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Bacterial Nanocompartments: Structures, Functions and Applications

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ABSTRACT

Increasing efficiency is an important driving force behind cellular organization and often achieved through compartmentalization. Long recognized as a core principle of eukaryotic cell organization, its widespread occurrence in prokaryotes has only recently come to light. Despite the early discovery of a few microcompartments such as gas vesicles and carboxysomes, the vast majority of these structures in prokaryotes are less than 100 nm in diameter - too small for conventional light microscopy and electron microscopic thin sectioning. Consequently, these smaller-sized nanocompartments have therefore been discovered serendipitously and then through bioinformatics shown to be broadly distributed. Their small uniform size, robust self-assembly, high stability, excellent biocompatibility, and large cargo capacity make them excellent candidates for biotechnology applications. This review will highlight our current knowledge of nanocompartments, the prospects for applications as well as open question and challenges that need to be addressed to fully understand these important structures.

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INTRODUCTION

Historically, prokaryotes have long been considered simple, lacking much of the complexity that defines eukaryotic cells. However, discoveries during the last quarter century have challenged this simplified view of bacterial cell biology resulting in two major conceptual revisions. The discovery of FtsZ in the 1990s (1-3) ushered in the age of bacterial cytoskeletal research (reviewed in 4) culminating in the recognition that bacteria, in fact, possess a larger variety of cytoskeletal proteins than eukaryotic cells (5). Likewise, the discovery of ever-increasing numbers of organelle-like structures in bacteria and archaea (Fig. 1) demonstrated that compartmentalization, a key feature of eukaryotic cells is ubiquitously used in prokaryotes. In contrast to bacterial cytoskeletal proteins, the first microcompartment was discovered more than a century ago in aquatic cyanobacteria (6; for a recent review 7). What made the early discovery of gas vesicles possible was their sheer size. Individual structures can have diameters of 120 nm and reach lengths of 1400 nm, making them the only known bacterial compartment visible in a compound light microscope. The advent of transmission electron microscopy in the 1950s led to the discovery of carboxysomes (8). Initially called polyhedral bodies, carboxysomes are 40-200 nm large (quasi)icosahedral protein shells that contain a two-protein enzymatic core formed by carbonic anhydrase and RuBisCO and are essential for carbon fixation (9). Similar microcompartments were later found in *Salmonella* when grown on 1,2-propanediol (10, 11) or ethanolamine (12, 13) and subsequently in a large number of bacterial phyla (14). In contrast to carboxysomes, these metabolosomes are catabolic organelles that detoxify short-chain aldehydes (14, 15). In 2008, serendipity helped discover another type of bacterial compartment, the encapsulins (16). Encapsulins are composed of 20-40 nm wide shells and

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internalized cargo proteins and because their size is smaller than 100 nm they are classified as nanocompartments (17, 18). The first observations of these structures were made in 1994, when high molecular weight protein aggregates were observed in the culture supernatant of *Brevibacterium linens,* noted for their pH stability and bacteriostatic properties (19). It took more than a decade until the nanocompartment nature of these "aggregates" was revealed and the atomic structure of the encapsulin from *Thermotoga maritima* solved using X-ray crystallography (16). Since then, many other encapsulins have been identified and some studied in great detail including encapsulins from *Mycobacterium tuberculosis* (20), *M. smegmatis* (21), *Rhodococcus jostii* (22), *Myxococcus xanthus* (18), *Quasibacillus thermotolerans* (23), *Synechococcus elongatus* (24), and the archaeon *Pyrococcus furiosus* (25). In fact, increasingly sophisticated comparative genomics has identified encapsulin-encoding genes in at least 31 out of 35 bacterial and 4 out of 5 archaeal phyla (24, 26-28), indicating that these structures are far more widespread than initially thought. Like all bacterial compartments, encapsulins are composed entirely out of protein, no nucleic acid, lipid or carbohydrates have been detected in any of these structures so far, and although encapsulins possess virus-like icosahedral morphologies their phylogenetic relationship to virus capsids is unclear with recent discussions suggesting a possible caudoviral origin (28). What is however clear is that the numerous bacterial compartments strongly support the idea that the principle of cellular compartmentalization predates the origin of eukaryotes and, in fact, appears to be among the first innovations that made primordial cells more efficient (29). Here, we will discuss the recent progress in our understanding of the structure and function of encapsulins as well as ongoing attempts to develop nanotechnological applications for these uniquely versatile structures.

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The structure of encapsulin shells. Geometrically, encapsulins are icosahedral shells that spontaneously self-assemble from multiple copies of a single protomer that can oligomerize into pentamers and hexamers (16, 25). Topologically, a minimum of twelve pentamers is required, while larger shells can be formed through the addition of variable numbers of hexamers. As a result, encapsulin shells, like virus capsids, are scalable structures that can vary in size (Table 1). To quantitatively characterize this complexity, the triangulation number T is used. Initially introduced to quantitatively describe the geometry of icosahedral viruses (35), T is useful not only to characterize the size of encapsulins but to group them based on their shell architecture. The simplest encapsulins, T=1 icosahedrons are found in *T. maritima* and *Mycolicibacterium hassiacum,* where 60 protomers form ca. 24 nm large shells (16, 36). This T=1 geometry is also present in the highly abundant family 2 encapsulins that are found among others in the freshwater cyanobacterium *S. elongatus* and are distinct from all other so far studied encapsulins that are grouped into family 1 (24, 28). The main difference between family 1 and family 2 encapsulins is the lack of an extended N-terminal helix in the latter, instead family 2 encapsulins possess a shorter N-terminal helix with an extended N-terminal arm. However, whether these differences are also present in the cNMP-binding domain-containing family 2 encapsulins remains to be seen. Family 1 encapsulin shells of *Sulfolobus solfataricus, P. furiosus* and *M. xanthus* are 32 nm wide T=3 icosahedrons that are assembled from 180 protomer subunits (18, 25, 37). Whilst, the encapsulin of *Q. thermotolerans* shows T=4 symmetry, possessing 240 protomer subunits and a diameter of 42 nm (23). This encapsulin possesses the largest atomically resolved shell so far creating an internal cargo space that is 530% larger than that of the T=1 capsids, and

