

Titles: Harnessing physicochemical properties of virus capsids for designing enzyme confined nanocompartments

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Abstract

Viruses have drawn significant scientific interest from a wide variety of disciplines beyond virology because of their elegant architectures and delicate functionalities. A virus-like particle (VLP), a noninfectious protein cage derived from viruses or other cage-forming proteins, has been exploited as a nano-scale platform for bioinspired engineering and synthetic manipulation with a range of applications. Encapsulation of functional proteins, especially enzymes, is an emerging use of VLPs that is promising not only for developing efficient and robust catalytic materials, but also for providing fundamental insights into the effects of enzyme compartmentalization commonly observed in cells. This review highlights recent advances in employing VLPs as a container for confining enzymes. To accomplish larger and more controlled enzyme loading, various different enzyme encapsulation strategies have been developed; many of these strategies are inspired from assembly and genome loading mechanisms of viral capsids. Characterization of VLPs' physicochemical properties, such as porosity, could lead to rational manipulation and a better understanding of the catalytic behavior of the materials.

Introduction

Virus capsids are typically constructed from multiple copies of a few structural proteins *via* a self-assembly process. The capsids as a system are delicately balanced to enable multiple and potentially conflicting functions including encapsulating viral genomes, protecting them from the external environments, delivering their genomes to the host cells, and releasing them for amplification. The sophisticated mechanism of capsid assembly and their delicate functionalities have garnered interest among scientists beyond virology and cell biology[1]. Scientists are repurposing the functionalities of capsids to design gene vectors and vaccines, and are also expanding their use as nanoplatforms for synthetic manipulation[2-6]. In this regard, they have exploited a virus-like particle (VLP), which is a noninfectious protein cage derived from viruses or other cage-forming proteins, to encapsulate non-nucleic acid cargos such as proteins, polymers, and inorganic nanoparticles, for a range of applications from medicine to nanotechnology.

Among the many cargo molecules that have been encapsulated inside of VLPs, the confinement of enzymes has recently drawn particular interest[7,8]. The interior of a viral capsid is a unique environment, spatially separated from the bulk exterior environment by the capsid shell, in which an enzyme can be sequestered to create well-defined nanoreactors that are capable of catalyzing a wide range of chemical transformations, conceptually resembling bacterial microcompartments[9,10]. Enzyme compartmentalization into a confined environment could confer the ability to control fluxes of substrates, intermediates, and products across compartments allowing for control over enzymatic reaction efficiency[7,11-13]. VLPs are typically comprised of a single type of coat protein (CP) and could serve as a simplified model system to study the effect of enzyme confinement in a CP microcompartment in contrast to bacterial microcompartments whose shells are generally composed of multiple homologous proteins[11-17]. Additionally, virus capsids serve as a shield for cargos from protease digestion, thermal degradation, and other external disturbances[18-20]. Furthermore, enzyme encapsulated VLPs have been

used as building blocks for construction of catalytically active protein arrays[21-24]. Altogether, encapsulation of enzymes within VLPs is a promising approach towards creating efficient catalytic materials and aiding in the understanding of the effects of enzyme confinement and crowding on their activities.

Throughout millions of years of evolution, viruses have evolved a variety of different mechanisms for their genome encapsulation and capsid assembly. Scientists have harnessed these mechanisms to explore encapsulation of enzymes and other cargos inside of VLPs. The first part of this review gives an account of several different approaches developed to encapsulate enzymes and other proteinaceous cargos. The second part of this review discusses some recent studies on physicochemical properties of virus capsids relevant to designing VLP-based nanoreactors. Characterization and rational manipulation of physicochemical properties of VLPs, such as molecular diffusion across the capsid shell, are critical components for understanding the catalytic behavior of enzymes confined inside of VLPs. Rational manipulation of these properties could lead to designing more efficient nanoreactors.

Passive encapsulation

The CP of many single-stranded RNA viruses can assemble into capsids *in vitro* even in the absence of their genomic RNA[25,26]. For instance, cowpea chlorotic mottle virus (CCMV) can be disassembled into subunit dimers at a near neutral pH (pH 7.5), and upon lowering the pH (pH 5.0), it subsequently assembles into a T=3 capsid[27,28]. One of the initial demonstrations of enzyme encapsulation inside of a VLP exploited this *in vitro* assembly capability of a CCMV capsid. Cornelissen *et al.* reconstituted CCMV subunits into a capsid in the presence of a horseradish peroxidase (HRP) enzyme and achieved encapsulation of a single HRP per CCMV VLP (Figure 1a)[16]. While this encapsulation is stochastic in nature and not particularly efficient, this study demonstrates an important concept that paves the way for further studies. The enzyme entrapped within the capsid was still highly active, indicating that the capsid

shell is permeable for small molecule substrates and products, whereas the enzyme itself remains trapped inside of the VLP (Figure 1b).

