Encapsulins, a new versatile class of nanocompartments and its applications in biotechnology

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Abstract
Compartmentalization is found in almost all life. Although bacteria and archaea do not have organelles they do have proteinaceous compartments, one of which is the encapsulin nanocompartment. Encapsulins were recently discovered by Sutter *et al.* in 2008 and are found widespread in bacteria and archaea. More than 900 putative encapsulin genes were found among fifteen bacterial and two archaeal phyla. Even though much is still unknown about them, such as the self-assembly pathway, physiological role and origin, encapsulins have been shown to be promising for a variety of purposes. In this review the properties of encapsulins will be described such as cargo loading by a selective short peptide sequence at the C-terminal of the cargo, easy disassembly and reassembly *in vitro* and ease of shell modification. Examples of the application of encapsulins as imaging probes for specific (mammalian) cells, targeted therapeutic drug delivery and nanoreactors will be given to show the versatility and practicality of encapsulin nanocompartments.

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Introduction

Compartmentalization is crucial for life to exist. Compartments guarantees that chemicals, metabolites and crucial enzymes stay localized and do not diffuse away. This was most likely crucial for the origin of life. Eukaryotes developed organelles such as the mitochondrion, Golgi body and the peroxisome. All these structures have a lipid bilayer separating them from the rest of the cell. Prokaryotes do not have organelles. However, they do employ compartmentalization in bacterial microcompartment (BMC) [1]. These are organelle like structures have a protein shell as opposed to the lipid bilayer of organelles. They can enhance the turnover rate of a certain reaction by increasing the substrate concentration and protect the cell from toxic by-products or intermediates formed [2]. One well known BMC is the carboxysome, first observed well over 60 years ago in a cyanobacterium [3]. Later it was discovered that the carboxysome plays a crucial role in the Calvin cycle, namely the carbon fixation [4]. Carboxysomes hold ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which uses CO_2 and O_2 as substrate [5]. Because of the similar affinity of RuBisCO to O_2 and CO_2, carboxysomes are used to concentrate the CO_2 and so increase the desired turnover rate of carbon fixation [6].

The bacterial nanocompartment discussed in this review is bacterial encapsulin. First described in 2008 by Sutter *et al.* as a virus capsid-like nanocompartment from *Thermotoga maritima*, involved in the protection from oxidative stress [7,8]. Genetic analysis has shown that encapsulins are widely distributed in nature with more than 900 putative gene spread out over 15 bacterial and 2 archaeal phyla [9]. Encapsulins are made up out of one type of shell protein opposed to several as is the case in many BMCs [10]. This allows for easy modification and heterologous expression of encapsulin shell proteins. Cargo proteins of encapsulins are encapsulated using a selective C-terminal sequence attached to the cargo proteins [11,12]. Their native cargo is often involved in the oxidative stress response of the cell such as ferritin-like proteins (Flp) and DyP-type peroxidases [7,8,13,14]. The physiological role of the encapsulin nanocompartment is not yet known however, their cargo proteins indicate it is involved in protection from oxidative stress [7]. Encapsulin has an exterior loop and several exposed residues which can be modified for selective targeting of cells or binding of therapeutic drugs [15,16,17,18]. This indicates encapsulins adaptability which can be exploited for various purposes. Until now ferritins and virus-like particles have been used for vaccine development [19], therapeutic drug delivery [20,21], nano reactors [22] and imaging probes [23]. In this review encapsulins will be discussed as a potential candidate for various biotechnology purposes. First their discovery and notable characteristics will be discussed, followed by a few examples of encapsulins applied in various biotechnology fields such as specific cell targeting, nanoreactors and imaging probes.

Encapsulins

Discovery and characterisation

The first encapsulin nanocompartment was observed in 1994 but was mistaken for a linocin-like protein with bacteriocin activity towards several gram-positive bacteria which form aggregates of >2 Mda [24].This was corrected in 2008 by Sutter *et al.*. It was determined that the supposed linocin-like protein was actually an encapsulin nanocompartment. Furthermore, the encapsulin nanocompartment of *T. maritima* was characterised which was first described as a multimeric protease that possess antimicrobial activity homologous to the linocin-like protein of *Brevibacterium linens* [25].

The crystal structure of *T. maritima* encapsulin protein was determined (PDB entry 3DKT) by Sutter *et al.* [8] (figure 1c). On basis of molecular weight it was expected that the encapsulin monomer forms a 60 subunit shell so the structure of the nanocompartment was solved (figure 1a,b). It formed a icosahedral shell with a triangulation number of 1 (T=1). The outer diameter is 240-230 Å with a shell thickness of 20-25 Å (figure 1a,b). The 31 kDa monomer shared structural homology with viral capsid

