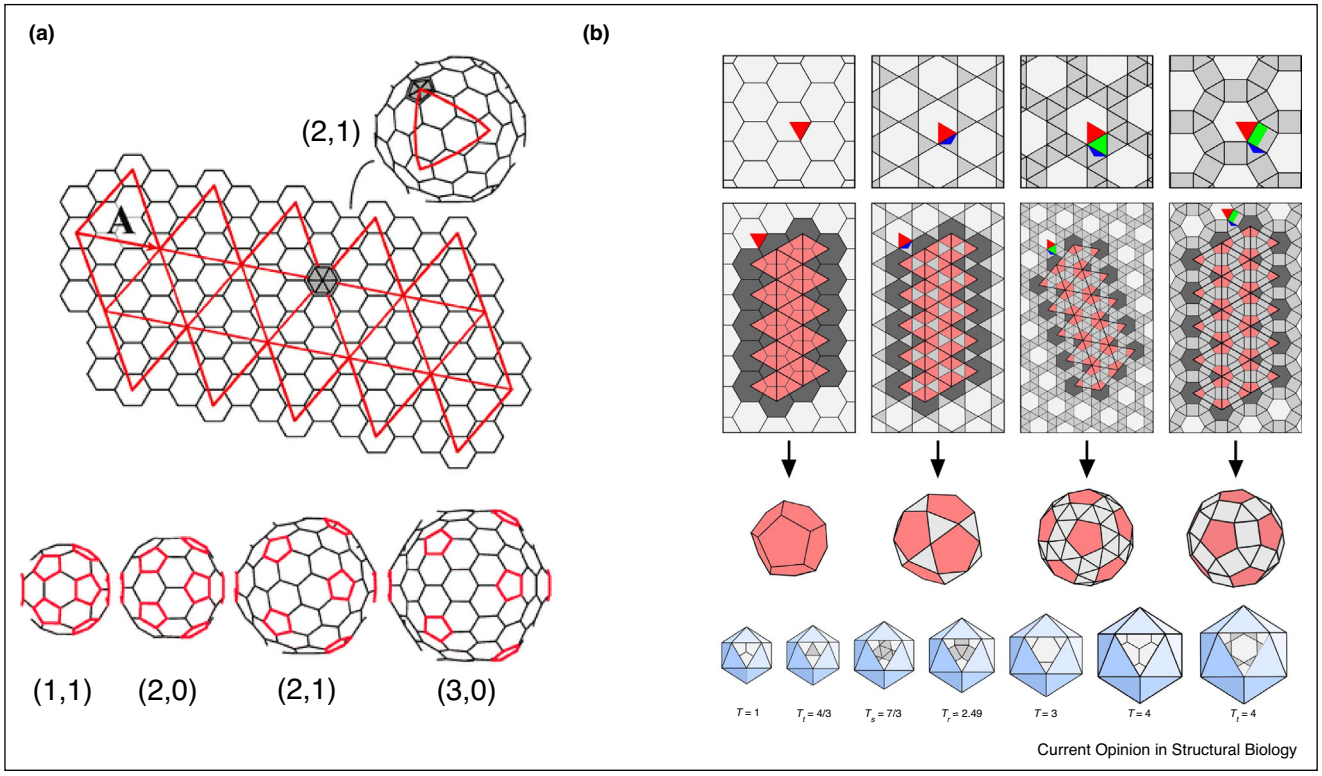


(a) Location of protein cage modifications. Encapsulin (PDB 3DKT) is used as an illustrative cage. Cages can be modified at 3 locations, the interior surface (for example to include a protein cargo as exemplified by GFP (green PDB 1GFL), the interface between protein subunits (each subunit shown in a different colour) and the external surface (red 'hooks'). **(b)** (top) Encapsulation of [NiFe]—hydrogenase within the bacteriophage P22 capsid for H_2 production using methyl viologen ($MV+^{\bullet}$) as an electron donor. Reprinted by permission from Springer Nature [48]. (middle) Cancer cell death caused by doxorubicin loaded ferritin cages. Reproduced from Ref. [6]—Published by The Royal Society of Chemistry. (bottom) Three-dimensional crystal lattice made of ferritin cages loaded with lysozymes. Reprinted by permission from ACS: [7•] https://pubs.acs.org/doi/10.1021/acs.nanolett.9b01148.

it is constructed of 12 pentagons, each consisting of five protein subunits with each pentagon locating on each of the 12 vertices of an icosahedron, giving 60 proteins in total. The capsid diameter can be increased by distancing the pentagons and filling in the 'gaps' with hexagons. The geometrical patterns are classified by the distance between pentamers, defining the triangulation numbers (Figure 2a).