Electrostatic interaction mediated encapsulation

Although many single-stranded RNA viruses can assemble into capsids *in vitro*, even in the absence of their genomic RNA, capsid formation is typically more efficient in the presence of their genomic RNA[26,29]. Strong electrostatic interactions between the CP and the RNA provide the initial driving force for capsid assembly and genomic cargo encapsulation[30]. Similarly, by introducing a complementary charge interaction between cargo molecules and the CPs of capsids, the loading efficiency of cargo molecules into capsids can be enhanced[31-35]. For example, Hilvert *et al.* genetically engineered a mutant of the lumazine synthase protein cage derived from *Aquifex aeolicus*, which has a negatively charged internal surface (AaLS-neg), and a green fluorescence protein tagged with a positively charged decaarginine tag (GFP-R10). Co-expression of AaLS-neg and GFP-R10 in *E. coli* led to the formation of an AaLS-neg cage loaded with GFP-R10 (Figure 2a)[34]. Electrostatic interactions between CPs and cargos have also been applied to encapsulate non-proteinaceous cargos, such as pre-formed inorganic nanoparticles inside of VLPs derived from several different viruses[36-38].

RNA packing signal mediated encapsulation

Some single-stranded RNA viruses such as MS2 and Q β are known to use specific sequences in their genomic RNA, which have stem-loop structures, to bind to the coat proteins and thereby direct both capsid assembly and RNA encapsulation[26,39,40]. For example, the genomic RNA of MS2 has a 19-nucleotide translational repressor (TR) fragment, which is known to serve as a packaging signal due to specific interactions with the CP[26,39]. Stockley *et al.* pioneered a method to exploit this assembly mechanism to encapsulate a cargo protein inside of an MS2 capsid[41]. They covalently attached a

protein, ricin A chain (RAC), to the TR oligonucleotides and successfully directed RAC to the inside of the capsid while the CP assembled into a capsid *in vitro*.

Finn *et al.* employed a similar strategy to achieve *in vivo* encapsulation of enzyme cargos inside of a Q β capsid while co-expressing these components in *E. coli* (Figure 2b)[18,42,43]. A chimeric single-stranded RNA was used as a linker to mediate the encapsulation of the cargo proteins. This RNA linker is composed of three segments, a hairpin (hp) that associates with interior-facing residues of the Q β CP, an RNA aptamer sequence (α -Rev apt) that binds to an arginine-rich peptide (Rev-tag), and an mRNA for the Q β CP that additionally serves as the spacer between the other two segments. A cargo protein was genetically fused with the Rev-tag so that the α -Rev apt segment of the RNA linker interacts with the cargo protein, whereas the hp segment associates with the CP and directs the cargo into the Q β capsid. Thus, the transcribed mRNA can simultaneously serve as the CP transcript and as the cargo encapsulation mediator. Finn *et al.* demonstrated that co-expression of the CP/RNA mediator with the tagged protein cargo is a versatile approach to encapsulate a number of enzyme cargos (10 – 36 cargos per capsid) within the Q β capsid[42].

Scaffolding protein (SP) mediated encapsulation

Large double-stranded RNA or DNA viruses, including some bacteriophage, typically require scaffolding proteins (SP) or an inner core to template proper capsid assembly[44-46]. Bacteriophage P22 is one of the most well-studied model viruses whose capsid is formed through SP mediated assembly of CPs[47]. VLPs derived from P22, which is assembled from 420 copies of CPs into a T = 7 icosahedral capsid with the aid of 100 – 300 copies of SPs, can be produced by a heterologous expression system in *E. coli*. Douglas *et al.* have harnessed the SP mediated capsid assembly process to direct the encapsulation of enzymes inside of P22 VLPs in a highly efficient manner[17,20,21,48-52]. The 303-residue-long SP can be severely truncated to an essential helix-turn-helix scaffolding domain located near the C-terminus