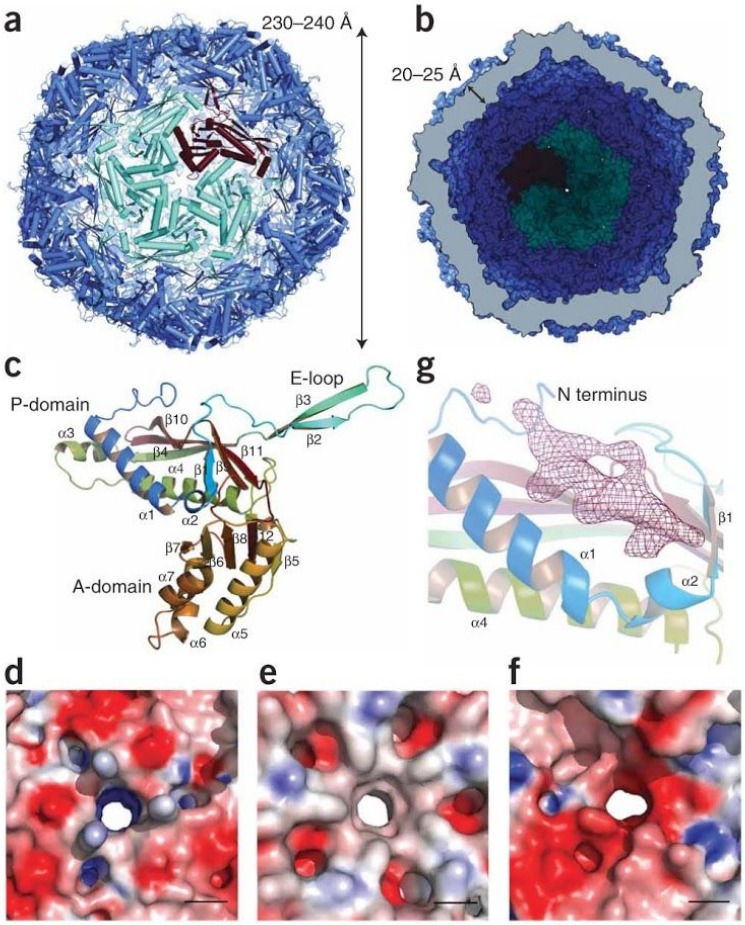

Figure 1. Structure of the T. maritima encapsulin. (a) View from the outside on the five-fold symmetry axis. One pentamer is highlighted in cyan, with one monomer in red. (b) View to the inside of the shell, which is cut open in the middle and shown in a surface representation. (c) T. maritima encapsulin monomer showing the A- and P-domains and the protruding E-loop. The monomer is colored in a rainbow scheme from the N terminus (blue) to the C terminus (red), the domains are named according to the homologous gp5 major capsid protein of the HK97 virus (PDB entry 1OHG) [26,27]. (d-f) Electrostatic surface representation of the holes in the shell of T. maritima encapsulin, colored with a blue to red gradient from +10 kTe^{-1} to -10 kTe^{-1}. The bar length corresponds to 5 A°. (g) Additional density shown in red is from a ten-fold-averaged F_0 - F_c omit map contoured at 6.5 σ. Adapted from Ref. [8]

proteins however, it does not share high sequence homology. The viral capsid is a capsid of the HK97 virus (PDB entry 1OHG) [26]. This lambdoid bacteriophage is known to infect *Escherichia coli* [28]. Gp5 forms a icosahedral capsid (T=7) from 415 monomers of 41 kDa [29]. The mature capsid is ~600 Å in diameter and has a thin shell. Furthermore, the encapsulin of *T. maritima* has structural similarity with a previously determined structure of a virus-like particle of *Pyrococcus furiosus* (PfV) (PDB entry 2E0Z)[30]. *P. furiosus* is a hyperthermophilic archaea with an optimal growth temperature of more than 90°C [31]. The virus-like particle in *P. furiosus* is assembled from 180-subunits of 38.8 kDa to a total molecular mass of ~7 MDa [30]. The nanocompartment it forms is icosahedral (T=3) with a maximum diameter of 363 Å along the five-fold symmetry axis.

Due to the similarities with virus capsids, especially with gp5 capsid protein of HK97, it has been suggested that encapsulins could have viral origins even though there is no sequence homology [8,30,32,33]. As stated by the title of Abrescia *et al.* "Structure unifies the viral universe", sequence homology is not crucial for related capsids when talking about virus capsids [32]. The physiological roles are very different. Viral capsids contain the viral DNA and serves as a protective shell while encapsulins physiological function is yet unknown, but thought to be involved in the oxidative stress response [7,14,34]. Encapsulins are widespread in the bacterial and archaeal kingdom which means if encapsulins have a viral ancestor, it most likely have happened early in evolution allowing it to be in such diverse organisms [9]. The lack of

viral genes surrounding the encapsulin gene would suggest the contrary. However, it is possible the bacteria and archaea discarded those genes throughout evolution as they had no use for them [8].

Giessen and Silver proposed in 2017 that there are four distinct families of encapsulins based on sequence analysis of all putative encapsulin genes [9]. Furthermore, this classification orders the encapsulin shell proteins on the different size of assembled nanocompartment, cargo proteins and their putative function. The families are: Flp-fusion encapsulins, T=1 encapsulins, T=3 encapsulins and T3-like encapsulins. This analysis was based on putative encapsulin genes of which the majority have not been characterized, meaning this is still susceptible to change. The T=3 and T3-like clusters contain larger encapsulin nanocompartments, usually around 320 Å in diameter made up out of 180 subunits such as the encapsulin of *Myxococcus xanthus* [7]. The encapsulin of *M. xanthus* also contains three more proteins which are three different cargo proteins (EncB, EncC and EncD). EncB and EncC show a ferritin-like domain and a conserved sequence, ExxH, which can bind iron. The encapsulin can store iron with a much greater capacity than normal ferritins, around 10-fold more. Moreover, it was found that some encapsulin systems have multiple cargo proteins, one core cargo protein and up to three secondary cargo proteins based on the C-terminal targeting sequence [9]. The core cargo protein being the gene within the operon of the encapsulin and secondary cargo containing the C-terminal sequence while not present in the operon.

During the sequence analysis multiple novel encapsulin systems were found with new cargo proteins, three of which were expressed and characterised [9]. Firstly, haemerythrin was discovered as new core cargo protein of the encapsulin of *Sporichthya polymorpha* DSM 43042. This encapsulin system protects the cell from both oxidative and nitrosative stress. This was tested by exposing *E.* coli, in which the encapsulin was expressed, to hydrogen peroxide (H_2O_2) and nitric oxide (NO) and observing if it leads to a decrease in growth when expressing the encapsulin with haemerythrin cargo, encapsulin alone or haemerythrin alone. When exposed to H_2O_2 only when expressing the encapsulin with haemerythrin resulted in a strong protective effect. Encapsulin with haem had the biggest effect when exposed to NO however, unlike when exposed to H_2O_2, only haemerythrin also saw an increase in survival compared to the control of empty encapsulins. This gives more evidence for the putative physiological of encapsulin to protect the cell from oxidative stress.

The second new cargo type characterised is a new four-helix bundle protein (IMEF) often found in Firmicute bacteria. This four-helix bundle protein was found to mineralise iron and sequester it inside the encapsulin nanocompartment. Interestingly a second protein, Ferredoxin (Fer), was found directly downstream in 61% of all the IMEF encapsulin systems. Fer also contained the conserved targeting sequence however, it was located on the N-terminus of the protein. This is the first cargo protein to have the targeting sequence on the N-terminus. The IMEF encapsulin system stores iron in the encapsulin nanocompartment, although Fer is not essential for iron storage, it does help in efficiency.

