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Engineering encapsulin nanocages for drug delivery

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Encapsulins are a widely distributed and functionally diverse class of protein compartments found across diverse bacterial and archaeal phyla involved in various aspects of microbial metabolism. They self-assemble into cargo-loaded protein shells between *ca.* 20 and 50 nm in diameter with either $T = 1$, $T = 3$ or $T = 4$ icosahedral symmetry. Encapsulin nanocages possess several key features that make them an attractive engineering platform for creating nanocarrier-based drug delivery systems. This includes a modular and efficient cargo loading mechanism for the facile encapsulation of proteins of interest, diverse physicochemical characteristics and high stability, and robust genetic and chemical strategies of shell modification. For these reasons, encapsulins have garnered significant interest as platforms for various engineering ventures in biomedicine and biotechnology. In this review, we summarize recent advances in engineering encapsulins for drug delivery applications, highlighting their engineerability as a platform technology, innovative strategies employed to enhance their therapeutic potential, and recent concrete drug delivery applications based on engineered encapsulin nanocarriers.

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1. Introduction

Drug delivery systems, particularly nanocarriers, represent an innovative approach in modern medicine for the targeted delivery and controlled release of therapeutic agents.[1] For this review, we define nanocarriers as drug carrier systems consisting of nanosized particles ranging in size from *ca.* 1 to 500 nm able to transport a drug or bioactive substance to a site of interest. These systems are designed with the primary goal of enhancing the efficacy, safety, and bioavailability of drugs while minimizing side effects. A wide array of platforms for efficient drug delivery has been explored, including liposomes,[2] polymers,[3] micelles,[4] and virus-based systems,[5] each with its own merits and drawbacks. In general, nanocarriers are engineered at the nanoscale to improve pharmacokinetics and biodistribution, ensuring that therapeutic agents reach their intended targets with precision. They are capable of delivering a diverse range of molecules, from small molecule drugs[6] to larger biomolecules such as proteins,[7] nucleic acids,[8] and peptides.[9] The overall goal of drug delivery technologies is to overcome biological barriers and achieve site-specific delivery, thereby maximizing therapeutic outcomes while minimizing adverse effects. As the field continues to evolve, new materials and approaches are being explored to meet the growing demands of precision medicine.

Protein nanocages possess a number of distinctive properties that make them attractive engineering targets for various biomedical applications related to drug delivery.[10,11] These properties include, but are not limited to, their inherent biocompatibility, facile genetic functionalization, molecularly defined and uniform structures, self-assembly, modularity, and overall stability. Unlike lipid-based compartmentalization systems, protein nanocages are genetically encoded which allows precise and predictable rational engineering with single amino acid resolution.[12] This ease of modification at the genetic level has been exploited for the selective encapsulation of cargo molecules, including proteins,[13] nucleic acids,[14] and small molecules,[15] yielding defined host–guest complexes. Through genetic engineering, protein nanocages have also been imbued with specific targeting capabilities[16] and other useful properties for targeted delivery like triggered disassembly.[17] Various types of protein nanocages, including virus-like particles (VLPs),[5] ferritin,[18] lumazine synthase,[19] and computationally designed protein cages,[20] have been used as programmable drug delivery devices. For example, by taking advantage of their inherent cell penetrating capabilities, VLPs have been engineered to deliver therapeutic RNAs,[21] proteins,[22] or small molecules[23] to target cells. Ferritin's ease of shell modification and reversible pH-induced disassembly behavior has long been utilized for loading various therapeutic molecules *in vitro* for subsequent delivery.[24] Lumazine synthase has been engineered to display cell-specific targeting peptides and covalently

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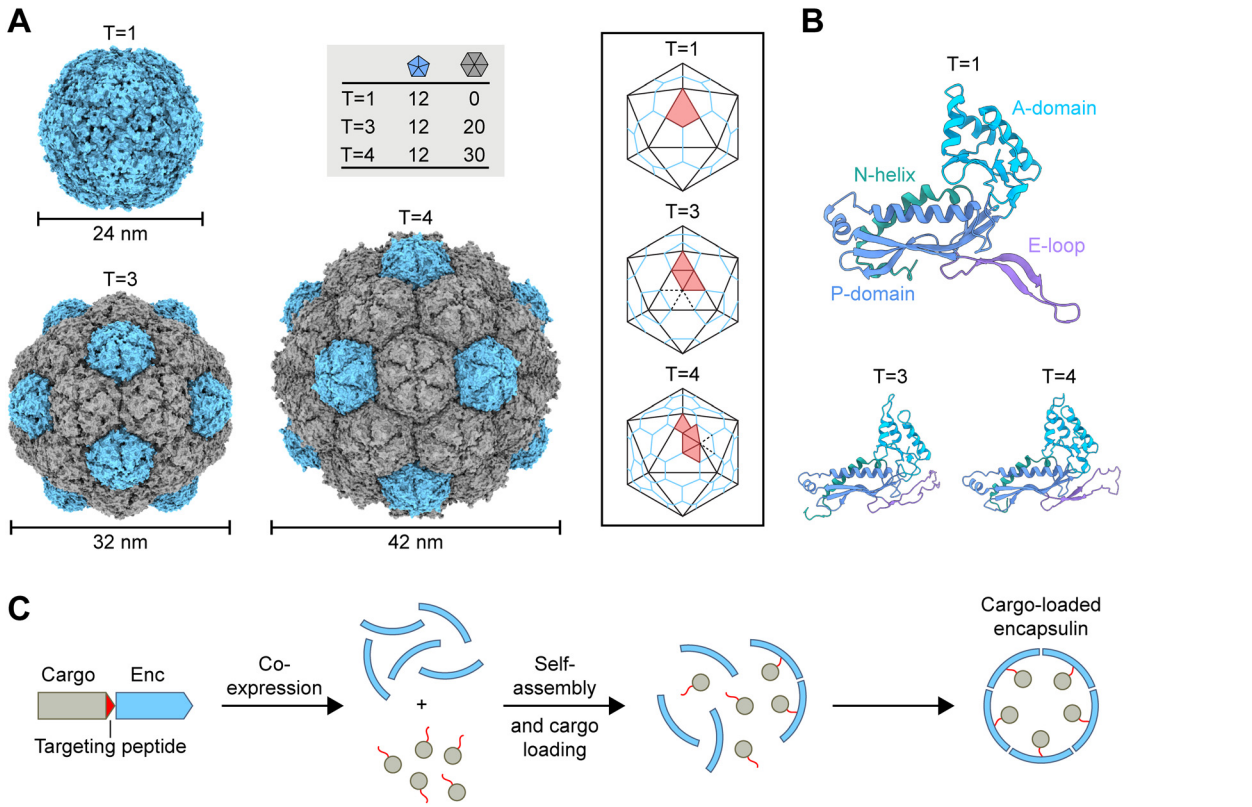