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220% larger than that of the T=3 capsids. Of note, the number of bioinformatically identified possible T=4 encapsulins is so far less than the numbers of T=1 and T=3 shells, which may indicate that among encapsulins like phage capsids larger structures are evolutionary disadvantaged and therefore less common in nature. Two additional less numerous families of encapsulins have been recently identified using computational approaches (28). However, no high-resolution structures exist of any family 3 natural-product-biosynthesis encapsulins or family 4 "truncated A-domain" encapsulins, in which only the compact, five-fold symmetry interface contact-mediating C-terminal A-domain of the HK97-fold is present and all other domains are missing. Thus, it remains to be seen how different their capsids are from solved structures.

Notably, the mutual resemblance between encapsulins and icosahedral phage capsids is not only the consequence of their shared geometry but the result of a similar molecular structure of their shell protomer proteins. All encapsulin protomers have the same HK97-like fold as the mature 31 kDa large viral gp5* phage main capsid protein (38), despite a lack of sequence similarity (16, 39). This prominent fold was first observed in the lambdoid Hong Kong 97 (HK97) bacteriophage (39), and has since been found in other tailed phages (40, 41), in herpes viruses (42, 43), in the archaeal *Haloarcula sinaiiensis* tailed virus 1 (HSTV-1; 44), and in several domains of double stranded DNA viruses (45). Structurally, the HK97-like fold is characterized by the presence of the "spine" α–helix, the peripheral (P-) domain, the axial (A-) domain and the β–hairpin elongated (E-) loop (39) with the E-loop showing the most sequence variability between different protomers (16, 18, 27, 46). While the shell proteins of *P. furiosus* and *M. xanthus* closely match the domain structure of the HK97-fold (18, 25), the *T. maritima* protomer's homology is limited to the A- and E-loop domains (16).

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The E-loop is essential for the interfacing between subunits, and the relative orientation of it defines the triangulation number and size of the capsid. *T. maritima* encapsulins possess E-loops that are shorter and more rotated than those of larger T=3 capsids. This alteration appears to allow the protomers to form tight β-sheets with their neighbors resulting in the smaller T=1 capsids (16). In contrast, the T=4 encapsulin shells of *Q. thermotolerans* show a non-covalent chain mail topology, a structural feature commonly found in virus capsids. Here, the E-loop and P-domain of each capsid monomer are arranged head to tail, forming interlocking concatemeric rings, which provide the structure with increased level of thermostability (23). This structural motif has also been observed in the T=1 encapsulins of *S. elongatus*, however in this instance, it is an extended N-terminal arm which interlocks with the neighboring subunit to create the chain mail topology (24).

Despite their structural similarity, encapsulins and viral capsids differ with respect to a number of important features. To start, encapsulins and capsids use different assembly mechanisms and pathways. Encapsulins self-assemble through the repeated addition of dimers (47), whilst virus capsids make use of more complex assembly processes (48-50). In particular, virus capsid assembly is usually guided by a scaffolding protein that is either N-terminally fused to the protomer (as in HK97) or provided as a separate protein (51). As all studied encapsulins effortlessly self-assemble upon expression in *Escherichia coli*, they do not rely on scaffold-mediated guidance which may be due to their lower T-numbers. Moreover, HK97 capsids undergo large-scale molecular rearrangements of their assembled protomers during capsid maturation (52). These conformational changes are necessary to increase capsid stability and expand cargo capacity. No such protomer movements appear to occur in encapsulins, which may be due to the very different osmotic properties of the

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encapsulated cargos. Although the assembly is different, the resulting structures are remarkably similar potentially suggesting that encapsulins may have evolved from HK97-like phages. Gradually, selection could have erased the integrated phage genome leaving only the gene for the capsid shell, the future encapsulin behind. Whether this scenario is correct is difficult to judge as very rarely genes encoding phage-like proteins are found in today's encapsulin operons (e.g., phage-like replicative helicase in *S. solfataricus*; 37). Nonetheless, the recent lab-based evolutionary conversion of lumazine synthase into an RNA-containing virus-like capsid supports the plausibility of the relatedness of these self-assembling structures (53).