Lastly, the new characterised encapsulin were present in four anammox bacteria, *Jettenia caeni Kuenenia stuttgartiensis*, *Brocadia fulgida* and *Brocadia sinica*. Anammox bacteria are responsible for converting organic nitrogen (NH_4^+ and NO_2^-) to inorganic nitrogen (N_2) [35]. Two associated proteins were found in this encapsulin system, a copper oxidase-hydroxylamine oxidoreductase (HAO) fused with a nitrite reductase (NIR) referred to as NIR-HAO. This fusion protein did not contain a targeting peptide for incorporation. However, as it was closely tied in the encapsulin operon it was thought to be a cargo protein. The second associated protein was a N-terminal dihaem cytochrome domain which was predicted to line the interior of the encapsulin nanocompartment. Both proteins copurified with the encapsulin nanocompartment. Less copurified encapsulin and NIR were found when the dihaem cytochrome domain was not coexpressed indicating this domain helps with cargo loading.

Although the physiological function of the encapsulin nanocompartment is unknown, encapsulins are widespread among bacteria and archaea. They have diverse cargo proteins ranging from DyP-type peroxidases to NIR-HAO showing the divergence in the protein class. Moreover, the different families allow for more choice when encapsulins are desired for a certain purpose.

Encapsulin characteristics

Encapsulins are simple nanocompartments in bacterial and archaeal microorganisms. However, they have interesting characteristics which can be employed for other purposes.

The simplicity of encapsulins is one of their strongest characteristics. Made up out of just one monomer, encapsulins are easily heterologously expressed and modified. Assembly of the nanocompartment does not require scaffolding proteins, they self-assemble in solution. Moreover, encapsulins also do not require cargo to properly fold [12,36], empty encapsulins can be expressed and purified from host organisms [12,36–38]. Next, empty nanocompartments can be loaded with desired cargo, be it a protein or a small peptide. The single protein shell of encapsulins allows for easy modification of both the outer as well as the inner surface of the protein shell.

Cargo loading

The gene of the core cargo of encapsulins is very close to the encapsulin shell protein gene, often in the same operon [8,9]. This indicates close translational coupling as they are transcribed together. The encapsulin protein of *P. furiosus* is even fused with its respective cargo showing extremely tight coupling of encapsulins [30,39]. In most other encapsulins cargo is loaded by a C-terminal sequence often referred to as targeting peptide (TP) [8]. This TP is around 30 residues long and is highly conserved among cargo proteins [8,9]. In figure 2 the additional electron density seen in the crystal structure of the encapsulin of *T. maritima* is filled in with part of the extended C-terminal sequence, the TP, of the Flp cargo protein [8]. This is the assumed binding of the cargo inside the encapsulin shell at a pentamer of the shell proteins. When the TP is removed from the cargo proteins it results in empty encapsulins that do not associate with any proteins. Once it is assembled the encapsulin cannot be loaded with cargo protein. The self-assembly pathway is unknown however, it is known that cargo binding is not essential as encapsulins still form lacking cargo on the interior [12,36]. Moreover, the structural integrity of the nanocompartment is not dependent on cargo [36]. In fact, a slight decrease in indentation force was observed when the encapsulin of *B. linens* was loaded with its native DyP cargo protein when compared to empty encapsulin, possibly due to the binding of the C-terminal sequence. This binding may disrupt local symmetry thus decreasing the stability of the nanocompartment resulting in a decreased breaking force. Cassidy-Amstutz *et al.* identified the minimal peptide length of the peptide tag for *in vivo* and *in vitro* encapsulation of a desired protein [12]. It was found that for efficient incorporation *in vivo*, the TP needed to be at least 15 residues long. Scrambling the native TP randomly or having no TP showed minimal incorporation of cargo. *In vitro,* selective loading of cargo was achieved using only a 5 residues TP. Moreover, a random TP and no TP saw no loading while *in vivo* they did show minimal loading. The success of the 5 residues TP could be due to the higher concentration of cargo when loading *in vitro*, as it can be reasoned that the 5 residues TP has lower affinity for the binding pocket. The higher concentration would push the equilibrium between bound and unbound TP to it binding thus increase the incorporation of the cargo in the nanocompartment. Furthermore, *in vivo* the TP is in competition with other peptides to bind while this is not the case *in vitro*.

The extent of cargo protein loaded into the encapsulins appears to be constricted by the size and shape of the cargo protein. The native cargo of the encapsulin of *B. linens* is DyP-E, this forms a trimer of dimers inside the encapsulin nanocompartment [40]. Although the volume of hexameric DyP-E is far less than that of the encapsulin interior, the shape of the enzyme complex forbids more cargo from being encapsulated. When packaging a smaller protein such as teal fluorescent protein (TFP), twelve monomers were loaded [41]. In an effort to improve cargo loading, mutation were introduced which promote dimerization. It was shown for cowpea chlorotic mottle virus (CCMV) the loading of cargo was improved by introducing five mutations (D144E, A145P, R149I, K162S, and K164S) which promote dimerization of TFP [42]. This however, did not increase cargo loading of the encapsulin, still twelve TFPs were loaded.

