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Biocatalytic application and structural elucidation of robust bacterial protein nanocages†

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Encapsulins, bacterial protein nanocompartments, have emerged as promising platforms for enhancing biocatalyst stability. This study presents the identification and structural characterization of two encapsulins: ArthroEnc from *Arthrobacter* sp. SLBN-53 and DendroEnc from *Dendrosporobacter quercicolus*. Both bacterial encapsulins were successfully overexpressed in *Escherichia coli* and purified. Cryo-electron microscopy revealed that ArthroEnc assembles into a 20 nm $T = 1$ icosahedral capsid composed of 60 subunits, with its structure determined at 2.9 Å resolution, whereas DendroEnc forms a 40 nm $T = 4$ icosahedral complex with 240 subunits, resolved at 3.4 Å resolution. Both structures exhibit the characteristic HK97 phage-like fold. To explore their functional potential, the encapsulins were used to pack two distinct enzymes: CyanoPOX, a heme-containing peroxidase, and PTDH-mFMO, a fusion enzyme combining phosphite dehydrogenase with a flavin-containing monooxygenase. DendroEnc packed with CyanoPOX exhibited enhanced proteolytic stability, effectively shielding its cargo from chymotrypsin degradation. Activity assays with ABTS and guaiacol confirmed that encapsulated CyanoPOX retained enzymatic function within DendroEnc. Finally, mutating the pore of DendroEnc resulted in a fivefold reduction in activity, demonstrating the potential for tuning substrate diffusion. This study advances our understanding of encapsulin diversity and highlights their potential as versatile platforms for enzyme stabilization, biocatalytic and other biotechnological applications.

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Introduction

Compartmentalization is a fundamental characteristic of all cells. It enables the segregation and coordination of physiological functions in a regulated and orderly manner.¹ To meet these diverse requirements, cells have evolved mechanisms that include lipid- and protein-based organelles.² Eukaryotic cells are primarily known for their reliance on lipid-based organelles, whereas prokaryotic cells predominantly employ protein-based compartmentalization strategies.^{3,4} In the past, bacterial microcompartments have been recognized as the primary class of protein-based organelles in bacteria.⁴ These large protein shells, often exhibiting a diameter larger than 100 nm, encapsulate and organize specific metabolic enzymes within a selectively permeable protein shell. This arrangement enhances reaction efficiency, sequesters toxic

intermediates, and enables the development of new metabolic pathways in diverse bacterial species.^{4,5} A little over a decade ago, a second extensive class of bacterial protein nanocompartments known as encapsulins was characterized.⁶ Encapsulins are robust icosahedral protein structures formed *in vivo* through the self-assembly of 60 to 240 protomer subunits that exhibit the HK97 (Hong Kong 97) phage-like fold.⁷ Structural analyses revealed that encapsulin protomers are composed of three highly conserved domains: the axial domain (A-domain), which forms the core of the subunit, the peripheral domain (P-domain), involved in inter-subunit interactions, and the extended loop (E-loop), which contributes to capsid assembly and stability.^{6,8} Encapsulins typically range from 24 to 42 nm in diameter with different triangulation numbers ($T = 1$, $T = 3$, or $T = 4$).⁹ A distinctive characteristic of encapsulins is their capability to encapsulate targeted cargo proteins, often enzymes.¹⁰ This action is facilitated through a selective loading mechanism mediated by short targeting peptides located at the N- or C-terminus of a cargo protein.¹¹ Genes that encode cargo proteins and encapsulin are organized in a co-regulated operon that defines the encapsulin system.^{12,13} The rationale governing the selective encapsulation of specific cargo proteins within bacterial microcompartments remains unclear. Predominant classes of cargo proteins identified within encapsulins include dye-decolorizing peroxidases (DyPs), ferritin-like proteins,