without losing the ability to direct the assembly of CPs into the capsid[53,54]. Genetic fusion of cargo proteins to either the N- or C-terminus of the truncated SP has been demonstrated to be a powerful and versatile approach for encapsulating a wide variety of functional proteins including enzymes[51,55]. In one of the initial studies of this approach, Douglas *et al.* demonstrated encapsulation of approximately 250 alcohol dehydrogenase (AdhD) per P22 capsid (Figure 2c)[49]. Based on the volume of the P22 interior cavity, this gives a local enzyme concentration of 7.16 mM or \sim 300 mg/mL, which is comparable to the reported value of macromolecular concentration inside cells[56]. Dense packing of enzymes within the cavities of the capsid achieved by this approach has allowed them to study enzyme behaviors in a crowded and confined environment such as enzymatic kinetics and protein stability.

Significant work has been done to establish the assembly of P22 VLPs *in vitro* from individually purified CPs and SPs[57,58]. Douglas *et al.* have adopted the *in vitro* assembly of P22 for encapsulating multiple populations of cargo proteins inside of the same capsid[14,59,60]. This *in vitro* assembly approach allows for control over the packaging stoichiometry of multiple cargos which is difficult to attain by the *in vivo* encapsulation approaches. For example, co-encapsulation of two different cargos, SP tagged AdhD (AdhD-SP) and wild-type SP (wtSP), with controlled stoichiometry, has been achieved by modulating the input ratio of these two components (Figure 3a, b)[14]. The turnover number of the AdhD enzyme decreased while increasing the number of co-encapsulated wtSP (Figure 3c). This result suggests that co-encapsulated wtSP can act as a molecular crowding agent to AdhD resulting in a reduction of activity of AdhD. This approach also has the capability to control the packing density of cargo enzymes, because wtSP can be selectively removed from the co-assembled capsids using 0.5 M guanidine hydrochloride[61], providing more space for other cargos in a confined VLP environment (Figure 3a). Selective removal of wtSP from the P22 VLP resulted in a similar turnover of AdhD regardless of the packing density of AdhD, suggesting that the encapsulated AdhD does not experience self-crowding even at a high packing density. The *in vitro* assembly approach would allow for further investigation of the

effect of confinement and crowding on the activities of enzymes encapsulated inside of a microcompartment.

Other non-covalent interactions mediated encapsulation

Other types of specific interactions, which are not involved in viral genome packing, have also been adopted to direct cargo into a capsid[62-64]. For example, a complementary pair of coiled-coil sequences was used to achieve higher cargo loading[62]. Cornelissen *et al.* demonstrated that a positively charged K-coil peptide was genetically fused to the CCMV CP and the complementary E-coil peptide was introduced to lipase B (PalB) from *Pseudozyma antarctica*. Introduction of the coiled-coil peptide allowed non-covalent interaction between the cargo and the CP prior to pH-mediated capsid assembly, resulting in encapsulation of one to four PalB per capsid.

A new class of cage-like proteins called encapsulins has recently been identified in bacteria and archaea[15,65-67]. Encapsulins exhibit an icosahedral symmetry and three different architectures corresponding to triangulation numbers of one, three, and four, have been found to date[68]. Interestingly, all encapsulins have an HK97-like fold despite sharing little sequence homology. As the name suggests, encapsulins involve packing cargo proteins within their interior cavity. For example, the encapsulin in *Thermotoga maritima* encapsulates ferritin-like proteins, which possess ferroxidase activity, and is likely to serve to mitigate oxidative stress[66,69]. The protein encapsulation is typically guided by a short targeting peptide (TP) which is located at the C-terminus of a cargo protein and interacts with the interior side of the encapsulin protomer. This specific interaction between the TP and encapsulin has been utilized to direct encapsulation of heterologous proteins as cargos[15,70,71]. For example, Savage *et al.* demonstrated that a non-native cargo protein can be loaded into the encapsulin from *Thermotoga maritima* both *in vivo* and *in vitro* by the fusion of the 30 amino acids TP or the 15 amino acids truncated TP to the cargo protein[15].