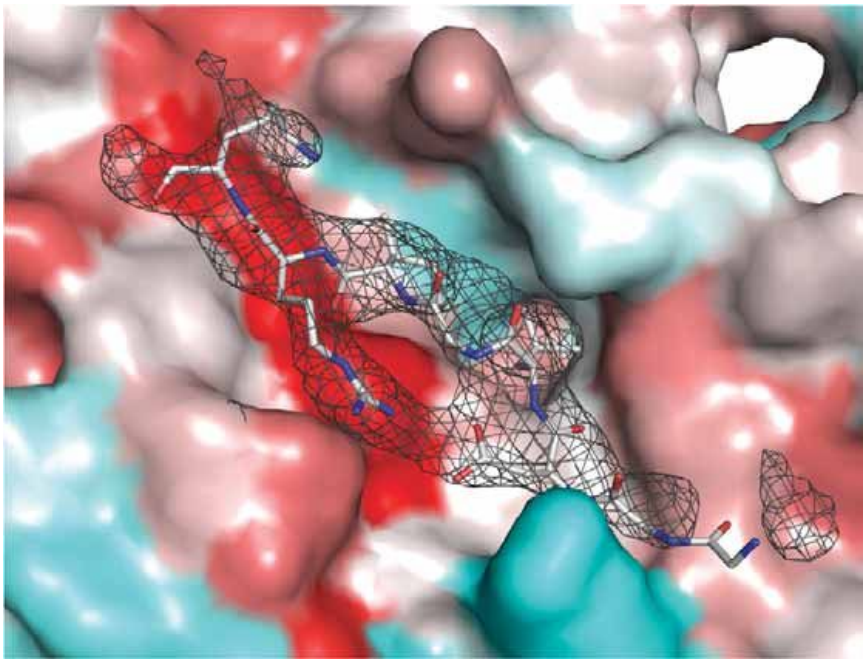

Figure 2. Part of the C-terminal extension of the Flp cargo protein of T. maritima overlaid on the additional electron density seen in figure 1g. Ten-fold–averaged omit electron density map shown at a σ cutoff of 5. The peptide model is shown in stick representation with labeled N and C termini. The T. maritima encapsulin surface is colored according to conservation from cyan (low) over white (medium) to red (high). Adapted from ref. [8]

This corresponds well with the number of pentamers which form the encapsulin shell. This could suggest that the cargo loading is highly coordinated with each cargo protein binding a pentamer of shell proteins.

To conclude, the highly conserved C-terminal sequence incorporates the cargo of the encapsulin nanocompartment. The extent of incorporation is determined by the size and shape of the cargo. Non-native proteins can be easily incorporated by adding a TP to the C-terminal of the desired cargo allowing for great versatility.

Assembly and disassembly

Encapsulins are very stable in a wide range of pH and at elevated temperatures however, they can be disassembled *in vitro*. This can be be helpful when you want to load the encapsulin which cannot be expressed or made by the cell, such as a therapeutic drug.

Disassembly is done by incubating at low pH (<3), high pH (>12) or with 7M guanidine hydrochloride (GuHCl) [11,12]. When disassembled using extreme pH values the encapsulins easily reassemble when brought back to neutral pH conditions. The success of reassembly is dependent on the disassembly conditions. When the encapsulin of *T. maritima* was disassembled with alkaline conditions or using GuHCl, less of the encapsulins correctly reassembled back into the nanocompartment in comparison to disassembly under acidic conditions. All encapsulins correctly reassembled under acidic conditions [12]. Using circular dichroism (CD), a negative peak at 215 nm was observed after disassembly under acidic conditions (pH 1), indicating the presence of β-sheets in the sample. Native encapsulin, under neutral conditions, showed a strong signal for α-helixes. This behavior was not observed when encapsulin was disassembled under alkaline conditions or using 7M GuHCl. These results suggests that, when encapsulin is disassembled under acidic conditions (pH 1) the proteins takes up an alternative fold/formation containing β-sheets, stabilizing the structure, which could be responsible for the very successful reassembly under acidic conditions. *In vitro* loading of cargo had the best results using 7M GuHCl as disassemble method. Using acidic or alkaline pH results in minimal loading of the reassembled

encapsulins opposed to robust loading employing 7M GuHCl. Disassembly using extreme pH values could induced a different pathway of self-assembly once it is brought back to neutral pH, not exposing the TP binding place properly. The accessibility of the TP binding place is crucial for incorporation of the cargo into the interior of the encapsulin. The loading of cargo *in vivo* or *in vitro* is still unknown. However, it is known that cargo loading is performed during assembly of the nanocompartment. The native self-assembly pathway is still unknown, it has been suggested that it goes by addition of dimers of shell protein for the encapsulin of *B. linens* [36] as a relatively large 58-mer population was observed. The, in smaller amounts present, of only even numbered population further suggests it too. Contradictory 59-mer encapsulins were observed by Rurup *et al.* [41], suggesting monomer addition and deduction is also possible. This shows the lack of insight in the self-assembly pathway of encapsulins which needs to be further research before the applications can be truly realised.

Encapsulins are easily disassembled and reassembled *in vitro* allowing for loading of foreign cargo using the TP but more research needs to be done to gain more insight in the self-assembly pathways and TP binding.

Cargo activity

Not only do encapsulins protect the interior enzymes from proteolytic activity and over a wide range of temperature and pH values [11], the encapsulated enzymes retain activity. The pores in the nanocompartment allow for metal ions or small molecules to enter. This does limit the size of substrates that can enter the encapsulin nanocompartment[8]. A large part of the cargo enzymes of encapsulins are Flps or DyPs [9] whose substrates are often small enough to pass through the pores present in encapsulins. The physiological role of encapsulins is thought of to be a response to oxidative stress, by storing iron it helps to protect the cell from oxidative stress [7]. For these purposes the pore size is not a constraint as only a metal ion has to pass through. However, for encapsulated DyP this could be a problem. The physiological function of DyP-type peroxidases is still unknown. However, activity towards lignin and varies dyes is shown [43,44]. These substrates do not have easy passage through the 3-5 Å pores of encapsulin, especially not lignin. Nevertheless, encapsulated DyPB from *Rhodococcus jostii* RHA1 not only retained activity towards polymeric lignin, it increased eightfold [13]. Activity using 2,2′ -azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate was also preserved but slightly decreased to 70-75% of unencapsulated DyP [13,14]. ABTS is too big to pass through the pore of the encapsulin which is why the retained activity is puzzling. A proposed explanation says the pore a dynamic and flexible structure which can increase and decrease in size. The same has been observed for encapsulin of *Mycobacterium tuberculosis* [14]. Here it was attributed to positively charged residues around the pore which allows the negative ABTS to pass through. A possibility is that the encapsulin nanocompartment is a dynamic structure which can lose subunits as 58-mers and 59-mers have been observed as stated above [36,41]. This would allow larger substrate to enter the the nanocompartment and thus retain activity towards it. The abundance and longevity of these not-fully assembled encapsulins is not known, more research needs to be done to get insight in this. Moreover, it is unknown how encapsulated DyP retains activity towards such a big substrate as lignin not to mention increase activity. It has been proposed that encapsulins increase the localization of the enzyme by increasing the local concentration. A possible method to do so is that the encapsulins disassemble on the surface of lignin releasing their cargo and thus increasing the degradation of the lignin. This has yet to be confirmed which could possibly be done by measuring the amount of intact encapsulins after the reaction has run for a while. On native PAGE more species of lower molecular weight should be found if it disassembles. Although the entire mechanism of substrate import is not yet known, this illustrates how encapsulins can be used for nanoreactors as the cargo retains their function and increase in stability.