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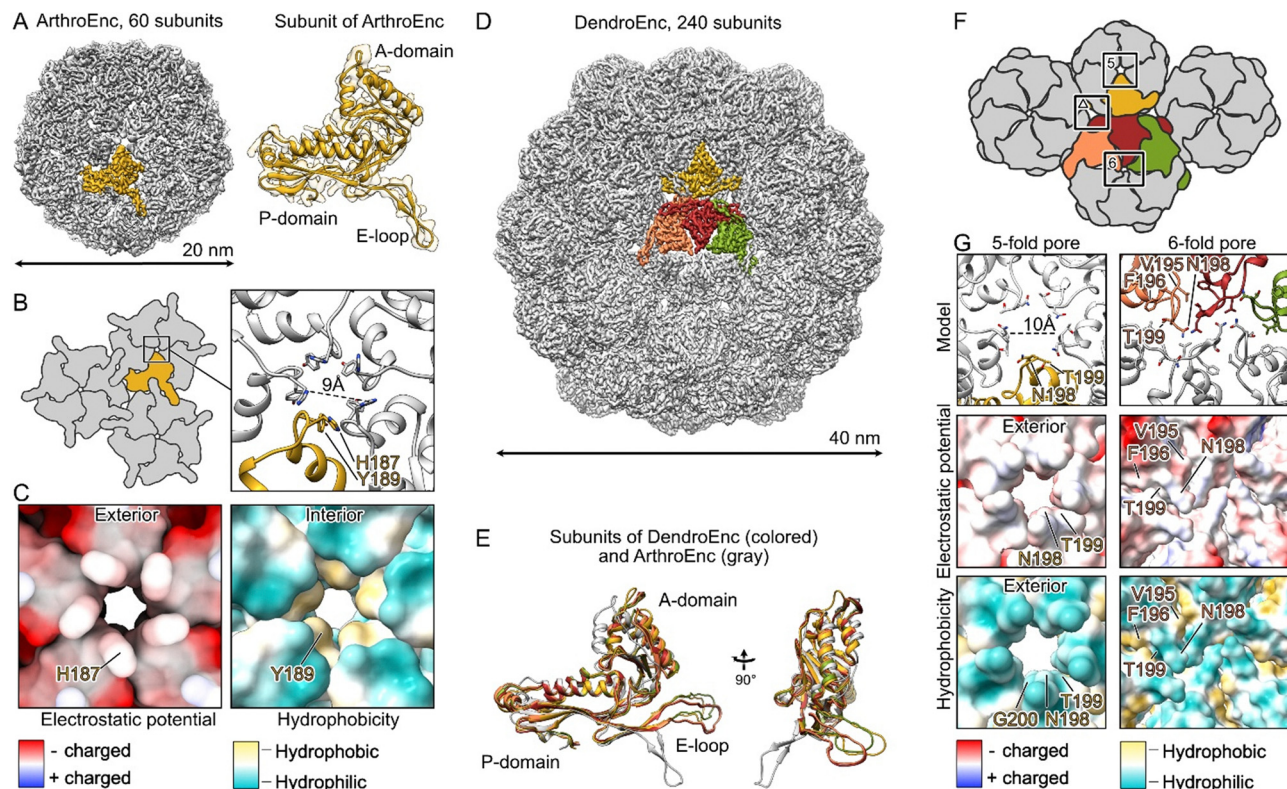


Fig. 1 Cryo-EM structures of ArthroEnc and DendroEnc encapsulins. (A) Cryo-EM reconstruction of ArthroEnc with $T = 1$ icosahedral symmetry with 60 subunits and a structure of its HK97-like fold domain. (B) Schematic organization of ArthroEnc and a close-up of its 5-fold pore. (C) Electrostatic surface and hydrophobicity of ArthroEnc's pore. (D) Cryo-EM reconstruction of DendroEnc revealing $T = 4$ icosahedral symmetry with 240 subunits. An asymmetric unit is shown in colors. (E) Structural alignment of four different subunits of DendroEnc (in orange, red, green and yellow) and a subunit of ArthroEnc (gray). (F) Schematic organization of DendroEnc and its 5-fold, 6-fold and triangular-shaped pores. (G) Structural organization of DendroEnc's 5- and 6-fold pores.