Covalent conjugation of cargo to VLPs

Covalent conjugation of a cargo protein to a CP is another approach to encapsulate the cargo inside of VLPs. The covalent conjugation can be achieved not only through genetic fusion[72-74] but also through post-translational protein ligation methods such as Sortase A[75,76], SpyTag/SpyCatcher[77], and split intein[78]. In a recent study, Giessen and Silver demonstrated *in vivo* encapsulation of various enzymes in MS2 capsid by using the SpyTag/SpyCatcher mediated protein fusion technique (Figure 2d)[77]. The SpyTag sequence (16 aa) was inserted into the CP of MS2 (MS2-Spy), which is still able to assemble into a capsid. Meanwhile, enzyme cargos were genetically fused with the SpyCatcher (116 aa) that recognizes the SpyTag and forms an isopeptide bond with it. Co-expression of MS2-Spy and a SpyCatcher fused enzyme led to covalent ligation of enzyme-SpyCatcher with the interior surface of the capsid *in vivo*.

Addressing physicochemical properties of enzyme encapsulated VLPs

The physicochemical properties of VLPs, such as possible diffusion limitations across the capsid shell, electrostatic potential around the capsid pores, and the pH inside the capsid are critical for understanding and predicting catalytic behavior for enzymes encapsulated in VLPs. However, many aspects of these properties have not yet been well characterized. For example, although high resolution structures obtained from X-ray crystallography or cryo-electron microscopy are available for many viral capsids, this structural information may not accurately predict the permeability of molecules across the capsid shell because of the dynamic nature of viral capsids in solution. To address this issue, Douglas *et al.* recently developed a methodology for studying diffusion across the protein shells of VLPs (Figure 4a)[11]. In this study, AdhD encapsulating P22 VLPs with two different morphologies, procapsid form and expanded form, were used as a model system. In both the infectious phage and the VLP, the procapsid is the initial structure with 56 nm in diameter formed *via* SP templated assembly[79]. The infectious phage undergoes a structural transformation by DNA packing, resulting in expansion of the procapsid to a more angular particle of 62 nm in diameter[80]. This morphogenesis can be recapitulated in the P22 VLPs with either

heat[81,82] or chemical[83] treatment and the resulting particles are referred to as the expanded form. To investigate diffusion across procapsid and expanded forms of P22 VLPs, Douglas *et al* conjugated NADH, a cofactor for the AdhD enzyme, to six different sized PAMAM dendrimers ranging from 1.3 – 7.8 nm in diameter. These conjugated cofactors were used as a molecular probe to investigate their diffusion across the porous protein shell layer of P22 VLPs by monitoring NADH oxidation to NAD⁺ catalyzed by AdhD. The pore size of the procapsid and expanded forms were revealed to be 4.4 nm and 2.7 nm, respectively. This study provides a generalizable method to analyze diffusion limitation across a viral capsid and many other porous nanoparticles.

Several recent studies have illustrated that electrostatic interactions between substrates and protein cages have a significant effect on the transportation of substrates and products across protein cages and hence the overall catalytic reaction[11-13,84]. For example, Tullman-Ercek *et al.* demonstrated that for alkaline phosphatase encapsulated inside of MS2 capsid the k_{cat} and K_M could be varied by modulating the surface charge around pores at the 5-fold and quasi-6-fold axes of the capsid through point mutation of the CP. As enzyme activity itself should not be altered by the mutation to the CP, the change in the catalytic parameters is likely due to changes in substrate and product fluxes through the pores. This result supports the hypothesis that the shell layer of protein compartments could modulate the transport of small molecules into and out of the compartments and thus influence catalytic reactions.

The pH inside of a viral capsid could be significantly different from that of the bulk solution outside of the capsid because of the high concentration of charged residues exposed on the interior cavity surface[74,85,86]. As pH is a critical parameter in the role of enzymatic reactions, it is important to assess the pH condition inside of a virus capsid. Cornelissen *et al.* have addressed this issue by encapsulating a negatively charged pH-responsive polymer probe within the CCMV VLP[85]. The study indicated that the pH inside the CCMV VLP is around 0.5 pH units more acidic than the outside environment.