Fig. 1 Structures and assembly principles of encapsulins. (A) Left: Family 1 encapsulin shells. T = 1, T = 3, and T = 4 shells are shown. Pentamers are colored in blue and hexamers in grey. The number of pentamers and hexamers needed to form a closed shell with a given triangulation (T) number is shown. Right: Schematic organization of icosahedral encapsulin shells with different T numbers highlighting their asymmetric units (red). (B) Structures of family 1 encapsulin protomers. Examples of T = 1, T = 3, and T = 4 protomers are shown. Conserved domains of the encapsulin shell protein (axial (A)-domain, peripheral (P)-domain, N-terminal (N)-helix, and extended (E)-loop) are highlighted. (C) Schematic depiction of the targeting peptide-mediated assembly of a single-cargo encapsulin system.

modified with small molecule drugs, resulting in targeted drug delivery systems.[19] More recently, diverse *de novo* designed protein nanocages with useful properties like stimulus-responsive disassembly have also emerged as promising tools for drug delivery.[25]

The inherent difficulty of targeted drug delivery, in particular when it comes to delivering therapeutic macromolecules like proteins or nucleic acids, makes the continued discovery, design and engineering of novel nanocarrier systems an important strategy to advance the field. One of the more recently discovered and employed nanocarrier systems are encapsulin nanocompartments, naturally occurring protein cages found across many prokaryotic phyla.[26–29] Encapsulins self-assemble into icosahedral cargo-loaded protein compartments ranging in size from *ca.* 20 to 50 nm in diameter with triangulation numbers of T = 1 (60 subunits), T = 3 (180 subunits), or T = 4 (240 subunits) (Fig. 1A and B). The biological functions of encapsulins are diverse and systems involved in oxidative stress resistance,[30–32] iron[33–37] and sulfur storage,[38–41] and secondary metabolite biosynthesis,[42,43] have been characterized. While multiple distinct encapsulin types have been discovered, so far, only family 1 encapsulins have been used as engineering platforms.[44] Therefore, when referring to encapsulins in this review, we are specifically alluding to family 1 encapsulins. Encapsulins possess several key features that make them attractive engineering targets. First, encapsulins can specifically sequester dedicated cargo proteins.[45,46] All native cargos contain N- or C-terminal targeting peptides (TPs) necessary for efficient cargo loading during shell self-assembly (Fig. 1C). This feature—a dedicated and modular protein loading mechanism—has been widely utilized to package non-native cargo proteins into encapsulin shells *via* simple genetic fusion of TPs to proteins of interest.[47–50] Second, encapsulins exhibit diverse physicochemical properties useful for a variety of biomedical applications. For instance, encapsulins found in thermophilic organisms typically show extreme thermostability,[51] while encapsulins encoded by acidophiles possess high acid tolerance.[52] Lastly, many encapsulin shells are highly robust and can be modified through chemical conjugation or genetic insertions and fusions without disrupting shell assembly or cargo loading.[14,53–58] Overall, encapsulins represent a promising platform for the creation of multifunctional protein nanocages.

In this review, we will focus on the use and potential of encapsulins as targeted drug delivery devices. We will initially detail engineering approaches to modify encapsulin systems and focus on cargo loading, nanocarrier targeting, and cargo

release. We will then discuss recent applications of engineered encapsulins as drug delivery systems and conclude with a discussion of current challenges and the future potential of encapsulin nanocarriers.

2. Engineering encapsulins for drug delivery

2.1. Cargo loading

One of the key features a nanocarrier needs to be suitable as a drug delivery vehicle is a means of attaching or loading therapeutic molecules to the carrier.[59] Encapsulins natively possess a modular mechanism for the specific loading or encapsulation of cargo proteins *in vivo*. Encapsulin cargo loading is mediated by the interaction of targeting peptides (TPs), also called cargo-loading peptides, present at the N- or C-terminus of native cargo proteins, with the interior of the encapsulin shell during the self-assembly process (Fig. 1C).[45] One of the benefits of TP-based cargo loading is its modularity and simplicity. With a typical length of 7 to 12 residues, TPs can be easily genetically fused to either the N- or C-terminus of essentially any non-native cargo protein that can be physically accommodated within the interior of the corresponding encapsulin shell. Thus, TPs can be essentially treated as modular protein tags. Due to the specificity of the TP interaction with its binding site located on the luminal face of each shell protein subunit, *in vivo* loading of TP-fused cargo proteins is straightforward, eliminating the need to disassemble and reassemble protein nanocages *in vitro*. The efficiency of *in vivo* cargo loading can be modulated by adjusting the relative expression levels of shell and cargo proteins, or by TP mutagenesis to adjust TP affinity to its binding site.[60] Other factors that influence cargo loading are the size and oligomerization state of the intended cargo—with larger proteins or complexes resulting in lower overall loading—and the length and flexibility of the linker connecting the TP to the globular cargo domain or complex.[60]

Utilizing the inherent cargo loading capabilities of encapsulin systems has been the primary approach of constructing encapsulin-based nanocarriers (Fig. 2A). For example, a number of TP-tagged cytotoxic enzymes, such as mini-singlet oxygen generator (miniSOG; cargo loading capacity: 7–9%)[61,62] and nitroreductases,[63] have been successfully encapsulated inside