In conclusion, cargo proteins encapsulated by the encapsulin nanocompartment retain their activity. However, substrate specificity is higher and activity may be lower than unencapsulated enzyme.

For specific substrates such as lignin enzymatic activity can be higher due to increasing localization but this is most likely not the case for most substrates. Nanoreactors can, for these reasons, be designed around encapsulin nanocompartments although, with a limited substrates range and enzymes inside.

Specific targeting as image probes or drug delivery

Targeted therapeutic drug delivery is highly sought after. It has been examined using different nanocompartments such as ferritin [45]. Their small size increases their permeability and ease of surface modification allows for specific binding or drug binding. Selective binding can as well be used as an imaging probe for fluorescence or electron microscopy. Here a few studies will be discussed, showing the versatility of encapsulins in applications of specific targeting as therapeutic drug delivery or imaging probe. It has been shown that encapsulins can be handily labeled using various fluorophores [15–18,46], allowing for the positional study of encapsulins *in vivo* which will further enhance our understanding of their physiological role and function. This is also paving the way for *in vivo* super resolution of fluorescence spectroscopy [46]. Moreover, the study shows that the lysines on the surface of encapsulins are available for modification allowing for another way to introduce being peptide of fluorophores.

Encapsulins can also be genetically modified to serve as selective targeting tool for either imaging or therapeutic drug delivery. One such study by Moon *et al.* made encapsulins into a multi-purpose system, a selective targeting system which releases a therapeutic drug or serves a targeted imaging probe (figure 3) [16]. The encapsulin of *T. maritima* was expressed with a extra insert of 5 glycines, 6 histidines and 5 glycines again which showed improved thermostability and eliminated nucleic acid binding to the exterior of the nanocompartment. In this study fluorescein-5-maleimide (F5M) was used as fluorescent for confocal fluorescence microscopy (CFM). The F5M was covalently linked to the encapsulin protein via a surface cysteine. The cysteine exposed on the exterior of the nanocompartment was also used to bind a SP94 peptide (SFSIIHTPILPL) which is a known targeting probe for hepatocellular carcinoma cells (HCC cells) [47]. HCC is the most prevalent liver cancer in the world and is one of the leading causes of death in cancer patients [48]. First Sp94 peptide was chemically bound the to surface of the encapsulin nanocompartment using Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to confirm it retains its ability to bind HepG2 cells, an immortalized HCC cell line. Once this was confirmed it was genetically incorporated allowing more control as opposed to the cascade of reactions from chemically binding it. This was done in a loop region, between residues 138 139, with two glycines added on each end of the insert. The structure was confirmed using TEM and DLS showing only slightly larger hydrodynamic diameter of 29.1 nm, which was assigned to the SP94 peptide insert. The genetical insertion of the SP94 peptide showed similar binding to the HepG2 cells as the chemical attachment did. Now that encapsulins could bind target cells the drug delivery capacity was tested of these modified encapsulin proteins. A prodrug against HCC, 6-maleimidocaproyl hydrazone of doxorubicin (Aldox), was chemically bound to the encapsulin containing SP94, via cysteine 123 (SP94EncAldox). It was linked by a acid sensitive hydrazine link which allows the release of the doxorubicin under acidic conditions (pH 4.5-5.5). The cytotoxicity of HepG2 was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which indicates the viability of cells. When HepG2 cells were treated with SP94EncAldox it demonstrated a increase in cytotoxicity similar to that of free doxorubicin, SP94Enc lacking the Aldox did not show an increase in cytotoxicity, showing the encapsulins themselves have no negative effect on the cells. This study illustrates encapsulins can be used for targeted drug delivery by modification of the protein shell which may reduce the side-effects of treatments as the full body concentrations stay low. Furthermore, it illustrates the imaging capabilities of the encapsulin nanocompartment as the encapsulins specificly bound the cells illuminating them on the confocal fluorescence microscopy.