It is well studied that genetic materials affect the mechanical stability of viral shells[87]. In some cases, the viral genome structurally reinforces the shell through structural interactions with the shell[88], whereas in other cases, it destabilizes the shell by increasing internal pressure[89]. Similarly, heterologous cargos encapsulated inside of VLPs could affect the stability of the capsids. Caston and Pablo *et al.* investigated mechanical properties of proteinaceous cargo-loaded P22 with two different morphologies, procapsid and expanded forms, by using cryo-electron microscopy and atomic force microscopy (Figure 4b)[90]. Their study indicated that the presence of cargos stiffens the P22 capsid, but the underlying mechanism could be different between the two capsid morphologies. As discussed in the previous section, in the case of the procapsid form, the cargo is constrained to the interior of the capsid lumen *via* the C-terminus of SP. Therefore, the procapsid is structurally reinforced by a cargo-SP fusion protein (Figure 4b left). On the other hand, in the case of the expanded form, cargo-shell interactions are lost, thus the cargo-SP is detached from the shell. The cargos are still trapped but become free inside the capsid (Figure 4b right). The different concentration of cargo proteins between the inside and the outside of the shell creates osmotic pressure and this pressurization increases the rigidity of the capsid. The estimated osmotic pressure could be up to 3 MPa, which is comparable to the DNA induced pressurization in natural viruses. A better understanding of the stabilization mechanism of cargo-loaded VLPs could lead to the development of a method to optimize the mechanical properties of VLP based materials.

Conclusion

This review followed recent studies regarding the encapsulation of enzymes within VLPs from a simple passive stochastic approach to actively guided approaches. Studies on virus assembly have provided scientists with inspiration and means to develop more efficient and better controlled enzyme encapsulation methods. Sequestration of enzymes and other proteins within microcompartments has been observed in many biological systems and is believed to confer enhancement of specific metabolic

processes. Enzyme encapsulation within VLPs could be used as a model system for better understanding the effects of enzyme confinement on its activity as well as designing more efficient catalytic materials.

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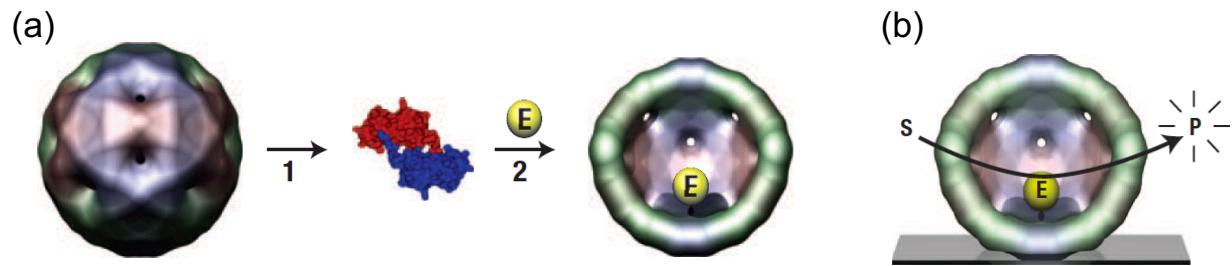


Figure 1 (a) Schematic illustration of a passive enzyme encapsulation process in a CCMV capsid. Step 1: A CCMV capsid is disassembled into dimers at pH 7.5. Step 2: A guest enzyme (E) added to the sample is encapsulated when the CCMV dimers are reassembled into the capsid by lowering the pH to 5. (b) A substrate molecule (S) diffuses into the capsid and are subsequently converted to a product molecule (P) by the enzyme. The images were reproduced from ref. 16 with permission from Springer Nature.