Although the rigid encapsulin shells form formidable permeability barriers, they contain multiple pores through which small molecules, ions or organic compounds can enter the encapsulin lumen (22, 54-56). Structurally, these pores are located at the sites of symmetry (3- and 5-fold pores) and at the interface between protomers (2-fold pores) ranging 3-7 Å in size (16, 18, 23, 57). The structures of the two- and five-fold pores appear often to be conserved, whilst the three-fold pores show high levels of variability possibly to accommodate specific substrates (17). Usually, two-fold pores are lined with negatively charged residues, while five-fold pores, like the ones from *T. maritima,* are often uncharged but surrounded by a ring of histidine residues, which may coordinate and help translocate metal ions like iron across the shell (16). Additionally, it has been proposed that the interaction between the shell residues and potential substrates may influence the activity of the encapsulated cargo proteins (16). Compared to *T. maritima*, the encapsulins in *Q. thermotolerans* possess 3-fold pores that are larger (7.2 Å) and negatively charged due to the presence of aspartate and glutamate residues, which may facilitate iron uptake (23).

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Remarkably, in this bacterium's encapsulin the two-fold pores appear to be closed. However, the fact that two flexible asparagine side chains are present at the expected site of the pores points to the possibility that the pores may be gated to provide control over substrate permeability (23). Such gating has recently been observed for the 5-fold pores of the *Haliangium ochraceum* encapsulin which can widen from 9 to 24 Å (58).

By and large, the size and physicochemical properties of the pores control access to the interior of the encapsulin, permitting small molecules while blocking larger ones. However, the maximum molecular cut-off for access is currently unknown. For example, in *R. jostii* RHA1, the encapsulated dye-decolorizing peroxidase (DyP) degrades nitrated lignin, which is several magnitudes larger than any known pore and it is currently unclear whether and how lignin can actually enter the encapsulin (16, 22, 59). To solve this conundrum, it has been proposed that the encapsulin is either a dynamic structure, able to disassemble upon recognition of the substrate (22) or that the currently unknown pore architecture in this encapsulin is large enough to allow the passage of lignin polymers (see 58). Undoubtedly, pores are essential for encapsulin function allowing cytosolic substrates access to the encapsulated proteins. Thus, pores represent interesting targets for bioengineering to potentially fine-tune substrate access and selectivity (60).

Encapsulin cargo proteins. The initial clue that encapsulins contain cargo proteins came from unaccounted electron densities in X-ray images of the *T. maritima* shell (16). Eventually, eight amino acid residues could be resolved that matched the C-terminal part of a ferritin-like protein encoded in the same operon next to the gene of the encapsulin shell protein. In contrast to its C-terminus, the rest of this ferritin-like protein was too variably

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arranged as to be resolved in the electron density maps. Since then *in-silico* research has shown that many encapsulin cargo proteins possess similar peptide sequences termed targeting or cargo loading peptides (CLP) that specifically target them to the encapsulin. Fortuitously, CLPs are often sufficiently conserved so that they can be used to bioinformatically identify potential cargo proteins (24, 27). By combining this approach with a genome neighbourhood analysis-based strategies thousands of previously unknown potential cargo proteins have recently been identified (28). However, validating cargo proteins and measuring their stoichiometry is more challenging and has e.g. been achieved using STEM measurements of purified natively assembled encapsulins (18). Sequence-wise, CLPs of cargos of family 1 encapsulins are C-terminal peptides (16, 54-56), while CLPs of family 2 and 3 encapsulins are often highly-disordered N- or C-terminal aa sequences (24, 28). Like in *T. maritima*, cargo proteins are usually expressed in co-regulated operons with their corresponding shell protein and it is generally assumed that they are loaded co-translationally although experimental evidence for this packaging mode is currently lacking (16, 27). However, there are exceptions to this rule. In *P. furiosus*, the cargo gene is fused with that of the shell protein and they are expressed as a single polypeptide (25). Other bacteria possess additional cargo proteins and these 'secondary cargos' are not encoded in the encapsulin operon (18, 61). The alternate genetic loci of these secondary cargos challenge a simple co-translationally packaging model during assembly (54, 55). How the cells solve this problem is currently unclear.

Research has shown that CLPs are both necessary and sufficient for the encapsulation of cargo proteins (16, 27, 54-56, 61, 62). The deletion of the CLP from the C-terminus of DyP, a cargo protein in *T. maritima,* prevented encapsulation (16). While in *R. erythropolis N771*,

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the addition of CLPs to non-native cargos such as enhanced green fluorescent protein (eGFP) and luciferase facilitated efficient packaging (54). This flexibility of packaging is one of the key features that make encapsulins attractive for bioengineering.

As space is limited inside encapsulins, cargo protein loading is restricted raising questions of how many cargo proteins can fit into a single shell and how they are organized. In theory, each capsid protomer has one CLP binding site and therefore could bind one cargo protein but in practice the number of cargo proteins must be lower than that of protomers. Steric hindrance among the cargo proteins, the need for proper folding and shell closure all constrain cargo encapsulation (16, 47). For example, it has been shown that DyP oligomerizes into hexameric rings upon encapsulation in *B. linens*, with one hexamer being assembled in each shell (47). In the same shell, twelve molecules of GFP can be loaded weighing 400 kDa compared to the 240 kDa of the DyP hexamer. Thus, the spatial arrangement of the molecules is more important than the size when determining packaging into encapsulins. Ferritin-like proteins have been shown to oligomerize into decamers during packing, with each decamer being significantly smaller than a DyP hexamer (56). This difference in size means 120 ferritin-like proteins fit within a T=1 shell compared with the 6 DyPs, thus drastically increasing the cargo to shell ratio (47). Cargo loading is further complicated in multi-cargo encapsulins. As described, *M. tuberculosis* and *M. xanthus* need to package three or four different cargo proteins, respectively thereby increasing the risk for steric clashes (18, 61, 63). Perhaps, these bacteria use currently unknown mechanisms for regulating packaging of heterologous cargos, as a high level of selectivity for cargo is maintained as the complexity of cargo increases (18, 47, 55). Maybe cargo protein oligomerization plays a not yet understood role in the control of cargo packaging. Another