While high-resolution structural studies of capsids have been achieved using X-ray crystallography [10], the large highly symmetrical nature of protein cages makes them ideal candidates for structural investigation using cryo-EM single particle reconstruction techniques. Numerous cryo-EM structures have now been solved of both viruses [11] and other natural protein cages [12,13]. This structural information allows us to modify both the exterior and interior of such cages in a rational manner, leading to useful outcomes. For example, exterior modification of protein cages can be utilized to produce artificial vaccines. This is because a single cage contains multiple repeating monomer units and thus genetic fusion of an antigen to one monomer results, after assembly, in it being displayed on the surface multiple times with a high density. This is ideal to enhance the immune response. Indeed, viral capsids and other protein assemblies have been extensively exploited as display vehicles for vaccine development [14,15]. Additionally, the interior of protein cages can be utilized for accommodating other molecules and by protecting guests from the outside environment, they can potentially serve as useful carriers for drug delivery purposes [16]. Structure-based engineering of naturally existing protein cages is a powerful strategy to encapsulate guests inside their lumen. Such an approach has been recently exemplified by circularly permutated lumazine synthase that was engineered to relocate the native sequence termini at the cage exterior to the lumen. By genetic fusion to the topologically rearranged subunit,

Figure 2



(a) Geometric representation of Caspar and Klug theory which enumerates possible icosahedral designs by mapping its 20 triangular faces on a 2D-lattice. The lattice is presumed to consist of regular hexagons, with the exception that each vertex from the triangulation should be a pentagon. This results in a curving of the hexagonal matrice into a sphere. The CK capsids are described with two integers, h and k which are related to T as follows: $T = h^2 + hk + k^2$ and the number of protein subunits is 60 in the $T = 1$ state. Bottom row shows CK structures with $T = 3$, 4, 7 and 9. Reproduced from Ref. [49] with permission from the PCCP Owner Societies. **(b)** (top) Representation of 4 Archimedean lattices permitting Caspar–Klug construction of 4 Archimedean solids with icosahedral symmetry by changing 12 hexagons into pentagons (middle), (bottom) Smallest polyhedral structures which differ in size from classical Caspar and Klug designs or represent an alternative arrangement for equal size capsids. Reproduced from Ref. [20••].

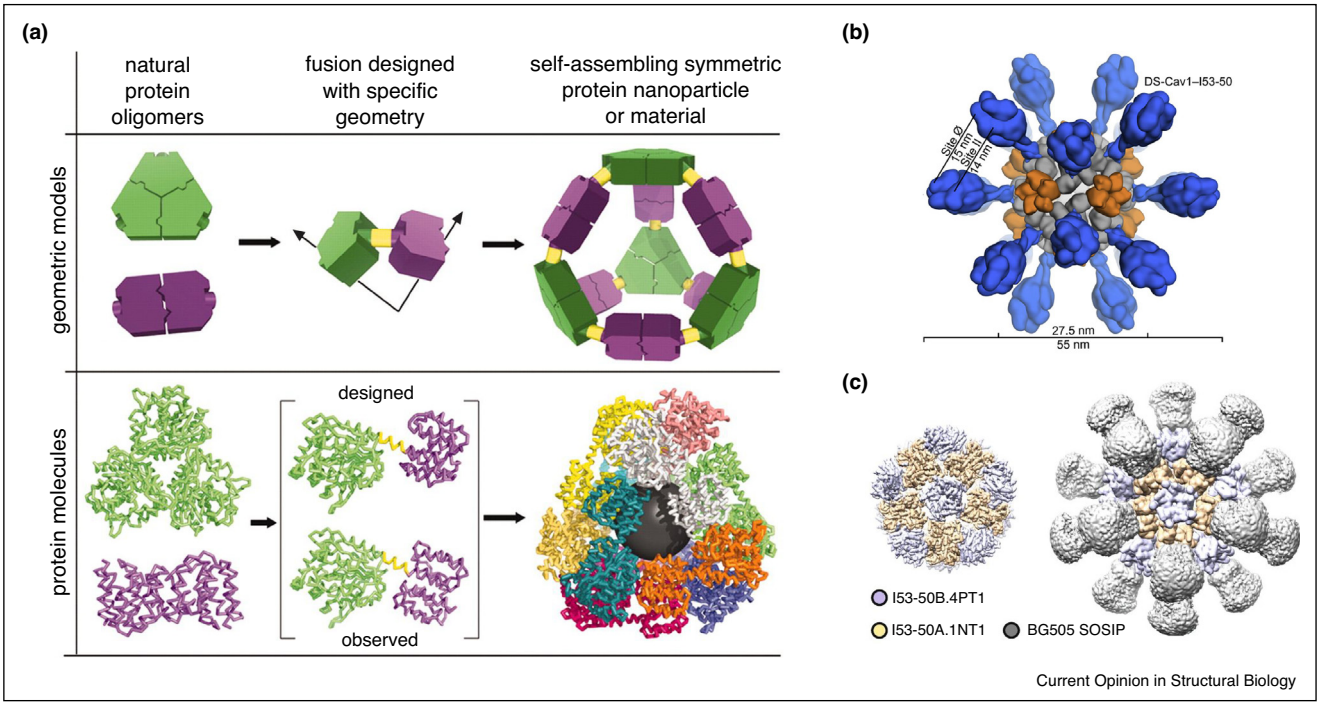
target proteins or short peptides that bind other molecules can be displayed on the interior, affording a facile means for encapsulation of guest macromolecules [17,18].