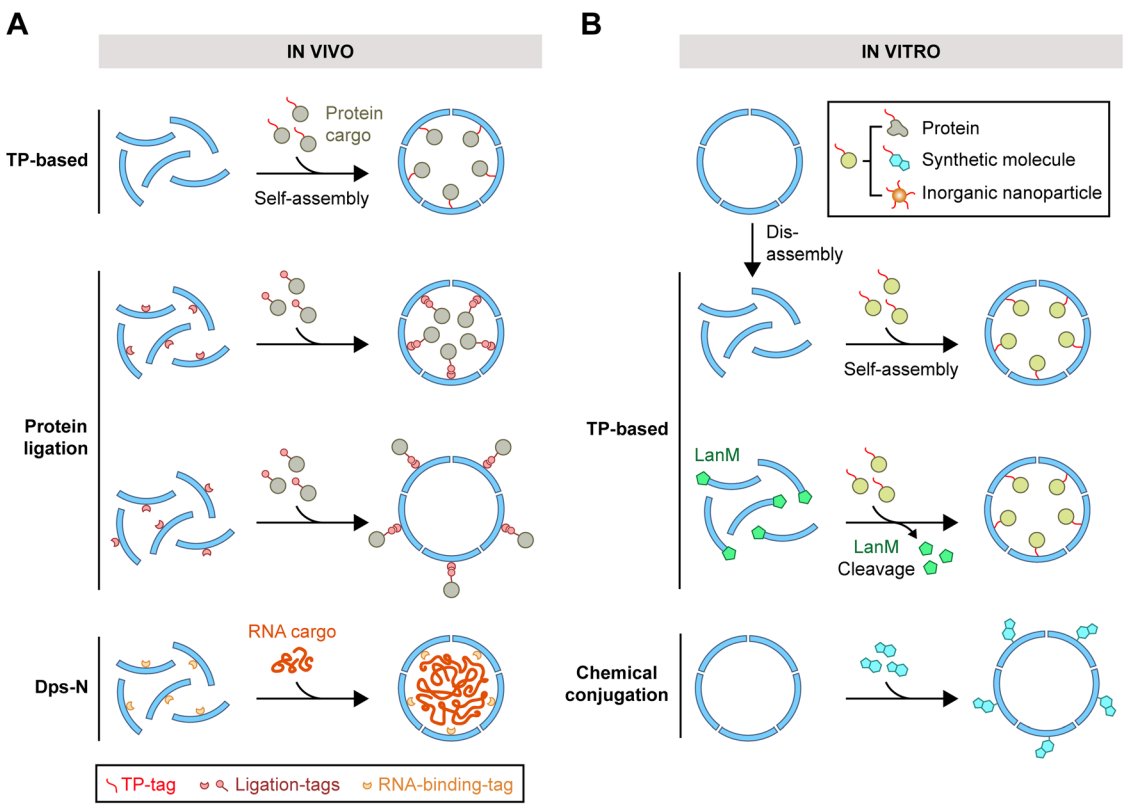

Fig. 2 Cargo loading strategies for encapsulin nanocarriers. (A) Conceptually different, so far experimentally explored, single-step *in vivo* strategies for loading encapsulin nanocarriers with protein or RNA cargo. They can be classified based on the mechanism of cargo loading or attachment into targeting peptide (TP)-based, protein ligation-based, or nucleic acid binding-tag-based (Dps-N) methodologies. Further, cargo can either be internally encapsulated or externally attached to the shell. Encapsulin shells and protomers are shown in blue. (B) Conceptually different *in vitro* cargo loading strategies. Both TP-based and chemical conjugation-based protocols have been successfully employed. Encapsulin shell disassembly is usually induced *via* extreme pH shifts or the use of chaotropic reagents. Protein cargo, synthetic small molecules, and inorganic nanoparticles have been encapsulated using TP-based strategies. LanM fusion shell proteins can be purified as individual protomers while proteolytic LanM cleavage triggers shell self-assembly and TP-based cargo loading. LanM: lanmodulin.

Thermotoga maritima and *Mycobacterium smegmatis* encapsulin shells *in vivo* to produce therapeutic nanocarriers for anti-cancer therapy. Besides TP-based cargo loading, covalent protein ligation strategies have also been explored for *in vivo* cargo encapsulation (Fig. 2A). Both split-intein-[56] and sortase A[55] -based ligation systems have been successfully utilized for loading GFP (40% loading) and NanoLuciferase (65% loading) into *T. maritima* and *Quasibacillus thermotolerans* encapsulin shells, respectively. While *in vivo* encapsulation is a straightforward and robust approach to load cargo proteins into encapsulin shells, orthogonal *in vitro* loading strategies have also been developed. As encapsulin shells are generally highly stable, any *in vitro* loading approach requires disassembling the protein shell under harsh conditions—using low pH, high temperature, or chaotropic reagents—followed by reassembly of the shell in the presence of TP-tagged cargo in physiological buffer (Fig. 2B).[64] Since the concentration of encapsulin shell and cargo can be freely adjusted during *in vitro* loading, this approach may offer more control over the amount of cargo loaded or the ratio of multiple co-encapsulated cargo proteins.[39,65] Downsides of *in vitro* loading approaches include the need to separately purify all components and the fact that disassembly under harsh conditions followed by shell reassembly can result in aberrant shell structures and the loss of substantial amounts of protein due to aggregation and precipitation.[64,65] Recently, a novel and innovative cargo loading strategy was described based on an encapsulin shell protein fusion that can exist as a monomeric protein (Fig. 2B).[66] Specifically, the lanthanide-binding protein lanmodulin (LanM) was fused to the N-terminus of the *Q. thermotolerans* encapsulin shell protein which prevented shell self-assembly. After monomeric fusion protein purification, LanM could be removed *via* protease treatment triggering *in vitro* assembly of the shell. If TP-tagged cargo was present during LanM cleavage, cargo loaded shells could be obtained. This approach allows controlled *in vitro* loading without the need for shell disassembly under non-physiological conditions.