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option, found in iron-mineralizing encapsulin-associated Firmicutes (IMEF) could be the use of both N- and C-terminal CLPs on different cargo proteins, which may provide higher levels of control over packaging (27). Whereas it is thought that the relative concentrations of the substrates in proximity to the binding site is used to control the loading in other systems. Additionally, there may exist transcriptional control systems that limit the relative production of each cargo protein, allowing for increased packaging efficiency. One way to address these issues would be *in situ* cryo-electron microscopy of the cargo complexes. Although data exist that indicate sub-stoichiometric occupancy of binding sites (21), the resolution in many tomograms is not yet high enough to definitively answer these questions (18, 58).

Biological functions of encapsulins. Although the biochemical properties of the cargo proteins are key to unravelling the biological functions of encapsulins, answers are not always straightforward. To start, most bacteria produce un-encapsulated versions of their cargo proteins (16, 27). In addition, functional assignments are often complicated by the lack of physiological data for the mostly bioinformatically identified encapsulin systems (28) and, finally, the observation of assembled encapsulins in culture supernatants have raised questions about the localisation of these structures (19).

Despite these challenges recent research has started to provide crucial information. Importantly, packaging of fragile proteins into stable shell structures may increase the life time of the cargo, as encapsulin shells have increased thermo- and pH-stability (16, 19, 22), as well as providing protection from proteases (54, 55). In fact, the prevalence of encapsulins in extremophiles suggests that cargo packaging may be particularly beneficial under harsh

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conditions (27). Interestingly, atomic force microscopy has shown that cargo-binding decreases the shell's mechanical stiffness due to conformational changes of the shell structure, which may have implications for the overall stability of encapsulins (47, 64).

Surprisingly, despite their intracellular assembly, it is not completely clear whether encapsulins are intra- and/or extracellular structures. Their initial discovery in culture supernatants pointed to possible extracellular functions (16, 18, 19, 22), however currently available evidence suggests that they are cytosolic and appear only in the supernatant after cell lysis because of their resistance to degradation (17). This hypothesis is also supported by the lack of any known bacterial transport system capable of translocating an assembled structure of this size (26, 65). However, this does not rule out that encapsulin-producing cells may undergo coordinated lysis to release large numbers of these structures to either control physicochemical parameters of the medium, degrade substances, poison other cells or provide metabolites for their kin.

Based on large-scale bioinformatics analyses of their potential cargos, encapsulins have been suggested to play roles in a wide range of physiological responses including stress resistance, toxin sequestration, natural product biosynthesis, catabolic and anabolic metabolism and anaerobic hydrogen production (26, 28). However, the validity of these assigned functions and the precise role of the encapsulin shell in these different processes has been studied in only a handful of instances.

One well studied function is their role in oxidative stress response, protecting the cell from peroxide-related damage. DyP cargo enzymes such as those found in *M. tuberculosis* are active against polyphenolic compounds such as azo dyes, although their natural substrates are unknown (56, 59, 61, 66). *In vitro* studies have shown that the peroxidase activity of DyP

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increases 8-fold upon encapsulation, perhaps due to the increased protection provided by the shell or by increasing the local substrate concentration akin to the accumulation of CO_2 in carboxysomes (67-70). The deletion of the shell protein, EncA, from *M. xanthus* resulted in a strain that was more sensitive to hydrogen peroxide than the wild type (18). Increased peroxide sensitivity has also been observed when the ferritin-like cargo protein is deleted from the *M. tuberculosis* system (71). Importantly, all other cargo proteins in *M. tuberculosis* appear to contribute to the overall oxidative stress resistance of the bacterium (61). In contrast, the DyP-containing encapsulin of *R. jostii* RHA1 has been implicated in a catabolic reaction. Deleting the *dypB* gene, results in a mutant which generated encapsulins that were unable to break down the substrate nitrated lignin (22).

Probably, the best studied function of encapsulins is the mineralization and storage of iron. Iron is essential for many cellular processes but in excess can cause oxidative damage. If iron homeostasis is not maintained, Fe(II) is oxidized to insoluble Fe(III) and reactive oxygen species like free hydroxl radicals are formed *via* the Fenton reaction that can damage cellular structures (72-74). Hence, it is hypothesised that iron-sequestering encapsulins constitute a back-up iron management system, alongside the traditional ferritin system, that becomes active during times of stress. For example, amino acid starvation of *M. xanthus* up-regulates the number of encapsulins fivefold (from 4-5 to 20-25 per cell) with each structure capable of storing ~30,000 iron atoms that form 5-6 nm granules within the organelle's 20 nm wide core (18). The observation that the more stress-resistant tan variants of this bacterium appear to not contain any assembled encapsulin during growth and, upon starvation, form only 4-5 particles per cell, the same number found in the more sensitive yellow strain during vegetative growth, highlights the fact that encapsulin assembly is not