Going beyond nature

While most natural protein cages conform to the CK theory there are some interesting outliers. One such example is dengue virus, where three E monomers in each icosahedral asymmetric unit do not have quasi equivalent symmetric environments in the external, icosahedral scaffold [19] falling outside CK theory and showing that the evolution of viruses may still not be fully understood. This has been addressed recently by Twarock's and Luque's work demonstrating that CK theory can be expanded by constructing the icosahedral architectures based on the Archimedean lattices that is, lattices in which all polygons are regular, and each vertex is surrounded by the same sequence of polygons [20••] (Figure 2b). Only a handful of Archimedean lattices contain a hexagonal sublattice (Figure 2b) with one of them being the basis for CK construction. Twarock and Luque used these tilings to derive and classify icosahedral polyhedra by replacing 12 hexagons with pentagons, obtaining icosahedrally symmetric Archimedean solids (Figure 2b). The structures produced via this process widen the spectrum of possible viral capsids architectures as CK theory describes only icosahedral structures built by repeats of an identical asymmetric unit and excludes capsids built from proteins of different sizes which is possible in Twarock and Luque theory and which may inspire new protein cage designs.

Artificial protein cages are a relatively new area of protein-cage research. Early work by Lai *et al.* demonstrated the idea using a symmetry engineering technique whereby proteins with different rotational symmetries were fused together to produce a building block capable of self-assembly into a platonic solid [21] (Figure 3a). More recently the Baker group has been able to utilize computational approaches to design the interface between