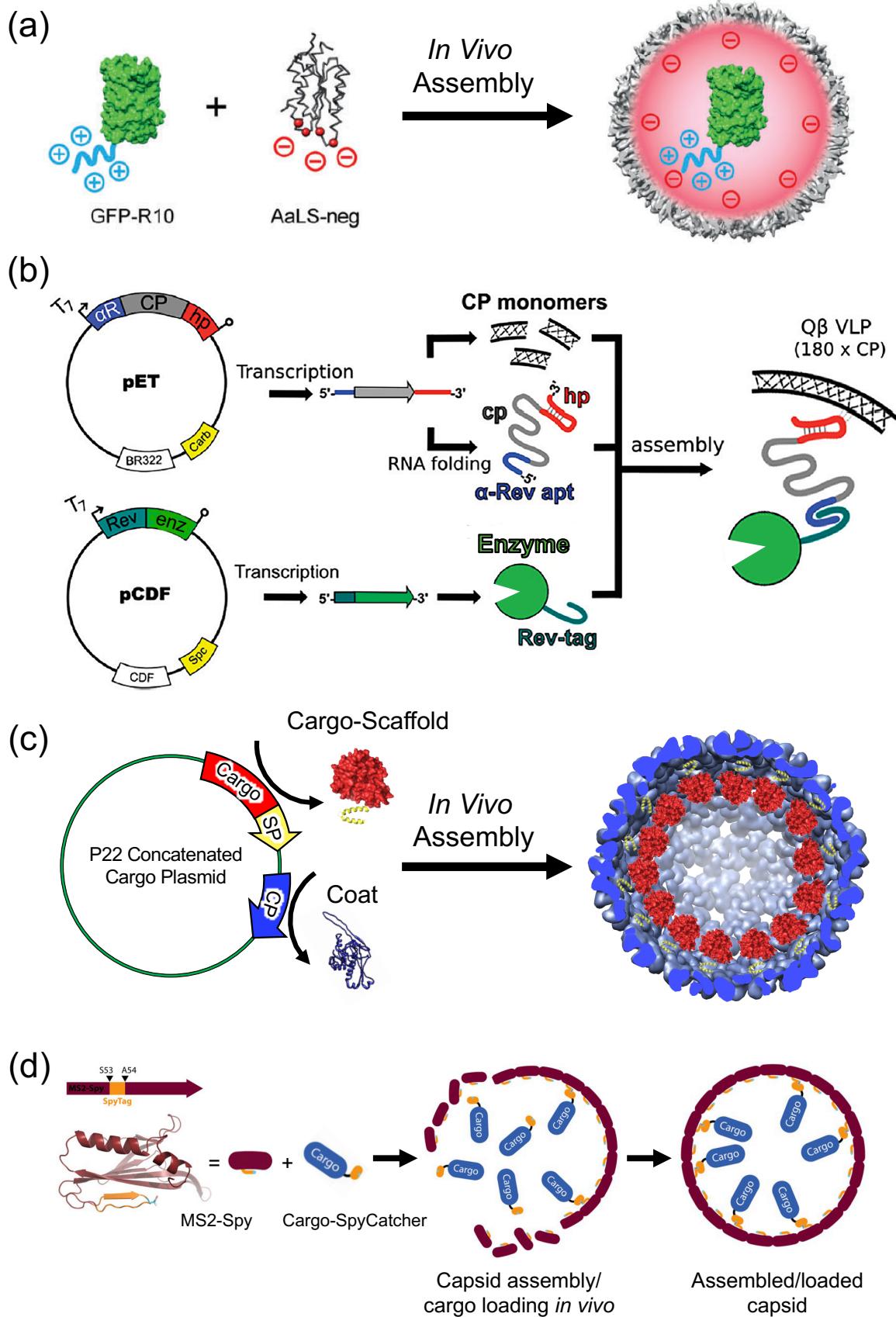


Figure 2 Examples of directed encapsulation of cargo proteins inside of capsids *in vivo*. (a) GFP fused with positively charged tag is directed to inside of the AaLS-neg cage *via* electrostatic attraction. The image was reproduced from ref. 32 with permission from the Royal Society of Chemistry. (b) A Rev-tagged enzyme is directed to bind to the interior of the Q β capsid *via* an RNA linker which possesses a Rev aptamer and a Q β genome packaging hairpin domain. The image was reproduced from ref. 18 with permission from Wiley. (c) Fusing a cargo protein to the N-terminus of a truncated P22 SP leads to directed encapsulation of the cargo to the P22 capsid. The image was reproduced from ref. 49 with permission from the American Chemical Society. (d) A cargo protein fused with the SpyCatcher is covalently conjugated to the SpyTag inserted MS2 CP *via* isopeptide bond formed between the SpyCatcher and the SpyTag, while the CP assembles into the capsid. The image was reproduced from ref. 77 with permission from Wiley.