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only dependent on environmental conditions but also controlled by strain-specific factors (unpublished results and 75). In contrast to *M. xanthus*, *Q. thermotolerans* lacks traditional 'ferritins' and therefore appears to use encapsulins as their primary system for iron homeostasis (23). As the larger size means that each *Q. thermotolerans* encapsulin can store up to ~83,000 iron atoms (23). Interestingly, it appears that all encapsulin proteins are necessary for efficient iron storage. Research using *Bacillaceae bacterium* MTCC 10057 encapsulin expressed in *E. coli* determined that only when the shell and both cargo proteins were expressed was iron mineralized efficiently *in vivo*, despite one of the cargo proteins, Fer, not being essential for iron mineralization (23). Furthermore, *E. coli* only showed increased resistance to hydrogen peroxide when the iron content of the medium was increased, indicating that iron storage and oxidative stress response are linked (27). Another type of cargo protein linked to oxidative stress are hemerytherins (27). These metalloproteins are capable of reversibly binding O_2 through an iron atom bound by an oxo bridge (75). In encapsulins, they are found exclusively in T=1 capsids organised into 20 sets of dimers (63, 77, 78). Expression of the hemerythrin-encapsulin system from *Streptomyces sp*. AA4 in *E. coli* showed that for optimal levels of protection both, the shell and cargo were necessary (27).

Encapsulins have also been identified as metabolically important in anaerobic ammonium oxidising (anammox) bacteria (27). Anammox bacteria oxidise ammonium with nitrite creating dinitrogen gas as part of their metabolism (79, 80). Using growth curve assays, it has been shown that encapsulins aid anammox bacteria in resisting hydroxylamine-related stress (27, 81, 82) by sequestering the toxic intermediate hydrazine (80, 83), through a nitrite reductase-like (NiR)/hydroxylamine oxidoreductase (HAO)-like fusion cargo protein (82,

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83). The NiR-like cargo has been hypothesised to be a laccase, a multicopper enzyme which oxidises aromatics using oxygen (81). Intriguingly, anammox encapsulins could also function extracellularly, as their NIR-like cargo can help maintain an anaerobic environment, a prerequisite for the bacterium's survival (81). However, due to the difficulties of working with anaerobes and the lack of genetic tools no work has so far been done exploring this possibility.

Biotechnological applications of nanocompartments. Using biological structures to solve medical and engineering problems has long been a goal of nanotechnology. Thus far, various biological nanostructures have been explored including micelles (84), liposomes (85), polymer nanoparticles (86), virus-like particles (87), DNA origami structures (88), and many different protein-based cages (89, 90). Despite being functional, these nanostructures lack the ability to self-assemble *in vivo,* which is one of the great advantages of encapsulins. Although exceptionally well suited, encapsulins have, however, limitations that remain to be addressed. One such limitation is their small pore size of 3-4 Å, which is ideal for the transfer of ions or small substrates, but severely limits access of larger molecules (16, 36, 57). Encouragingly, site directed mutagenesis of the pore-forming loop region of the *T. maritima* shell protein has recently tripled the pore size to ~11 Å. This modification resulted in a 7-fold increase in the rate of diffusion across the pores (57). Hence, additional modifications could further improve diffusion and the recent discovery of naturally occurring larger pores (5-9 and 24 Å) in the encapsulins of *M. hassiacum* (36) and of *H. ochraceum* (58) respectively indicates that this goal may be achievable with smaller alterations. Another area for research is cargo packaging. While the well-defined CLP tags

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have been successfully used to package a wide range of heterologous cargo proteins (54, 55, 91), inorganic molecules like gold nanoparticles have only recently been explored as cargos (92). Moreover, recent systematic investigations of the CLP-shell interaction (93), shell stabilization and purification strategies (94, 95), and the design of an on-demand reversible assembling encapsulin (96) may increase control over the assembly process. Despite this overall progress, there are a number of open questions: How do the disordered packaging signals work and can they be used akin CLPs? What is the best strategy to package multi-protein complexes? How are these complexes organised inside the protein cage and how does this impact functionality (97)? Can the procedures for T=1 capsids simply be scaled up for the larger T=3 and T=4 shells? And finally, are there restrictions to the hosts in which encapsulins can be introduced? While prokaryotic hosts appear to be generally permissive (27, 56), few eukaryotic hosts such as yeast, mice and select mammalian and insect cell lines have so far been tested for the production of encapsulins (98-100). To highlight the nanotechnological potential of encapsulins, we will in the following briefly discuss four of the many theoretically possible applications (Fig. 2) that are currently moving from concept to commercialization:

Vaccines. For more than 40 years live or recombinant derivative adenovirus vaccines have been used for immunization (101, 102). So, it's no wonder that the virus-like nature of encapsulins has attracted attention as vaccine platform. In particular, their ability to package immunogenic cargos offers a second, very different mode of antigen presentation besides the surface display of adenovirus vaccines. The potential of both modes of presentation to induce an immune response has been shown through the production of a novel influenza A vaccine (103). For this vaccine, the *T. maritima* encapsulin was functionalized to display the

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matrix protein 2 ectodomain, an immunologically important epitope from influenza A on its surface whilst encapsulating GFP as a reporter. Following assembly, studies in mice showed specific antibody production against both the surface-displayed epitope and the GFP cargo (104). Likewise, encapsulin surface-associated OT-1 antigen has been used to activate CD8+ T cells for tumour rejection. Upon uptake of the OT-1-presenting encapsulins by phagosomes, corresponding antigen-specific T-cells were produced that significantly suppressed a B16-OVA melanoma (105). Consequently, encapsulin could become valuable alternatives to currently existing vaccines, while their lack of nucleic acid could increase vaccine acceptance.