Figure 3

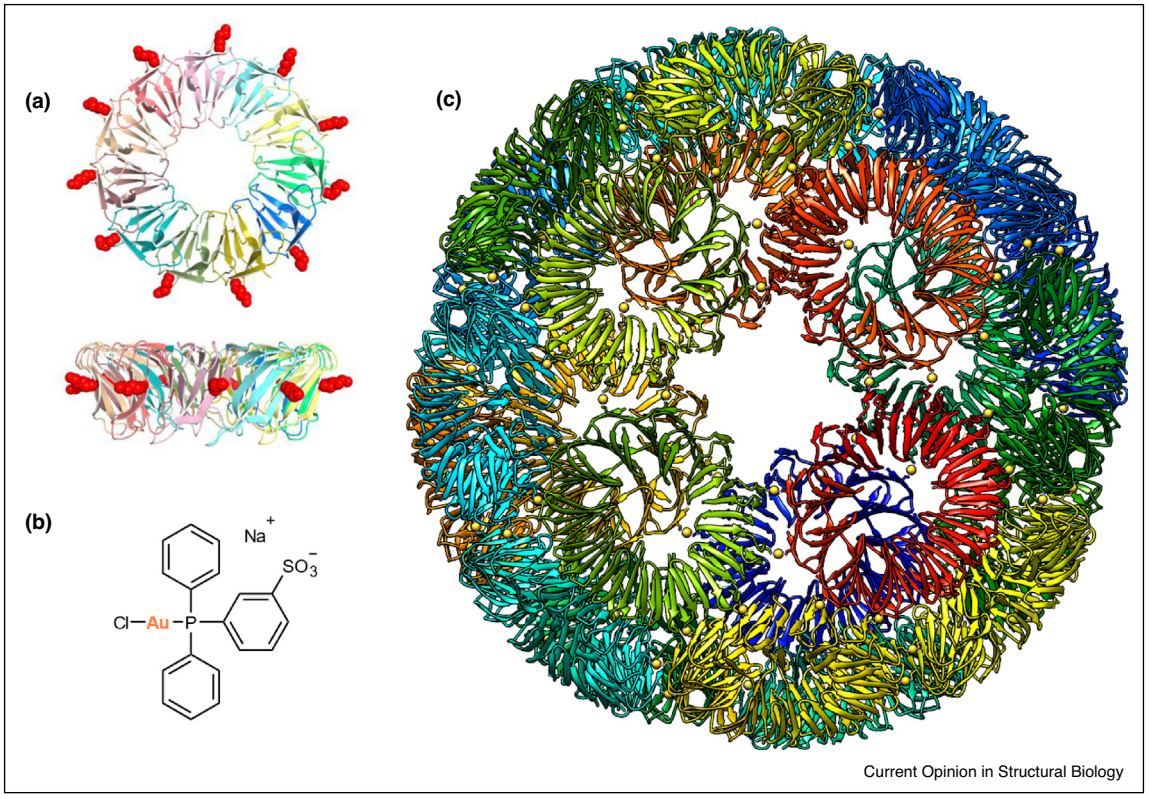


Structures of artificial protein cages.
(a) (top) Principles of designing protein nanocages by fusion strategy represented by geometric models. Fusion of individual subunits (middle) which naturally oligomerize into dimers or trimers (left) results in assembly of tetrahedral protein cage (right), (bottom) crystal structures of natural protein subunits (left), comparison between designed model and observed crystal structures of individual subunits fusion (middle), crystal structure of tetrahedral self-assembled cage (right), From Ref. [21], reprinted with permission from AAAS. **(b)** Model showing proposed structure of I53-50 protein cage with displayed Ds.-Cav1 trimer. Adapted from Ref. [25••] **(c)** Details of artificial protein cage I53-50 (left) and Gaussian filtered map at low contour showing docked mimics of Env trimers (right). Adapted from Ref. [26•].

protein building blocks of artificial cages, doing away with the necessity of preexisting interactions [22–24]. This has resulted in production of a number of cages corresponding to platonic solids. The cryo-EM structures of several have been determined including a number further modified for potential therapeutic (vaccine) use. For example, a computationally designed protein cage made of the two-component protein complex I53-50 [24] was decorated on its exterior by genetic fusion with a variant of F glycoprotein trimer (DS-Cav1) to produce a nanoparticle immunogen against respiratory syncytial virus (RSV) [25••]. The antigen-displaying protein cage (Figure 3b) was shown to induce a neutralizing antibody response 10-fold greater than DC-Cav1 alone. I53-50 was also used to present recombinant mimics of viral envelope glycoproteins from human immunodeficiency virus on its surface showing its efficacy as primary immunogen [26•] (Figure 3c).

Artificial protein cages have the potential to extend beyond what is typical in nature in terms of both the geometry and the chemistry whereby cages are formed. As such, we have recently described an artificial protein cage composed of a toroidal, 11-mer protein, trp RNA-binding attenuation protein (TRAP) from *Geobacillus stearothermophilus* [27,28,29••]. This unusual cage ('TRAP-cage') is made from 24 copies of the 11-mer (Figure 4). Monomers in the ring connect to monomers of neighbouring rings via thiol-gold-thiol coordination. Despite the apparently ordered cage-like assembly, a regular-faced convex polyhedron (excluding prisms and antiprisms) cannot be made from such a polygon in theory [30]. Cryo-EM single particle reconstruction studies revealed how this geometrical 'trick' was achieved: In the TRAP-cage, the rings arranged themselves to approximate an Archimedean solid. Although this geometrical solid is also 'forbidden' for hendecameric building blocks, the deviation is likely small enough to be accommodated by malleability of the protein structure. If similar levels of errors are allowed, other types of protein 'polygons' such as heptamers should also be able to make apparently regular protein cages, despite being strictly disallowed [30]. This realisation, that small errors can be accommodated, opens up a large library of potential protein cage building blocks which may not have previously been considered.

Figure 4



(a) The crystal structure of the TRAP ring protein (PDB 4v4f). Each monomer is shown in a different colour. Red spheres highlight the position of K35 which was mutated to cysteine in the cage-forming variant. **(b)** Monosulfonyl triphenylphosphine gold(I) (shown in orange) used for inducing TRAP cage formation. **(c)** TRAP-cage consisting of 24 TRAP rings with each ring connected to 5 neighbours via –S–Au–S– bonds involving opposing cysteine side chains. Each TRAP ring is shown in a different colour with gold(I) ions as yellow spheres.