In addition to loading target proteins into the interior of encapsulins, the exterior of their shells can also be utilized to attach proteins of interest to the nanocarrier system (Fig. 2A). Exterior tethering of cargo proteins can be achieved in various ways. For example, protein ligation systems such as SpyTag/SpyCatcher can be used for covalently tethering proteins to the exterior of the encapsulin shell.[56,58,67,68] SpyCatcher-fused target proteins have been successfully attached to an appropriately SpyTag-modified encapsulin shell where the SpyTag was genetically inserted into the encapsulin shell protein, either at the externally accessible C-terminus[56,58,68] or a solvent-exposed external loop.[67,68] Using this approach, various proteins, including glutathione-*S*-transferase,[56] dihydrofolate reductase,[58] and the cancer-targeting affibody EGFRAfb[56,67] have been successfully attached to and displayed on *T. maritima* encapsulin shells. Additionally, shorter functional peptides can be genetically inserted into the shell protein—specifically the E-loop, A-domain, or C-terminus—and externally displayed without disrupting shell self-assembly. Examples include GALA peptides,[17] Fc-binding peptides,[69] and OT-1 peptides[57] being externally displayed by insertion into the E-loop of the *Q. thermotolerans* encapsulin, and the A-domain and C-terminus of the *T. maritima* shell, respectively.

While most encapsulin nanocarrier systems have been engineered to carry proteinaceous cargo, small molecules, inorganic nanoparticles, and nucleic acids have also been loaded into or displayed on encapsulin shells (Fig. 2A and B). Different chemical conjugation protocols—including click chemistry, thiol-maleimide-based strategies, and amine-succinimide chemistry—have been used to attach small molecules like the anti-cancer compound aldoxorubicin[54] or fluorophores like fluoresceins[69] and spiropyrans[53] to different encapsulin shells (90–95% conjugation efficiency). Alternatively, small molecules can be chemically conjugated to native TPs and subsequently loaded into encapsulin shells *in vitro* as demonstrated with the *Q. thermotolerans* LanM-fusion system.[66] Similarly, inorganic gold nanoparticles, chemically modified with TPs, have also been successfully encapsulated within the *T. maritima* encapsulin shell using an *in vitro* disassembly/reassembly protocol.[70] Finally, nucleic acids have recently been successfully encapsulated *in vivo* by fusing the nucleic acid-binding peptide Dps-N to the N-terminus of different encapsulin shell proteins without losing native protein cargo loading capabilities.[14] This opens up the possibility of co-encapsulating both therapeutic proteins and RNAs inside encapsulin nanocarriers for synergistic therapy. Applications include the co-delivery of siRNAs and inhibitory proteins for blocking a given target at both the mRNA and post-translational level and the co-delivery of antigens and immune adjuvants for vaccine enhancement.[71,72]

2.2. Nanocarrier targeting

Targeted drug delivery offers significant advantages over non-targeted or systemic administration of drugs.[73] Targeted delivery enhances therapeutic efficacy by concentrating the drug at the desired site, thereby increasing the drug's effectiveness while minimizing side effects and damage to healthy tissues. Targeted approaches can also allow for lower doses to be used, further reducing the risk of toxicity, resulting in improved patient outcomes.

To confer targeting capabilities to encapsulin shells, targeting moieties have to be displayed in an accessible manner on the encapsulin exterior (Fig. 3A). The aforementioned external cargo attachment strategies—genetic fusion, protein-based ligation, and chemical conjugation—can be similarly applied for displaying specific targeting moieties on the outside of encapsulin shells. For all these approaches, identifying appropriate modification sites within the encapsulin shell protein is crucial, as incorrectly chosen sites can disrupt shell self-assembly or even cause protein aggregation and insolubility.[68,74,75] Several sites in encapsulin shell proteins across different encapsulin systems, including those from *T. maritima*,[74,76] *Brevibacterium linens*,[76] *Myxococcus xanthus*,[68] and *Q. thermotolerans*,[17] have been successfully used for genetically inserting externally displayed peptides or protein domains while preserving the shell's ability to self-assemble. Either by direct genetic insertion or by using the SpyTag/SpyCatcher protein ligation system, diverse targeting moieties, including the brain-targeting PepC7 peptide,[76] a

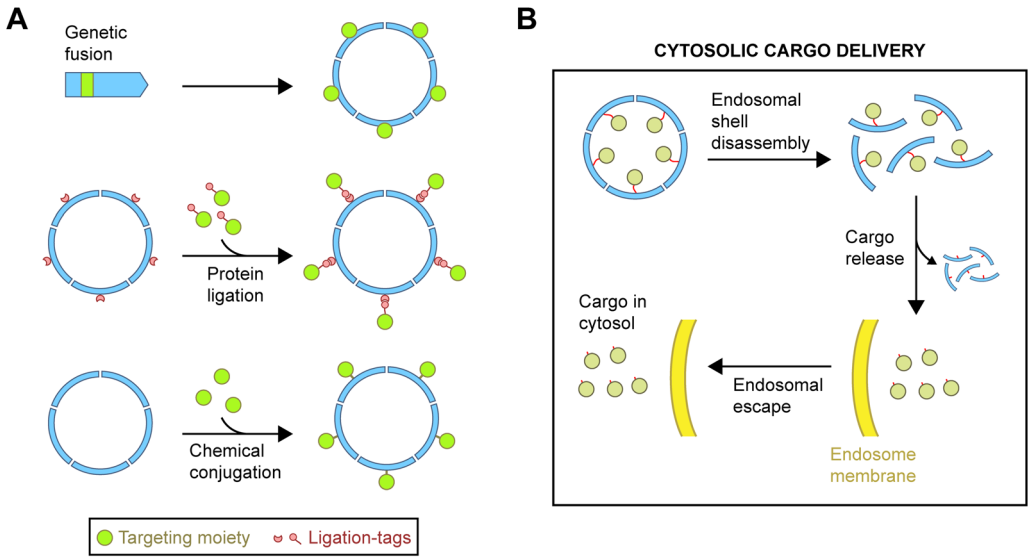

Fig. 3 Engineering targeting and cargo release for cytosolic drug delivery. (A) Different so far implemented targeting strategies for encapsulins are shown. These can be based on genetically integrating a targeting moiety into the encapsulin shell protein, on attachment *via* protein ligation, or on chemical conjugation techniques. (B) Overview of the necessary steps for successful cytosolic protein cargo delivery. After internalization *via* endocytosis, encapsulin shells need to disassemble followed by the release of cargo from the now disassembled shell. Finally, cargo needs to escape the endosome through the endosome membrane to reach its cytosolic target and exert its therapeutic activity.