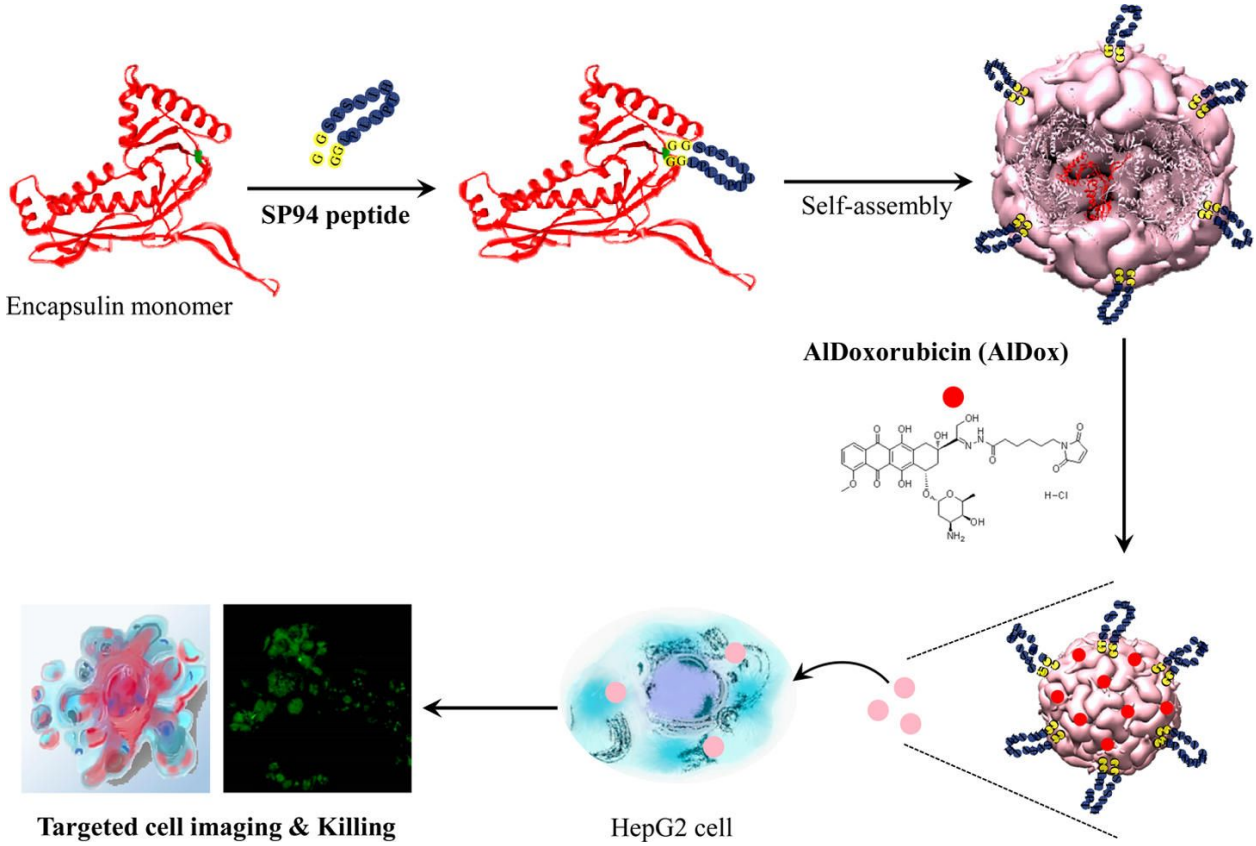

Figure 3. Schematic Representation of Encapsulin Utilized as a Versatile Modular Nanoplatform for the Targeted Delivery of Drugs and Fluorescent Probes[a]

[a]The position to introduce the SP94-peptide is indicated in green. SP94-peptide (blue) with linker (yellow) was genetically or chemically introduced onto the exterior surface of the assembled encapsulin to target surface markers of HCC cells. The corresponding anticancer drug aldoxorubicin (AlDox) was chemically attached to Encap_loophis42C123 (loophis42 indicating the position of the His-tag used for purification) and delivered to the target cells. Adapted from Ref. [16]

Another study showing the targeting abilities of modified encapsulin has also been done by Moon *et al.* [17]. In this study encapsulins were engineered as a targeting optical nanoprobe which could selectively recognize and bind squamous cell carcinoma (SCC) cells. SCC is the second most common skin cancer and accounts for around 20% of all cases [49]. First the ability to bind an antibody of a rabbit was shown to indicate encapsulins have the ability to selectively bind by inserts on the surface of the nanocompartment shell. A sequence (GGGGGGDCAWHLGELVWCTGGGGG) which has high affinity for the Fc-region of immunoglobulin G (IgG) of rabbits which was named Fc-binding peptide (FcBP), was genetically inserted into the surface loop between residues 138 and 139 of encapsulin (FcBP-encapsulin). The FcBP were displayed on the surface of the encapsulin which was confirmed using real-time quartz crystal microbalance (QCM). Subsequently rabbit IgG was added, followed by a washing step to wash away the non-specific bound molecules. Encapsulins with the insertion of FcBP showed a decrease in frequency while the encapsulins without insert remained unchanged. This indicates that the IgG of a rabbit binds the FcBP on the surface of the encapsulin nanocompartment. The encapsulins bound the IgG strongly as extensive washing did not dislocate them. Lastly, the ability of FcBP-encapsulin to bind SCC-7 cells was tested. To image this, F5M was covalently linked to the FcBP-encapsulins (fFcBP-encapsulin) by the cysteine residues on the surface on the shell which are known to be chemically active [16]. By confocal fluorescence microscopy it was confirmed fFcBP-encapsulin selectively bound

SCC-7 cells and was internalized. No selective binding was observed for Hela, HepG2, MDA-MB-231 and KB cells showing it is highly selective, further showcasing the potential of encapsulins to function as a targeting method for therapeutic drug delivery or as molecular imaging probe.

Additionally the encapsulin of *Rhodococcus erythropolis* N771 was successfully PEGylated [37]. PEGylation is the process of attaching poly(ethylene glycol) (PEG) to a compound or protein. This was done by attaching it to the exposed lysine residues via a PEG derivative, methoxy PEG succinimidyl carboxymethyl ester (methoxy-PEG-SCM). PEGylation increases the retention time of therapeutic compounds or proteins in the bloodstream [50]. The PEGylation of the encapsulin nanocompartment did not affect the structural integrity of the shell as well as their disassembly and reassembly *in vitro*. Successful PEGylation increases the use of encapsulin nanocompartments as therapeutic drug delivery or carrier systems. PEGylation was only tested for empty wild-type encapsulins, for encapsulins with cargo or with modification for targeting this has not been demonstrated yet.

Encapsulins show promising developments in the field of specific cell targeting but only for a very niche field. As a targeting peptide or compound for the cell needs to be known which should fit in a insert on the encapsulin protein shell.