As already noted, by analogy with natural protein cages, it would be of interest to be able to place molecular guests or 'cargoes' into artificial protein cages. Here, the knowledge obtained through development of packaging systems with natural cages can be transferred to artificial ones. A striking example has recently been reported by the Hilvert group, where a computationally designed protein cage, O3-33 [22] was mutated to possess a positively supercharged interior, a strategy previously exploited for lumazine synthase cage to encapsulate complimentarily charged molecules [31]. The resulting O3-33 cages have been shown able to capture siRNA and deliver it to mammalian cells [32••]. Moreover, another de novo designed protein cage, I53-50 [24] has been evolved in the laboratory to package its own mRNA genome in a similar manner as viral nucleocapsids, likewise an engineered lumazine synthase cage [33••,34].

Observing designed cages

The actual structure of artificial cages may not always match the design. For this reason, validation of design approaches and finalised structures using high resolution methods is essential. Both X-ray crystallography [24,35] and cryo-EM [36] have been widely used. Recent efforts have improved the resolution of protein cryo-EM to near-atomic level, propelling it out of the era of 'blobology' in the so-called 'resolution revolution' [37,38] and the technique is now ideally suited to detailed analysis of protein cages [25••,39•] (Figure 3b,c). Single particle analysis offers the opportunity to sample numerous structural states of the measured sample and determine its structure by combining images of the molecules in similar states [40]. Samples preparation varies but is typically achieved by applying them to an EM grid covered with a holey carbon film, followed by plunge-freezing in liquid ethane and liquid nitrogen and embedding in vitreous ice where the molecules may display different orientations [41].

For protein cages intended for cell delivery, it would be useful to be able to use high resolution imaging to understand if cell entry has been achieved and the intracellular fate of the cages. Protein cages are typically amenable to fluorescence labelling, allowing in-cell imaging using confocal microscopy [42]. This is still

challenging to achieve with high precision due to the small size of the cages. One solution may be to use in-cell cryo-electron tomography (cryo-ET) in which the biological material such as tissues or cells is flash frozen, cut into thin sections and imaged by an electron microscope [43]. Images in different orientations are captured by tilting the sections to obtain information in three dimensions. The multiple images are then aligned to reconstruct the 3D structure or tomogram of the object. This imaging technique is capable of obtaining nanometer scale information from the cellular environment of the samples in a near-native state. The bottleneck of cryo-ET structural analysis is the involved sample preparation step which was recently facilitated by new developments such as the application of focused ion beam (FIB) milling to thin samples to their ideal imaging thickness. This makes it possible to generate thin cell slices directly on the transmission electron microscope grids carrying the vitrified sample [44]. Another development which can help to visualize protein cages inside cellular compartments is the merging of fluorescent light microscopy with electron microscopy known as CLEM (correlated light microscopy and electron microscopy) having the advantages of both. Here, samples can be analysed in two modes: first by fluorescence imaging of the tagged proteins followed by fixation and EM analysis or the preparation of ultra-thin sections for EM still containing fluorescent labels and their analysis by both EM and LM [45]. As artificial cages move from basic to applied research, these techniques will be increasingly used to image them in cells.

Summary and perspectives

Using theoretical approaches and structural knowledge gained from natural protein cages as a start point, artificial nanometric protein cages are being developed with properties extending beyond those found in nature. These include altered physical properties such as programmability of cage assembly/disassembly with obvious benefits for 'triggered' drug release. Such cages would then be attractive for medical use, particularly as drug delivery systems and, as noted above, are already being developed as vaccines. Further modifications could provide cell targeting, immune stimulation, immune stealth and so on.

At this early stage, many questions and challenges remain. Understanding the kinetics of protein cage assembly is one example that may be met by using designed cages whose assembly is triggerable and something not seen in natural cages, which spontaneously assemble. Combining structural studies with extended chemistry and biotechnology toolboxes will be useful as the research moves towards applications. This will include exploitation of existing technologies for attaching additional (particularly biological) molecules to the interior and exterior of cages. Examples include click chemistry via orthogonal amino acid [46] approaches and protein/peptide tags for example, SpyTag/SpyCatcher [47]. The use of structural studies, increasingly cryo-EM to confirm that these engineered cages have the expected structure will continue to be important. Ongoing improvements in cryo-electron tomography will allow us to gain important snapshots of therapeutic protein-cages in the cellular environment allowing further refinement of them as useful medical tools.