Drug Delivery. Encapsulation of drug molecules increases efficiency of drug delivery and reduces the risk of side effects (106, 107). To explore encapsulins as drug delivery vehicles, *T. maritima* encapsulins displaying the hepatocellular carcinoma cell-binding peptide SP94 were chemically coupled to the anticancer prodrug aldoxorubicin. Efficacy wise the coupled prodrug showed the same killing efficiency as the free prodrug molecule, while the combination of a targeting ligand and therapeutic allowed site-selective delivery to HepG2 carcinoma cells (46, 108). Moreover, use of fluorescently labelled encapsulins of *T. maritima* (109) and *B. linens* (110) allowed images to be taken of interactions with their target cell populations highlighting their stability during the uptake process. More recently, PEGylation has been used to further improve the usability of encapsulins as drug carriers (111). PEGylation is a widely-accepted drug carrier modification, that increases the drug delivery efficiency by decreasing the visibility of the carrier to phagocytes and by preventing carrier aggregation. For example, using surface-exposed lysine residues, the encapsulins of *R.*

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403 *erythropolis* N771 were successfully PEGylated and encouragingly, the modification did not
404 interfere with the assembly and disassembly of the structure (111). However, inspection
405 revealed that the shells were empty, meaning that simultaneous cargo loading and
406 PEGylation may destabilize encapsulin even more than cargo loading does alone (47).
407 Nonetheless, successful PEGylation is likely achievable and would greatly improve drug
408 delivery.
409
410 **Biocatalysts.** Encapsulins are essentially biocatalysts that accelerate the kinetics of
411 biochemical reactions through an increase in substrate concentration within a confined space
412 (112). Given their theoretical promiscuity, any encapsulated protein should in practice be
413 able to catalyse the corresponding reaction. In a proof of principle study, five different
414 enzymes, a catalase, a monooxygenase, two oxidases and a peroxidase were successfully
415 packaged in the encapsulin of *M. hassiacum* (36). Four of the five enzymes were
416 enzymatically active, while the fifth protein, the flavin-containing monooxygenase was
417 inactive likely due to the 9 Å pores blocking access of the cofactor NADPH, again
418 highlighting the importance of pore size (57). Additionally, encapsulins have been used as
419 nanofactories for e.g. the production of silver nanoparticles and antimicrobial peptides.
420 Modifications to the *T. maritima* encapsulins yielded monodisperse silver nanoparticles that
421 possessed better antimicrobial activity than chemically manufactured ones (113). Another
422 functional nanoparticle resulted from the fusion of the antimicrobial peptide HBCM2 to the
423 N-terminus of the *T. maritima* shell protein. The recombinant purified particles showed
424 antibacterial activity against *E. coli* as a result of their surface-exposed antimicrobial
425 peptides (114). In essence however, all these examples are so far mostly very basic attempts
426 to harness the biocatalytic capacity of encapsulins (115). Next generation T=3 and T=4

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designer encapsulins should allow larger multi-protein cargos and the potential to perform highly complex metabolic reactions.

Imaging probes. Encapsulins' ability to sequester or to display light and electron microscopic imageable substances make them ideal imaging probes. A quality that is further enhanced by their robust stability, small size and ease of tissue penetration (109). In its simplest form, CLP-carrying GFP is used as cargo and visualized using fluorescent microscopy. However, photo instability and the low number of GFP molecules per capsid limit this method. Recently, surface-bound spiropyran flourophores have been used to boost fluorescence (116). As each *B. linens* shell monomer possesses four surface-accessible lysines, a total of 240 flourophores can be coupled to a single encapsulin. Experiments showed that the spiropyran was stable through at least five cycles of photo-isomerization creating a photo-switchable probe (117). To further improve this approach, dual functionalization of the shell surface has been introduced using the SpyCatcher system that allows combining fluorescent proteins and targeting peptides thereby creating targetable imaging probes (118). Importantly, these highly fluorescent probes can be used for single molecule imaging *in vivo* and could improve resolution in STORM and PALM microscopy. Finally, iron sequestering nanocompartments have been used as a probe for MRI scans (98). Normally, metalloproteins, ferritins or tyrosine are used as reporters during MRI because of their paramagnetic properties (119). However, iron-sequestering encapsulins produce paramagnetic particles large enough to be visualised during MRI (98) and can also be used for magnetic hyperthermia therapy (120). Excitingly, the high iron content of these

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encapsulins also allows their use as multiplex imaging probes in electron microscopy addressing a long-standing imaging need (99).

Outlook. Since their serendipitous discovery in 2008, encapsulin research has made remarkable progress. Cryo-electron microscopy has revealed the virus-like HK97-fold of their shells, while increasingly sophisticated bioinformatics has discovered that encapsulins of this fold type are found in almost all bacterial and archaeal phyla. Nonetheless there are many unanswered questions. Are HK97-fold encapsulins the only type of encapsulins or are their other structural archetypes awaiting to be discovered? What determines the size of encapsulins and how large can they get? Given the narrow range of sizes, do evolutionary constraints, like in viruses, favour certain sizes over others? And speaking of viruses what is the precise evolutionary relationship between these two structures? Are encapsulins, prokaryotic proteins turned viruses turned encapsulins or is their relationship more tangled? Another poorly understood aspect is the dynamics of encapsulins. How precisely do they assemble - in one step or from the inside out and once assembled can they spontaneously disassemble or do they need auxiliary molecules? Other unresolved questions relate to their functions. While bioinformatics has greatly helped identify potential functions through cargo proteins, experiments are needed to confirm them. This is particularly relevant for cargos with disordered targeting signals that are not encoded in core operons. Another important question is whether these extremely durable structures perform some of their functions extracellularly e.g. by manipulating the microbe's environment, producing signals, delivering toxins or simply providing metabolites for its kin? While the answers to these questions will undoubtedly inform our fundamental understanding, bioengineering will

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likely focus on more tangible aspects such as the recombinant large scale production of encapsulins, the packaging of novel cargos, the control of the pore size, shell stability and cargo capacity. Other relevant aspects are the functionalization of the capsid shell through ligands, probes, targeting molecules etc. Although currently mostly conceptual, these studies will become more and more applied as we begin to better understand these small but highly versatile structures.