Nanoreactor

The fixed architecture and size of the nanocompartment can be utilized for certain nanoreactor processes. As Giessen and Silver demonstrated the fixed shape and size the encapsulin nanocompartment can be used for the production of size dependent silver particles (figure 4) [38]. The encapsulin protein of *T. maritima* was genetically engineered to include a silver precipitation sequence, AG4 (NPSSLFRYLPSD). This was fused to the N-terminus (EncAG4) so it will be displayed on the interior of the nanocompartment. AG4 starts a nucleation site for Ag^+ to precipitate from solution inside of the encapsulin growing until the entire compartment is filled [51]. Ag nanoparticles show antimicrobial activity, most likely due to the release of Ag^+ ions which promote the generation of ROS [52]. Ag nanoparticles of 20 nm or smaller show an increase in antimicrobial activity [53]. This makes the encapsulin of *T. maritima* an attractive candidate as the expected size of the Ag nanoparticles formed in the encapsulin is between 13 and 15 nm in diameter. The regular structure of the encapsulin nanocompartment means the Ag nanoparticles will be uniform in

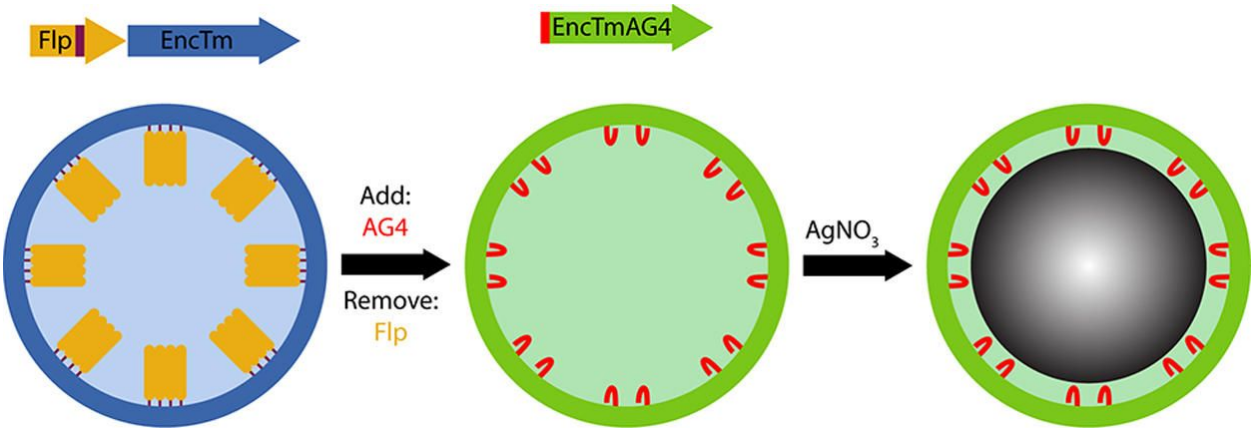

Figure 4. Engineering of the T. maritima protein compartment. Schematic depiction of capsid engineering. The ferritin-like protein (Flp, orange) was removed from the wild-type encapsulin system and the AG4 peptide (red) was fused to the N-terminus of the Enc yielding EncAG4, which would then allow the size-constrained synthesis of silver nanoparticles in its interior when exposed to silver ions. Adapted from Ref. [38]

size and shape. Using $AgNO_3$ as Ag^+ source, the nanoparticles were created with an average size of 13.5 nm with high uniformity. These synthesised Ag nanoparticles had increased antimicrobial activity over $AgNO_3$, citrate-capped Ag nanoparticles and the EncAG4 as negative control. Moreover, the Ag nanoparticles demonstrated the greatest antimicrobial activity when the protein shell of the nanocompartment had been removed. Giessen and Silver demonstrated that encapsulins can be used as nanoreactors by producing monodisperse population of Ag nanoparticles, made possible by the fixed size and shape of the nanocompartment.

Encapsulins have relatively small pores in their protein shell, 3-5 Å big, limiting the size of substrates that can enter the nanocompartment. Williams *et al.* tried to increase the pore size of the encapsulin of *T. maritima* by modifying or deleting the residues around one of the pores [54]. Williams *et al.* took a closer look at a six residues loop region (residues 184-189) that forms the pore on the 5-fold symmetry face. From sequence analysis it was found that only three of these six residues had a high rate of conservation among the encapsulin family, opening the door for substitution and deletion in effort to increase the pore size. Substituting all six residues to alanine (AlaALL) did not disturb the yield of encapsulin formation, nor impact the structural integrity verified by TEM. Several other deletion were tried as well, from two residues to eight residues, counting from the middle outwards. Deletion of two (**Δ**6Ala4) and four residues (**Δ**6Ala2) both flanked by alanines did not decrease the yield of encapsulins and did not show faulty structures. Deletion of all six residues (**Δ**6) did impair encapsulin formation and so did deletion of residue 182 up to 189 with a glycine for residue 190 (**Δ**9Gly). Robust encapsulins were again observed when residue 182 was made into a glycine (**Δ**9Gly2). The largest pore size were estimated computationally for **Δ**9Gly2 and **Δ**6Ala2 with a pore size of 11 and 6 Å, respectively. Experimentally the computationally estimated pore sizes were tested using a lanthanide binding tag (LBT). LBT is a 17 peptide long sequence which shifts its luminescence upon binding of lanthanide ions [55]. The LBT was associated with the encapsulin nanocompartment by fusion with the N and C-terminal (NLBT and CLBT) to incorporate it on the interior and exterior, respectively. $TbCl_3$ was used as lanthanide ion and the luminescence was measured using stopped-flow spectroscopy. Wild-type encapsulin with LBT fused with the C-terminus was used as positive control. CLBT-wild-type encapsulin reached half the maximum luminescence the fastest followed by NLBT-**Δ**9Gly2, NLBT-**Δ**6Ala2 and lastly NLBT-wild-type encapsulin. The same goes for the initial increase of luminescence, demonstrating the pores of encapsulin can be readily modified to increase the size to an estimated 11 Å in **Δ**9Gly2. Moreover, it illustrates that the increased pores also have an increased mass flow which could make nanoreactors faster or accept larger substrates. Larger substrates have not been tested however, the assumption can be made that a larger pore would increase the maximum size substrate that can pass through the pore.