Conflict of interest statement

JGH is named as an inventor on a patent application related to protein-cage assembly construction. He is also the founder of and holds equity in nCage Therapeutics LLC, which aims to commercialise protein cages for therapeutic applications.

CRediT authorship contribution statement

Izabela Stupka: Writing - original draft, Writing - review & editing. **Jonathan Gardiner Heddle:** Conceptualized, Funding acquisition, Writing - original draft, Writing - review & editing.

Acknowledgements

This work was supported by a National Science Centre (NCN, Poland) grant no. 2016/20/W/NZ1/00095 (Symfonia-4) to J. G. H. The authors would like to thank Yusuke Azuma, Artur Biela, Agnieszka Kowalczyk and Karolina Majsterkiewicz for useful critical reading of and useful comments on the manuscript and Agnieszka Kowalczyk for assistance with the graphical abstract.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Jin Y, He J, Fan K, Yan X: **Ferritin variants: inspirations for rationally designing protein nanocarriers**. *Nanoscale* 2019, **11**:12449-12459.

2. Nichols RJ, Cassidy-Amstutz C, Chaijarasphong T, Savage DF: **Encapsulins: molecular biology of the shell**. *Crit Rev Biochem Mol Biol* 2017, **52**:583-594.

3. Ladenstein R, Fischer M, Bacher A: **The lumazine synthase/riboflavin synthase complex: shapes and functions of a highly variable enzyme system**. *FEBS J* 2013, **280**:2537-2563.

4. Roos WH, Ivanovska IL, Evilevitch A, Wuite GJL: **Viral capsids: mechanical characteristics, genome packaging and delivery mechanisms**. *Cell Mol Life Sci* 2007, **64**:1484-1497.

5. Ren H, Zhu S, Zheng G: **Nanoreactor design based on self-assembling protein nanocages**. *Int J Mol Sci* 2019, **20**:25-27.

6. Yang R, Li Y, Wang X, Yan J, Pan D, Xu Y, Wang L, Yang M: **Doxorubicin loaded ferritin nanoparticles for ferroptosis enhanced targeted killing of cancer cells**. *RSC Adv* 2019, **9**:28548-28553.

7. • Chakraborti S, Korpi A, Kumar M, Stępień P, Kostiainen MA, Heddle JG: **Three-dimensional protein cage array capable of active enzyme capture and artificial chaperone activity**. *Nano Lett* 2019, **19**:3918-3924.
Article demonstrating triggerable self-assembly of *Thermotoga maritima* ferritin, with encapsulated lysozyme, into a 3 dimensional crystal lattice which resulted in increased enzyme themostability and activity.

8. Aumiller WM, Uchida M, Douglas T: **Protein cage assembly across multiple length scales**. *Chem Soc Rev* 2018, **47**:3433-3469.

9. Caspar DL, Klug A: **Physical principles in the construction of regular viruses**. *Cold Spring Harb Symp Quant Biol* 1962, **27**:1-24.

10. Rossmann MG: **Structure of viruses: a short history**. *Q Rev Biophys* 2013, **46**:133-180.

11. Jiang W, Tang L: **Atomic cryo-EM structures of viruses**. *Curr Opin Struct Biol* 2017, **46**:122-129.

12. Montemiglio LC, Testi C, Ceci P, Falvo E, Pitea M, Savino C, Arcovito A, Peruzzi G, Baiocco P, Mancia F *et al.*: **Cryo-EM structure of the human ferritin–transferrin receptor 1 complex**. *Nat Commun* 2019, **10**:1-8.

13. Putri RM, Allende-Ballestero C, Luque D, Klem R, Rousou KA, Liu A, Traulsen CHH, Rurup WF, Koay MST, Castón JR *et al.*: **Structural characterization of native and modified encapsulins as nanoplatforms for in vitro catalysis and cellular uptake**. *ACS Nano* 2017, **11**:12796-12804.

14. Heddle JG, Chakraborti S, Iwasaki K: **Natural and artificial protein cages: design, structure and therapeutic applications**. *Curr Opin Struct Biol* 2017, **43**:148-155.

15. Neek M, Kim TI, Wang SW: **Protein-based nanoparticles in cancer vaccine development**. *Nanomed Nanotechnol Biol Med* 2019, **15**:164-174.

16. Bhaskar S, Lim S: **Engineering protein nanocages as carriers for biomedical applications**. *NPG Asia Mater* 2017, **9**:1-18.