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933 **Figure legends:**

934

935 **FIG 1** Structures of selected bacterial proteinaceous compartments inside and outside of the
936 context of a bacterial cell. All structures within each context are drawn to scale to provide a
937 perspective of their relative size in a cell as well as to each other. On the right, atomic level
938 models of (A) the polyhedral shell of the CO_2-fixing carboxysome from *Synechocystis*
939 PCC6803 (T=75, 120 nm), the atomic structures of the iron-sequestering encapsulin shells of
940 (B) *Q. thermotolerans* (T=4, 48 nm, PDB 6NJ8), (C) *M. xanthus* (T=3, 32 nm, PDB 4PT2)
941 and (D) *T. maritima* (T=1, 24 nm, PDB 3DKT); the crystal structure of (E) ferritin from
942 *Rhodobacter sphaeroides* (12 nm, PDB 3GVY) and the structure of (F) the proteasome from
943 *M. tuberculosis* (15x11.5 nm, PDB 3MI0). Note, that for the long buoyancy-providing
944 spindle-shaped gas vesicle on the left currently no atomic model exists and it was therefore
945 omitted from the atomic structures. The carboxysome structure is reproduced from reference
946 70 with permission of the publisher.

947

948

949 **FIG 2** Schematic overview of various strategies for using encapsulin systems in bio-
950 nanotechnology. The four sections of the prototype encapsulin show how the interior,
951 surface and structure of the encapsulin shell can be modified to functionalize the particle. *In*
952 *vivo* packaging takes advantage of the fact that any heterologous CLP-tag-carrying protein
953 will be packaged as cargo (various colored shapes) that then can i.e. precipitate metals like
954 iron (red small circle). Surface labeling relies on the fact that each shell monomer can be
955 either chemically coupled *via* a linker to small molecules or proteins (green spheres, purple

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squares and yellow hexamers) or modified through the genetic-based incorporation of a peptide sequence into the shell protein itself (brown rods and blue triangles). Shell design aims at modifying properties of the encapsulin shell by changing the size, charge or gating of the shell pores as well as other physical parameters of the structure. Finally, *in vitro* packaging takes advantage of the ability of encapsulins to repeatedly disassemble and reassemble. During the reassembly, any externally present inorganic structure such as a metal particle (large red sphere), protein (pink pentamer) or small molecule (variable shapes) will be packaged as long as they allow the formation of the closed shell to occur. Recently achieved fine tuning of the disassembly and reassembly process may allow packaging under physiological conditions. Importantly, these cargos do not necessarily need to have a CLP.

Table legends:

Table 1 Structural characteristics for a representative selection of bacterial nano- and microcompartments. For the nanocompartments examples have been included containing shell proteins with (encapsulins) or without the HK97-fold (non-encapsulins). The assignment of encapsulin families is based on recently published data (28). When known, the corresponding T-values are listed. Of note, the only difference between nano- and microcompartments is their size – structures with a diameter smaller than 100 nm are classified as nanocompartments those larger than 100 nm as microcompartments.