Proteins can be easily incorporated into the encapsulin nanocompartment using a short C-terminal tag, allowing for straightforward way to engineer single enzyme nanoreactors. The encapsulin shell can then provide a higher stability to the encapsulated enzymes allowing to operate at higher temperatures or pH values. The encapsulin of *B. linens* containing DyP-E was able to be immobilized by a covalent bond between a glass surface containing pentafluorophenyl silicate and the lysine on the surface of the encapsulin nanocompartment [40]. This allowed for the attachment of the encapsulins to illustrate the catalysis on a surface. The immobilization did not result in a significant decrease in activity towards ABTS, compared to free encapsulated DyP-E, revalidating the adaptability of encapsulins as nanoreactors. This is enforced by the ease of which foreign enzymes can be incorporated into the encapsulin nanocompartment by making use of a short C-terminal sequence. In this experiment a two enzyme system was used, the H_2O_2 which is needed for DyP catalytic activity was created using glucose oxidase (GOx) [56]. GOx uses glucose and oxygen as substrate to form H_2O_2, used in the reaction of DyP-E with ABTS. Here encapsulins are shown to work in a multienzyme system performing a cascade reaction

however, no advanced cascade reactions have been tested thus far. More research should be done to examine the applicability of encapsulins here.

In short, encapsulins can serve a variety of purposes as nanoreactors from the production of silver nanoparticles to nanoreactors using one or two enzymes. This shows the encapsulin system can also work in cascade reactions as well as single enzyme reactions while increasing the stability of the encapsulated enzymes.

Non-pathogen disease vaccine

As has been shown, encapsulins are a versatile class of proteins which are easy to modify and mold into a certain function. Using a similar method as Moon *et al.* [16], encapsulins were used to activate cytotoxic T-cells as a vaccination [18]. In this study, a model system for melanoma of Ovalbumin and OT-1 was used to study antigen-specific immune response [57]. The OT-1 peptide (SIINFEKL) corresponds to residues 257-264 of the OVA protein which is presented to T-cells by dendritic cells which makes the T cell differentiate to OT-1-specific cytotoxic $CD8^+$ T-cells [58]. This OT-1 peptide is genetically introduced in the encapsulin proteins to determine induction of an immune response and the efficiency of the induction. Three positions were examined where the OT-1 peptide was introduced, both at the end of the N-terminus (OT-1-Enc-N) and C-terminus (OT-1-Enc-C) and between residues 42 and 43 in the loop region (OT-1-Enc-L). None of the inserts had a significant impact on the structure and architecture of the nanocompartment. Next it was demonstrated that OT-1-Enc-C could promote maturation of immature dendritic cells by incubation for 18 hours. The matured in turn promotes proliferation of naive $CD8^+$ T cells which are selectively activity towards the OT-1 peptide. Less proliferation was observed when the OT-1 peptide was inserted in the loop or N-terminal regions thus the following experiments were done only using the C-terminal extension. Next it was shown OT-1-Enc-C promoted the proliferation of cytotoxic T cells in mice which in turn targeted and killed OT-1 peptide bearing cells. Subsequently the ability to suppress tumor growth of B16-OVA melanoma was tested of the OT-1-Enc-C vaccination. Tumor growth suppression was observed when vaccinated beforehand with the OT-1-Enc-C vaccination, the vaccination successfully generated OT-1 peptide specific cytotoxic $CD8^+$ T cells. The position of the targeting peptide such as the OT-1 peptide should be considered when trying to design a targeting or antigen presenting system. As seen above, only efficient maturation was promoted when inserted in the C-terminal, as opposed to when inserted in the N-terminal or loop region.

This study shows the promising direction encapsulin nanocompartments applications can enter for vaccination for non-pathogen-induced diseases such as melanoma.

Encapsulins as orthogonal compartments in mammalian cells

Sigmund *et al.* showed the applicability and versatility of the encapsulin of *M. xanthus* in mammalian cells [15]. The encapsulin could be expressed and non-native cargo could be readily loaded into the nanocompartment. Using split luciferase parts, LgBit and SmBit, which were separately fused to EncB and EncC cargo proteins of the encapsulin, luciferase activity was observed when the encapsulin was formed, demonstrating intact and correctly incorporated encapsulins. Moreover, it was observed that the encapsulin were readily taken in by the mammalian cell into the cytosol. As a result of the native function of the encapsulin of *M. xanthus*, iron was sequestered into the encapsulin which made for an excellent reporter for EM. Employing previously described targeting methods by modification of the shell protein would create a useful localization tool for mammalian cells for EM.

Discussion

Encapsulins are a novel type of nanocompartments which share a similar fold to the capsid protein of HK97. Because encapsulins were only recently discovered not much is known about them such as, their

physiological role, origin and other characteristics. This however, has not stopped some to try to employ it in various ways. Encapsulins are easily modified to suit a desired function, from drug delivery to nanoreactor. The ease of which non-native cargo can be encapsulated by only attaching a small C-terminal sequence gives encapsulins an advantage of other methods currently used. This allows for easy production of nanoreactors and loading of cargo *in vivo* and *in vitro*. The pores of the encapsulins do limit the substrate range of the nanoreactor. By deletion the pore size has been increased but no studies have been done on how this affects the substrate range or enzymatic activity of the encapsulated enzymes. Encapsulins have multiple modification sites both on the interior and the exterior. This allows for example for, the production of tightly size controlled silver nanoparticles by a modification on the interior of the nanocompartment. Moreover, multiple compounds can be attached to the surface at once which allows for encapsulins to become multipurpose nanocompartments. As has been shown by Moon *et al.,* encapsulins can be made into targeting therapeutic drug delivery systems by inserting a binding peptide and therapeutic drug on the surface of the nanocompartent. This opens the door to a new method to fight diseases with less invasive methods. We are in the very early stages of this development for only a few studies have been performed to demonstrate this for encapsulins. More work needs to be done before it can become a contender in this field. for this role. The size and ability to be modified makes encapsulins useful as an imaging probe, both for confocal fluorescence spectroscopy and electron microscopy.

In conclusion encapsulin nanocompartment an adaptable class of nanocompartments which is widespread throughout bacteria and archaea. Much more research needs to be done to characterize new encapsulin shell proteins and use known encapsulins for various applications. The self-assembly pathway is yet unknown as well as the exact interaction between cargo and protein shell. Nevertheless encapsulins show progress in the fields of targeted therapeutic drug delivery, imaging probes and nanoreactors coming out as a promising candidate for further studies.

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