17. Azuma Y, Herger M, Hilvert D: **Diversification of protein cage structure using circularly permuted subunits**. *J Am Chem Soc* 2018, **140**:558-561.

18. Azuma Y, Edwardson TGW, Terasaka N, Hilvert D: **Modular protein cages for size-selective RNA packaging in vivo**. *J Am Chem Soc* 2018, **140**:566-569.

19. Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG *et al.*: **Structure of dengue virus: implications for flavivirus organization, maturation, and fusion**. *Cell* 2002, **108**:717-725.

20. •• Twarock R, Luque A: **Structural puzzles in virology solved with an overarching icosahedral design principle**. *Nat Commun* 2019, **10**:1-9.

Work putting forward a novel description of how icosahedral viral capsids can be constructed by expansion of Caspar and Klugs' theory, widening the spectrum of possible architectures.

21. Lai YT, Cascio D, Yeates TO: **Structure of a 16-nm cage designed by using protein oligomers**. *Science* 2012, **336**:1129.

22. King NP, Sheffler W, Sawaya MR, Vollmar BS, Sumida JP, André I, Gonen T, Yeates TO, Baker D: **Computational design of self-assembling protein nanomaterials with atomic level accuracy**. *Science* 2012, **336**:1171-1174.

23. Hsia Y, Bale JB, Gonen S, Shi D, Sheffler W, Fong KK, Nattermann U, Xu C, Huang PS, Ravichandran R *et al.*: **Design of a hyperstable 60-subunit protein icosahedron**. *Nature* 2016, **535**:136-139.

24. Bale JB, Gonen S, Liu Y, Sheffler W, Ellis D, Thomas C, Cascio D, Yeates TO, Gonen T, King NP *et al.*: **Accurate design of megadalton-scale two-component icosahedral protein complexes**. *Science* 2016, **353**:389-394.

25. •• Marcandalli J, Fiala B, Ols S, Perotti M, de van der Schueren W, Snijder J, Hodge E, Benhaim M, Ravichandran R, Carter L *et al.*: **Induction of potent neutralizing antibody responses by a designed protein nanoparticle vaccine for respiratory syncytial virus**. *Cell* 2019, **177**:1420-1431.

Exciting work demonstrating the usage of computationally designed artificial protein cage as a scaffold for docking of a glycoprotein variant from respiratory syncytial virus showing its promising potential as a structure-based vaccine.

26. • Brouwer PJM, Antanasijevic A, Berndsen Z, Yasmeen A, Fiala B, Bijl TPL, Bontjer I, Bale JB, Sheffler W, Allen JD *et al.*: **Enhancing and shaping the immunogenicity of native-like HIV-1 envelope trimers with a two-component protein nanoparticle**. *Nat Commun* 2019, **10**.

Demonstration of displaying native-like HIV antigens on a computationally designed artificial protein cage which resulted in enhanced immunogenicity, showing the potential as an immunogen platform.

27. Malay AD, Heddle JG, Tomita S, Iwasaki K, Miyazaki N, Sumitomo K, Yanagi H, Yamashita I, Uraoka Y: **Gold nanoparticle-induced formation of artificial protein capsids**. *Nano Lett* 2012, **12**:2056-2059.

28. Imamura M, Uchihashi T, Ando T, Leifert A, Simon U, Malay AD, Heddle JG: **Probing structural dynamics of an artificial protein cage using high-speed atomic force microscopy**. *Nano Lett* 2015, **15**:1331-1335.

29. •• Malay AD, Miyazaki N, Biela A, Chakraborti S, Majsterkiewicz K, Stupka I, Kaplan CS, Kowalczyk A, Piette BMAG, Hochberg GKA *et al.*: **An ultra-stable gold-coordinated protein cage displaying reversible assembly**. *Nature* 2019, **569**:438-442.

Article demonstrating an ultra-stable artificial protein cage with metal coordination bonds at the protein–protein interfaces in which assembly and disassembly can be easily triggered showing its unique structure and geometry.

30. Grunbaum B, Johnson NW: **The faces of a regular-faced polyhedron**. *London Math Soc* 1965, **40**:577-586.

31. Azuma Y, Edwardson TGW, Hilvert D: **Tailoring lumazine synthase assemblies for bionanotechnology**. *Chem Soc Rev* 2018, **47**:3543-3557.

32. •• Edwardson TGW, Mori T, Hilvert D: **Rational engineering of a designed protein cage for siRNA delivery**. *J Am Chem Soc* 2018, **140**:10439-10442.