human epidermal growth factor receptor 2 (HER2)-targeting designed ankyrin repeat protein,[61] the hepatocyte-targeting $PreS1_{21-47}$ peptide,[68] a HER2 affibody,[67] and an epidermal growth factor receptor-targeting affibody,[56] have been successfully displayed on encapsulin shells for target cell recognition. If a desired targeting moiety can be modified with reactive crosslinkers, chemical conjugation to the encapsulin shell offers an alternative route for targeting moiety installation.[54]

A key advantage of displaying targeting moieties on large multi-subunit protein shells like encapsulins is the enhancement of the avidity effect.[77] Although an individual targeting moiety might exhibit low affinity to a given target, the encapsulin shell functions as a scaffold that facilitates the display of 60, 180, or 240 targeting moieties in close proximity, thereby significantly enhancing the overall affinity of the nanocarrier to its intended target. In principle, multiple different targeting moieties can also be installed on the encapsulin shell *via in vivo* assembly of mixed differentially genetically modified shell proteins or multiplexed protein-based or chemical ligations *in vitro*.[67,78] The resulting modified shells, now displaying multiple different targeting moieties would have the potential to delivery drugs to multiple target sites or even bridge two distinct cell types, such as cancer cells and immune cells.[79]

2.3. Cargo release

Following cargo loading and precise delivery to a given target site, the next crucial step in nanocarrier-based drug delivery is the controlled release of cargo (Fig. 3B).[80] Cargo release is a pivotal process substantially influencing the therapeutic efficacy of a drug delivery system. To achieve triggered release, the release mechanism should be finely tuned to respond to specific physiological triggers or environmental conditions found at the target site—in terms of tissue or cell type, but also with respect to the specific cellular target. Such conditional triggers can include local pH,[81] redox state,[82] or the presence of certain enzymes or ligands.[83,84] As most nanocarrier systems are internalized into cells *via* endocytosis, the specific conditions found inside endosomes—acidic pH, endosomal redox state, or the presence of endosomal proteases—can be exploited for cargo release.[85] For example, for many viruses—in essence, naturally occurring nanocarriers—the process of virus uncoating, the disassembly of the virus capsid with concomitant release of the viral genome, is known to be initiated by the acidic environment within the endosome.[86] In antibody drug conjugates, the linker that attaches the antibody to the drug is typically designed to break in the reducing conditions of the endosome, releasing the cytotoxic payload inside the target cell.[82] Further, endosomal proteases, such as furin and cathepsins, can also serve as triggers for cargo release.[87] After cargo release has been achieved within the endosome, endosomal escape represents a further formidable challenge for macromolecular drugs while most small molecule drugs can efficiently cross the endosomal membrane. Achieving efficient endosomal escape for therapeutic macromolecules is challenging due to the lipid bilayer barrier of the endosome membrane and the possibility of eventual lysosomal degradation. Especially for protein- and nucleic acid-based drugs, which can be sensitive to the harsh conditions present in the endolysosomal environment, it is important to escape the endosome prior to losing their therapeutic efficacy. Several strategies have been developed to facilitate endosomal escape, inspired by natural mechanisms employed by viruses and other microorganisms. One common approach involves the use of pH-sensitive components that trigger membrane-disruptive activities in response to the acidic pH of the endosome. Fusogenic

peptides, such as the GALA[88] and INF7[89] peptides, mimic viral fusion proteins and can disrupt membranes in a pH-dependent manner by embedding themselves into the endosomal lipid bilayer, resulting in membrane destabilization. Alternatively, pore-forming proteins used by intracellular microorganisms could be harnessed to facilitate endosomal escape of macromolecular drugs. For example, listeriolysin O, a pH-regulated cholesterol-dependent cytolysin derived from the intracellular pathogen *Listeria monocytogenes*, is capable of forming transmembrane β-barrel pores within the endosomal membrane under acidic conditions.[90] Orthogonal approaches for endosomal escape involve exploitation of the "proton sponge" effect, where material that can buffer protons is accumulated in the endosome, leading to osmotic swelling and eventual rupture of the endosomal membrane. It has been shown that His-tags inserted into protein nanocages can promote endosomal escape through this "proton sponge" effect, leading to improved cytosolic nucleic acid drug delivery.[91]

Releasing cargo from encapsulin shells represents a significant challenge, as it necessitates both shell disassembly—at least for internalized cargo—and the subsequent release of TP-tagged or otherwise conjugated cargo molecules (Fig. 3B). While strategies for cargo loading and targeted delivery in encapsulin-based drug delivery systems have been extensively pursued, cargo release strategies remain largely unexplored. Ideally, encapsulins would need to be engineered to disassemble in response to specific relevant stimuli at the target site, as outlined above. Towards this goal, an engineered encapsulin shell was recently created by inserting the GALA peptide—able to undergo a coil-to-helix conformational transition upon acidification—into the externally exposed E-loop of the *Q. thermotolerans* shell, resulting in pH-triggered destabilization and shell disassembly.[17] Alternatively, histidine residues, which readily protonate under acidic conditions, can be strategically positioned at shell subunit interfaces leading to pH-triggered electrostatic repulsion and disassembly, as recently demonstrated in the P22 viral capsid.[92] Once a successful stimulus-responsive shell disassembly strategy has been developed, cargo still bound or tethered to the encapsulin shell protein needs to be released. For small molecule cargos, linkers that can be cleaved under reducing conditions could be employed to facilitate release within the endosome.[93] In the case of cargo proteins, which would be non-covalently bound to the encapsulin shell *via* their TP-tag, one release strategy could involve inserting specific protease cleavage sites between the cargo protein and the TP-tag. Developing effective strategies for cargo release and endosomal escape in encapsulin nanocarrier systems holds tremendous potential as it would maximize therapeutic efficacy by optimally leveraging encapsulins' substantial drug-loading capacity.