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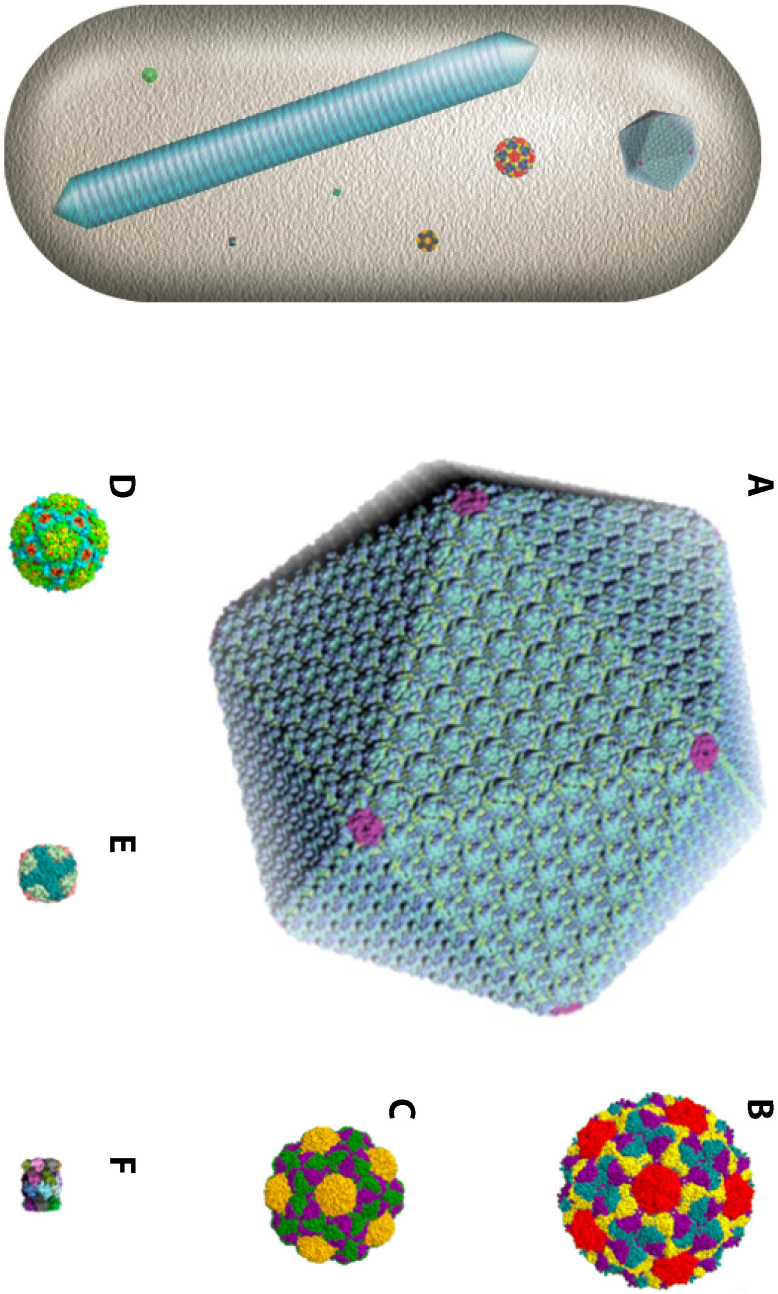

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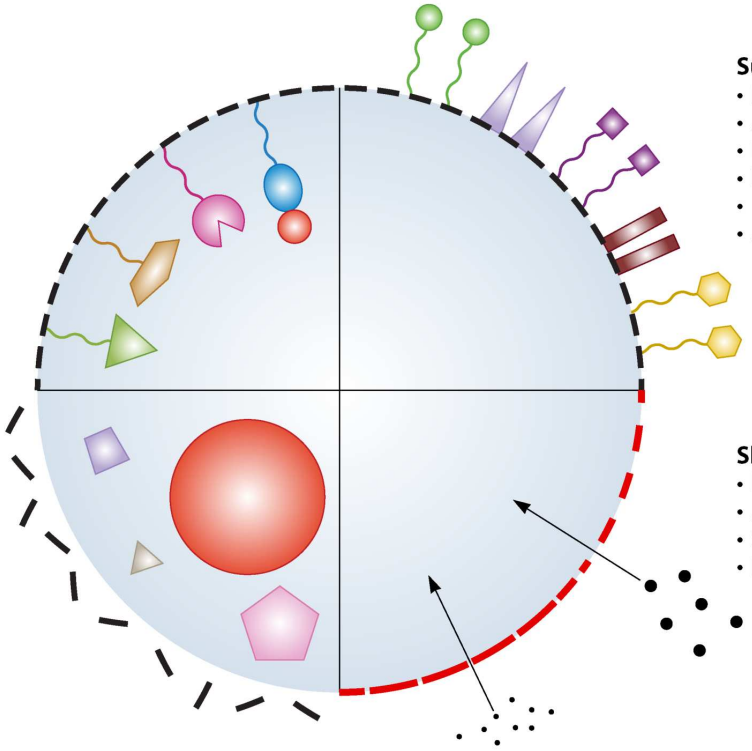
***In vivo* packaging:**
- Enzymes
- Metal-binding proteins
- Fluorescent proteins
- Antigens

Surface labelling:
- Purification tags
- Antigens
- Defensins
- Fluorophores
- Nanobodies
- Affimers



***In vitro* packaging:**
- Drugs and therapeutics
- Nanoparticles
- Proteins
- Quantum dots

Shell design:
- Pore size, charge and gating
- Access of metabolites
- Shell stability
- Increase of size

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	T-value	Monomers per shell	Diameter (nm)	Cargo	Encapsulin Family	Reference
Nanocompartments (size < 100 nm)						
Encapsulins						
Q. thermotolerans encapsulin	4	240	42	Ferritin-like protein	1	22
M. xanthus encapsulin	3	180	32	Ferritin-like protein	1	17
P. furiosus encapsulin	3	180	32	Ferritin-like protein	1	24
T. maritima encapsulin	1	60	24	Ferritin-like protein	1	15
M. smegmatis encapsulin	1	60	24	Dye- decolourising peroxidase	1	20
S. elongatus encapsulin	1	60	24.5	Cysteine desulfurase	2a	23
Non-encapsulins						
HK97 phage capsid	7	420	66	Double stranded DNA	-	30
Bacillus subtilis lumazine synthase	1	60	16	Riboflavin synthase	-	31
Microcompartments (size > 100 nm)						
Gas vesicles	-	-	45 – 120 Length - 100 – 1400	Gases	-	7, 32
α - Carboxysome	75	4500	120	Ribulose-1,5-bisphosphate carboxylase/oxygenase and carbonic anhydrase	-	70
β – Carboxysome	-	-	200-400	Ribulose-1,5-bisphosphate carboxylase/oxygenase and carbonic anhydrase	-	33
Pdu Compartment	-	-	80 – 120	Vitamin B 12-dependent propanediol dehydratase	-	34

Table 1

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