Article showing a simple approach towards RNAi loading inside a computationally designed protein cage by introducing positively charged amino acids inside its lumen showing effective RNAi packaging and cellular delivery.

33. •• Butterfield GL, Lajoie MJ, Gustafson HH, Sellers DL, Nattermann U, Ellis D, Bale JB, Ke S, Lenz GH, Yehdego A *et al.*: **Evolution of a designed protein assembly encapsulating its own RNA genome**. *Nature* 2017, **552**:415-420.

Shows a computationally designed nucleocapsid with positively charged interior subjected to laboratory-based evolution which resulted in its improved genome packaging and stability. This work shows that protein cages can acquire virus-like properties by simple laboratory evolution paths.

34. Terasaka N, Azuma Y, Hilvert D: **Laboratory evolution of virus-like nucleocapsids from nonviral protein cages**. *Proc Natl Acad Sci U S A* 2018, **115**:5432-5437.

35. King NP, Bale JB, Sheffler W, Mcnamara DE, Gonen S, Gonen T, Yeates TO, Baker D: **Accurate design of co-assembling multi-component protein nanomaterials**. *Nature* 2014, **510**:103-108.

36. De Ruiter MV, Klem R, Luque D, Cornelissen JJLM, Castón JR: **Structural nanotechnology: three-dimensional cryo-EM and its use in the development of nanoplatforms for: In vitro catalysis**. *Nanoscale* 2019, **11**:4130-4146.

37. Egelman EH: **The current revolution in Cryo-EM**. *Biophys J* 2016, **110**:1008-1012.

38. Subramanian R, Mayor S, Vinothkumar KR: **The resolution revolution reaches India**. *Science* 2014, **343**:1443-1444.

39. • Sasaki E, Böhringer D, van de Waterbeemd M, Leibundgut M, Zschoche R, Heck AJR, Ban N, Hilvert D: **Structure and assembly of scalable porous protein cages**. *Nat Commun* 2017, **8**:14663.

Describes two cryo-EM structures of engineered lumazine synthase variants with negatively charged lumenal surfaces able to package cargo.

40. Frank J, Goldfarb W, Eisenberg D, Baker TS: **Reconstruction of glutamine synthetase using computer averaging**. *Ultramicroscopy* 1978, **3**:283-290.

41. Taylor KA, Glaeser RM: **Electron diffraction of frozen, hydrated protein crystals**. *Science* 1974, **186**:1036-1037.

42. Rhee JK, Hovlid M, Fiedler JD, Brown SD, Manzenrieder F, Kitagishi H, Nycholat C, Paulson JC, Finn MG: **Colorful virus-like particles: fluorescent protein packaging by the Qβl capsid**. *Biomacromolecules* 2011, **12**:3977-3981.

43. Doerr A: **Cryo-electron tomography**. *Nat Methods* 2016, **14**:34.

44. Schaffer M, Pfeffer S, Mahamid J, Kleindiek S, Laugks T, Albert S, Engel BD, Rummel A, Smith AJ, Baumeister W *et al.*: **A cryo-FIB lift-out technique enables molecular-resolution cryo-ET**

within native *Caenorhabditis elegans* tissue. *Nat Methods* 2019, **16**:757-762.

45. De Boer P, Hoogenboom JP, Giepmans BNG: **Correlated light and electron microscopy: ultrastructure lights up!** *Nat Methods* 2015, **12**:503-513.

46. Khoshnejad M, Greineder CF, Pulsipher KW, Villa CH, Altun B, Pan DC, Tsourkas A, Dmochowski IJ, Muzykantov VR: **Ferritin nanocages with biologically orthogonal conjugation for vascular targeting and imaging**. *Bioconjug Chem* 2018, **29**:1209-1218.

47. Wang W, Liu Z, Zhou X, Guo Z, Zhang J, Zhu P, Yao S, Zhu M: **Ferritin nanoparticle-based SpyTag/SpyCatcher-enabled click vaccine for tumor immunotherapy**. *Nanomed Nanotechnol Biol Med* 2019, **16**:69-78.

48. Jordan PC, Patterson DP, Saboda KN, Edwards EJ, Miettinen HM, Basu G, Thielges MC, Douglas T: **Self-assembling biomolecular catalysts for hydrogen production**. *Nat Chem* 2015, **8**:179-185.

49. Šiber A, Božič AL, Podgornik R: **Energies and pressures in viruses: contribution of nonspecific electrostatic interactions**. *Phys Chem Chem Phys* 2012, **14**:3746-3765

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