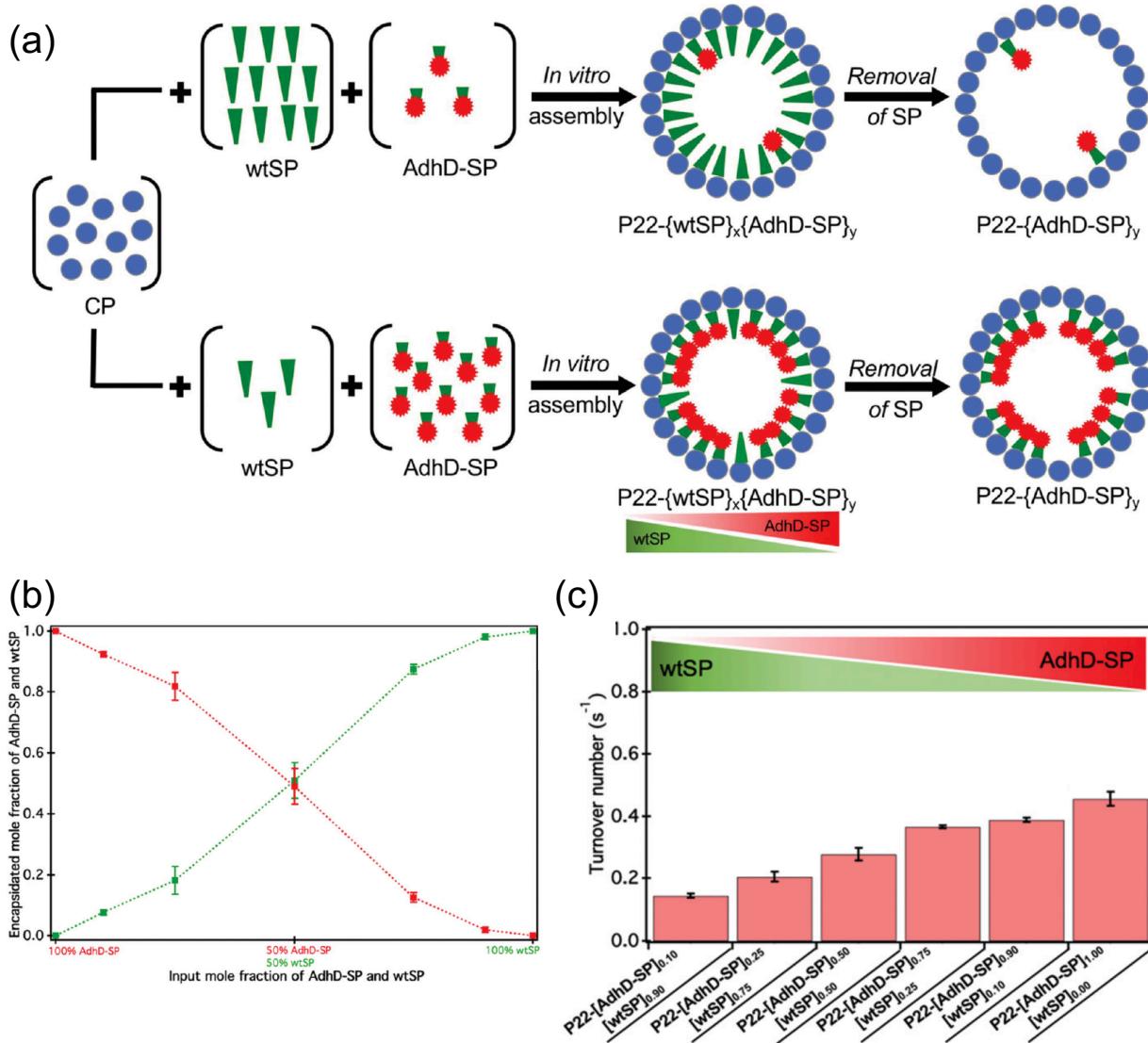


Figure 3 Controlled packing of multiple cargo proteins using *in vitro* assembly of P22 CP and SP into a capsid. (a) Schematic representation of co-encapsulation of AdhD-SP and wtSP. To control the stoichiometry of the two cargos, a constant amount of CP was added to the mixture of AdhD-SP and wtSP with a range of two-component ratios while keeping the total SP amount constant. (b) Plots of the encapsulation ratio of AdhD-SP and wtSP over a range of input stoichiometry ratios of the two components. (c) The turnover number (k_{cat}) of AdhD within P22 VLP co-encapsulated with wtSP. Error bars represent standard deviations of triplicate measurements. The images were reproduced from ref. 14 with permission from the Royal Society of Chemistry.