3. Drug delivery applications of encapsulins

As of writing this review, a relatively limited number of studies have been reported on engineering encapsulins as targeted drug delivery systems. So far, one of the primary application areas for these nanocarrier systems has been cancer therapy. Van de Steen *et al.* successfully developed a system, based on the *T. maritima* encapsulin, that uses a designed ankyrin repeat protein (DARPin9.29)—displayed on the shell exterior—as a targeting moiety, created *via* genetic fusion of DARPin9.29 to the shell protein C-terminus (Fig. 4A).[61] By C-terminally tagging miniSOG, a biological photosensitizer able to generate toxic reactive oxygen species (ROS) upon blue light exposure, with a TP-tag, efficient one-step *in vivo* assembly of the nanocarrier system could be achieved. DARPin9.29 was chosen for its specific binding affinity to HER2, which is overexpressed in certain breast cancer cell types. The engineered encapsulin nanocarrier exhibited specific binding to HER2-positive SK-BR-3 cells *in vitro* and triggered apoptosis upon blue light illumination, indicating its potential for targeted photodynamic cancer therapy. A similar miniSOG-encapsulin-based photosensitizer has been constructed by Diaz *et al.*, however, lacking the specific cell targeting capability of the abovementioned system.[62] It was demonstrated *in vitro* that this miniSOG-nanocarrier could be endocytosed by A549 human lung adenocarcinoma cells. Further, blue light exposure resulted in a phototoxic effect. Moon *et al.* also explored targeted delivery using a *T. maritima* encapsulin-based nanocarrier (Fig. 4B).[54] Hepatocellular carcinoma (HCC) cell-specific binding peptides (SP94) were either non-specifically chemically conjugated to the exterior of the encapsulin shell or displayed *via* genetic insertion into an external loop within the shell protein A-domain. Then, a fluorescent probe (F5M) or the anticancer prodrug aldoxorubicin (AlDox) was externally attached *via* a second—now specific—chemical conjugation step. Confocal microscopy confirmed the specific binding of SP94-conjugated encapsulins to HCC cells. Furthermore, *in vitro* cell culture experiments demonstrated the cytotoxic effect of doxorubicin (Dox) released from AlDox-loaded targeted encapsulins. Köhnke *et al.* used the *M. smegmatis* encapsulin to encapsulate a TP-tagged tandem nitroreductase (tdNfsB) *in vivo* to create a robust prodrug-activating nanoreactor (Fig. 4C).[63] The rationale was that the constrained environment within the encapsulin shell would enhance the activity and stability of the enzyme. The study demonstrated that the encapsulated tdNfsB exhibited significantly improved stability and comparable enzymatic activity against various nitroaromatic prodrugs as compared to the free enzyme. *In vitro* assays also showed that the tdNfsB-initiated cytotoxic effect of different prodrugs, specifically Nbzp and MA60, on H1299 lung carcinoma cells was akin to the cytotoxicity observed with free tdNfsB. The above findings highlight the modularity of encapsulins as a technology platform, allowing for facile surface modification with targeting moieties and the efficient encapsulation of various therapeutic and diagnostic molecules. The inherent enhanced permeability and retention (EPR) effect associated with nanoscale delivery systems like encapsulins, further enhances their potential for drug delivery applications by enabling potentially improved tissue penetration and longer circulation times.[94]

Applications of engineered encapsulins have also been explored in immunotherapy and vaccine development. Choi *et al.* used a genetically engineered *T. maritima* encapsulin for

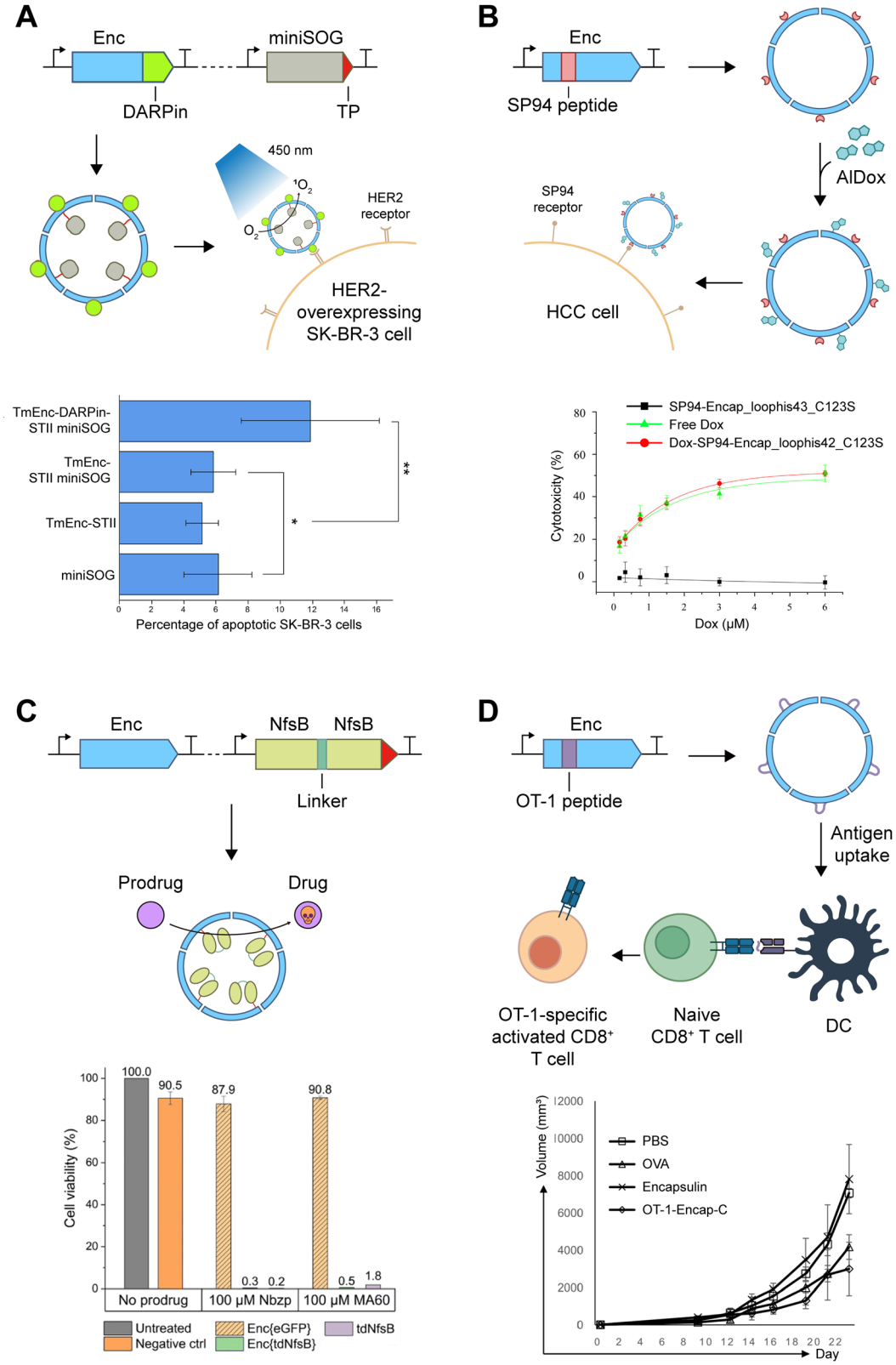

Fig. 4 Recent applications of engineered encapsulins for drug delivery. (A) Top: *T. maritima* encapsulin displaying the antibody mimetic protein DARPin9.29 (DARPin), loaded with the cytotoxic photosensitizer protein miniSOG. This drug delivery system specifically targets the HER2 receptor on breast cancer cells. Upon target binding and blue light illumination at 450 nm, miniSOG generates reactive oxygen species in the form of singlet oxygen ($^1O_2$) inducing apoptosis in targeted cells. Bottom: The cytotoxic effects of this drug delivery system on SK-BR-3 cells, compared to control samples, upon illumination, are shown. Enc: encapsulin. TP: targeting peptide. Adapted with changes with open access permission from ref. 60 *via* a creative

common license (https://creativecommons.org/licenses/by/4.0/). (B) Top: *T. maritima* encapsulin genetically engineered to display the HCC cell-specific binding peptide SP94 with the anticancer prodrug aldoxorubicin (AlDox) chemically conjugated to the shell exterior. Bottom: The dose-dependent cytotoxicity profiles of AlDox-SP94-Encap_loophis42, free AlDox, and SP94-Encap_loophis42 against HepG2 cells are shown. Adapted with permission from ref. 53. Copyright 2014 American Chemical Society (ACS). (C) Top: *M. smegmatis* encapsulin encapsulating tandem NfsB (tdNfsB) which can activate various nitroaromatic cancer prodrugs. Bottom: Cell viability assay results are shown demonstrating that encapsulated tdNfsB elicits comparable efficacy to the non-encapsulated tdNfsB control. Adapted with changes with open access permission from ref. 62 *via* a creative common license (https://creativecommons.org/licenses/by/4.0/). (D) Top: The mechanism with which the OT-1-modified *T. maritima* encapsulin (OT-1-Encap) provokes an immune response leading to the differentiation of OT-1-specific cytotoxic T cells. Bottom: B16-OVA tumor growth in mice vaccinated with PBS (phosphate buffered saline), ovalbumin (OVA), unmodified encapsulin (Encap), or OT-1-Encap-C is shown, illustrating the tumor suppressive activity of OT-1-Encap-C. DC: dendritic cell. Adapted with permission from ref. 56. Copyright 2014 American Chemical Society (ACS).

antigen delivery to dendritic cells (DCs) to induce antigen-specific cytotoxic T cell activation and tumor rejection (Fig. 4D).[57] In the study, a model antigenic OT-1 peptide was genetically fused to the N- or C-terminus of the shell protein or inserted into an externally displayed loop. The resulting encapsulin shell, now displaying OT-1 peptides, were effectively taken up by DCs, leading to the proliferation of antigen-specific $CD8^+$ T cells. Notably, the construct with the OT-1 peptide at the C-terminus (OT-1-Enc-C) elicited the most potent T cell activation. In a prophylactic vaccination study, mice immunized with OT-1-Enc-C showed significant suppression of B16-OVA tumor growth and increased infiltration of IFN-γ-secreting $CD8^+$ T cells into the tumors. This study highlights the feasibility of encapsulin-based nanoparticles as a platform for delivering antigens to the immune system, with potential applications in cancer immunotherapy and vaccines. Similarly, Kanekiyo *et al.* explored the use of engineered *T. maritima* encapsulin nanoparticles as a platform for displaying rationally designed Epstein-Barr virus (EBV) vaccine antigens.[95] The primary target of immunity is the EBV glycoprotein 350 (gp350) that enables attachment to B cells through complement receptor 2 (CR2/CD21). Different gp350 domains, including gp350 D_{123},were C-terminally fused to the encapsulin shell protein, resulting in externally displayed antigens after shell self-assembly. Immunization studies in mice and non-human primates demonstrated that D_{123}-encapsulin nanoparticles elicited significantly enhanced and more durable neutralizing antibody responses compared to the non-fused soluble antigen. A significant fraction of these antibodies targeted the crucial CR2-binding site on EBV, a key epitope for neutralization. This study provides compelling evidence for the utility of engineered encapsulin nanoparticles for developing highly effective subunit vaccines against viral infections.

The discussed studies collectively demonstrate the significant potential of encapsulins as a versatile and robust platform for a wide range of use cases in biomedicine. Their inherent properties, such as self-assembly, genetic programmability, and amenability to chemical modification, allow for the design of sophisticated encapsulin-based nanodevices with particular potential in macromolecular drug delivery, cancer immunotherapy, and vaccine development.

4. Challenges and future directions

The development of encapsulin-based drug delivery systems has garnered significant interest in recent years. While various ways of conferring targeting capabilities to encapsulins have been explored and successfully applied in cell culture settings, true cytosolic delivery—especially of macromolecular drugs—remains relatively unexplored. This is likely due to the formidable challenge posed by implementing an efficient way of endosomal escape, after cell targeting and cellular uptake. This challenge—engineering endosomal escape—is the primary reason for the generally observed low drug delivery efficacy in nanocarrier-based delivery systems, in particular when it comes to intracellular targets. For encapsulins, future research should focus on understanding the mechanistic details of encapsulin shell assembly and in turn disassembly. Developing efficient and robust stimuli-responsive shells that can disassemble after reaching the desired target site would represent a substantial advance. Innovative approaches such as engineering pH-sensitive, enzyme-responsive, or thermo/ultrasound-sensitive encapsulin shells may provide avenues towards realizing this goal. Additionally, a frequently overlooked but crucial factor in enhancing drug delivery efficacy in protein-based nanocage systems is drug release from the shell, after shell disassembly. In encapsulin-based nanocarriers, protein and small molecule drugs are loaded either through TP-tagging or chemical conjugation. Even if the encapsulin shell efficiently disassembles, therapeutic efficacy may be compromised if the drug remains tethered to the shell. This could impede endosomal escape or interfere with binding the intended target site, thus affecting the pharmacodynamic or therapeutic properties of the delivered drug. Therefore, exploring novel strategies to ensure complete release of the drug molecule from the encapsulin shell will be critical for maximizing the therapeutic potential of encapsulin-based delivery systems. With robust cargo release strategies implemented, it will also become possible to efficiently co-deliver both multiple different proteinaceous cargos or combinations of proteins and RNAs with many promising applications including synergistic therapy and vaccine enhancement.