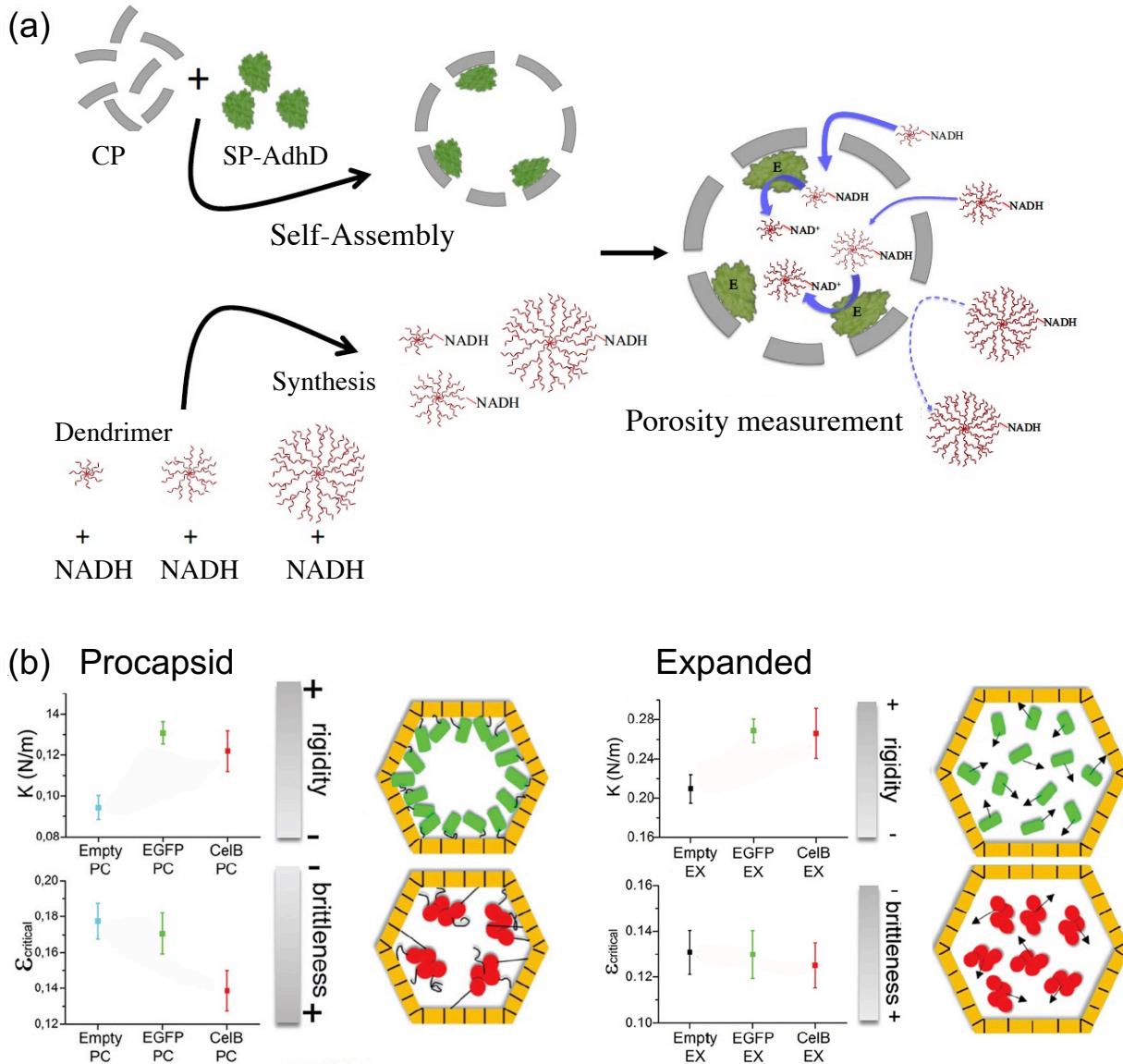


Figure 4 (a) Schematic illustration of an experiment for investigating molecular diffusion across a capsid shell. P22 VLP self-assembled with CP and SP-AdhD fusion proteins was used as a model capsid. NADH, a cofactor of AdhD enzyme, was conjugated to various sizes of dendrimers and used as a probe to assess the porosity of the P22 capsid. If the probe accessed the interior of the capsid, the redox reaction of NADH to NAD⁺ was observed. The images were reproduced from ref. 11. (b) Mechanical properties of empty and cargo proteins loaded P22 capsids. The average rigidity and brittleness of procapsid (left) and expanded (right) forms of P22 capsids loaded with GFP (green) or CelB (red) was compared with those of empty capsids. The cartoons present that GFP and CelB were bound to the interior surface of the procapsid shell, whereas those were detached from the surface and free in the expanded shell. The images were reproduced from ref. 90 with permission from the Royal Society of Chemistry.

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