Other crucial considerations for any drug delivery system, especially nanocarrier-based platforms like encapsulins, are immunogenicity, biocompatibility, and biodistribution. As for any system, the initiation of a severe immune response against the encapsulin shell protein or accessible targeting moieties could significantly hinder its utility and efficacy. Many factors can contribute to apparent immunogenicity including source organism, surface charge, corona formation, and genetic or chemical surface modifications. It is essential to investigate the immunogenic properties of encapsulins to be utilized as nanocarrier platforms thoroughly. So far, only two studies have

reported information on the immunogenicity of unmodified encapsulins, specifically of the model encapsulins from *T. maritima* (in BALB/c mice) and *M. xanthus* (in BALB/c and C57BL/6 mice).[96,97] While it was found that both the *T. maritima* and *M. xanthus* encapsulins exhibited good nano-safety profiles—no abnormal weight loss, gross pathologies, or prolonged increase in toxicity biomarkers was detected—some immunogenic properties could be observed, specifically the generation of IgM and IgG antibodies for the *T. maritima* system (no antibody subclass distribution reported) and of all four major IgG subclasses (IgG1, 2a, 2b/2c, and G3) for the *M. xanthus* system, characteristic of both T cell-dependent and -independent pathways. It was suggested that the formation of a dynamic serum-derived protein corona on the *T. maritima* encapsulin shell surface may play a role in immune recognition. Interestingly, the *Q. thermotolerans* encapsulin did not exhibit any antibody cross-reactivity in *in vitro* experiments compared to the *T. maritima* shell, potentially suggesting immune-orthogonality among encapsulin systems. This may be due to the high sequence variability found within encapsulin shell proteins and highlights that even among natural encapsulin shells, some systems may be well suited for drug delivery applications. It was further found that the *T. maritima* shell showed good colloidal stability, blood compatibility, and a biodistribution profile indicating sequestration from circulation by the liver and biodegradation within Kupffer cells. Direct comparison of the *M. xanthus* encapsulin with virus-like particles from the *Leviviridae* phage PP7 highlighted that the encapsulin proved to be significantly less immunogenic with an IgG2a/IgG1 ratio significantly higher for PP7 (>4) than for the *M. xanthus* encapsulin shell (<1) in BALB/c and C57BL/6 mice suggesting a Th2 anti-inflammatory immune responses upon encapsulin administration. The limited amount of available immunogenicity data for encapsulin shells prevents any broad conclusions that can be drawn at this point. However, it seems likely that some immune response is to be expected for most encapsulin shells given their bacterial origin. At the same time, substantial variability in encapsulin immunogenicity is likely given their often highly divergent sequences. Besides finding naturally occurring encapsulins with low immunogenicity, other strategies to engineer encapsulin variants with reduced immunogenic profiles include various chemical surface modification techniques—the most prominent one being polyethylene glycol (PEG) decoration[98]—or mutating the encapsulin sequence to remove surface epitopes resulting in decreased immune activation.

Clearly, the development of a generalized framework for the design of encapsulin-based nanocarriers as drug delivery systems will crucially rely on future studies that further investigate the basic properties of encapsulins. Nevertheless, a broad outline of key decisions to be made in any such engineering effort will follow. (1) Encapsulin selection and engineering: key aspects of choosing an initial encapsulin scaffold include the appropriate size of the shell (T = 1, T = 3, or T = 4) to accommodate the intended payload, shell stability, and modifiability (genetically or chemically) with respect to surface modifications or pore alterations. In addition, the immunogenicity of a given encapsulin should be considered and ideally tested before its use as a nanocarrier platform. (2) Cargo loading strategy: depending on the type of therapeutic molecule (protein/RNA *vs.* small molecule; single *vs.* multi-cargo loading), different *in vivo* and *in vitro* loading strategies are available and should be chosen based on loading efficacy, ease of nanocarrier production, and downstream application. (3) Targeting: both genetic and chemical strategies exist for installing specific targeting moiety on encapsulin shells and have to be chosen based on the particular therapeutic target in mind; multiple different targeting moieties can be installed at the same time and the inherent multivalency of encapsulin shells may be exploited for specific targeting applications to enhance avidity. (4) Cargo release: selecting the modality of cargo release—both from the encapsulin shell and subsequently from the endosome—is a crucial step for the efficacy of encapsulin nanocarriers. Possibilities include the use of pH- or protease-triggered cargo detachment from the shell and cargos modified with endosomal escape-promoting modifications, peptides, or domains. (5) Pharmacokinetics: while little information about the pharmacokinetics of encapsulin-based nanocarriers is available, it still represents a key consideration for real-world applications. In principle, the pharmacokinetics of encapsulins can be altered by surface modifications (*e.g.*, PEGylation), or genetic alterations. (6) Scalability: a final consideration for encapsulin nanocarrier application is the ability to produce them at scale. If compatible with a given application goal, *in vivo* production in bacterial hosts or yeast—both well established—likely represents the most robust and scalable way of producing encapsulin nanocarriers. While multi-step protocols may be necessary for *in vitro* cargo loading or chemical modifications, nanocarrier systems with proteinaceous cargo could ideally be produced through one-step fermentation approaches.

In conclusion, while encapsulin-based drug delivery is an innovative and programmable approach with substantial potential, addressing the challenges of delivery efficacy and nanocarrier immunogenicity, especially the lack of immunogenicity data in human-relevant models, as a key future challenge will be paramount for any future real-world application of encapsulin nanocarriers. In the near term, research should be focused on developing strategies for stimuli-responsive shell disassembly, drug release from the shell, and immune evasion. Advancing these areas will be essential in translating encapsulin-based drug delivery systems from bench to bedside in the future, with the potential to enable new therapeutic avenues and improve patient outcomes.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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