P-domains and E-loops of encapsulin subunits (Fig. 1F). This can be considered as a secondary entry point for substrate. Thus, DendroEnc pores are better designed to facilitate the transport of hydrophilic compounds, rendering it more suitable for potential biotechnological applications.

Proteolytic stability of DendroEnc constructs

Some encapsulins have been demonstrated to protect cargo proteins from proteolytic degradation.^{10,25} To determine whether this applies to DendroEnc, we evaluated its ability to shield cargo proteins from protease activity. Incubation of empty DendroEnc and CyanoPOX-loaded DendroEnc with chymotrypsin, followed by SDS-PAGE analysis, demonstrated limited proteolytic degradation of the encapsulin shell (Fig. S3, ESI[†]). Additionally, the encapsulated CyanoPOX remained intact after 24 hours of chymotrypsin exposure, whereas non-encapsulated CyanoPOX was degraded. These results confirm the protective function of DendroEnc, further validating its ability to effectively encapsulate and safeguard cargo proteins.

Activity of DendroEnc CyanoPOX in the presence of ABTS and guaiacol

To evaluate whether CyanoPOX retained enzymatic activity when encapsulated within DendroEnc, two known substrates

of this bacterial peroxidase,²² ABTS (2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)) and guaiacol, were tested. DendroEnc CyanoPOX exhibited activity toward both substrates. At 10 mM, the activity for ABTS and guaiacol was 0.02 U mg^{-1} and $0.59 \times 10^{-3} \text{ U mg}^{-1}$ respectively. No detectable activity was observed in empty DendroEnc, confirming that the enzymatic activity originated solely from the encapsulated CyanoPOX. These results indicate that the pores of DendroEnc are sufficiently large to allow diffusion of relatively bulky substrates, such as ABTS, enabling efficient enzymatic catalysis within the nanocompartment. Table S4 and eqn (S1) (ESI[†]) outline the calculation procedure used to determine specific activity.

Engineering the 5-fold pore of DendroEnc

With established protocols for the recombinant production of DendroEnc carrying specific cargo, we explored the effects of modifying its pores. Structural insights into the DendroEnc pore architecture guided the design of a mutation aimed at narrowing or closing the pore. Using an encapsulated enzyme with substrates of varying sizes allowed us to assess the impact of this modification on substrate diffusion.

Specifically, we targeted residue N198, a key component of the 5- and 6-fold pores architecture (Fig. 1G). By substituting N198 with tryptophan, a bulky amino acid, we aimed to restrict



substrate entry into the encapsulin. Co-expression of the DendroEnc N198W variant with CyanoPOX successfully yielded CyanoPOX-loaded DendroEnc N198W. To assess the structural consequences of this mutation, we determined its structure to a 3.6 Å resolution using single-particle cryo-EM analysis, providing insights into the impact of pore engineering on encapsulin architecture.

Structural analysis of the DendroEnc N198W CyanoPOX complex revealed that, similar to wild-type DendroEnc, the 6-fold pore remains fully closed with a tight hydrophobic barrier (Fig. 2). Moreover, the introduction of tryptophan at position N198 resulted in a further reduction in the diameter of the 5-fold pore. The five tryptophan residues align precisely at the pore, constricting its diameter to 5 Å and effectively limiting molecular passage in and out of the encapsulin. Importantly, the N198W mutation did not change the overall architecture of encapsulin, showing its ability to tolerate significant mutations while preserving its integrity, further highlighting its potential for engineering tailored molecular gating properties.

The activity of the DendroEnc N198W CyanoPOX mutant was tested using ABTS as a substrate (0.004 U mg^{-1}), revealing a 5-fold reduction in activity compared to the CyanoPOX packed in wild-type DendroEnc. This decrease aligns with structural insights showing that the mutation reduced the diameter of the 5-fold pore.

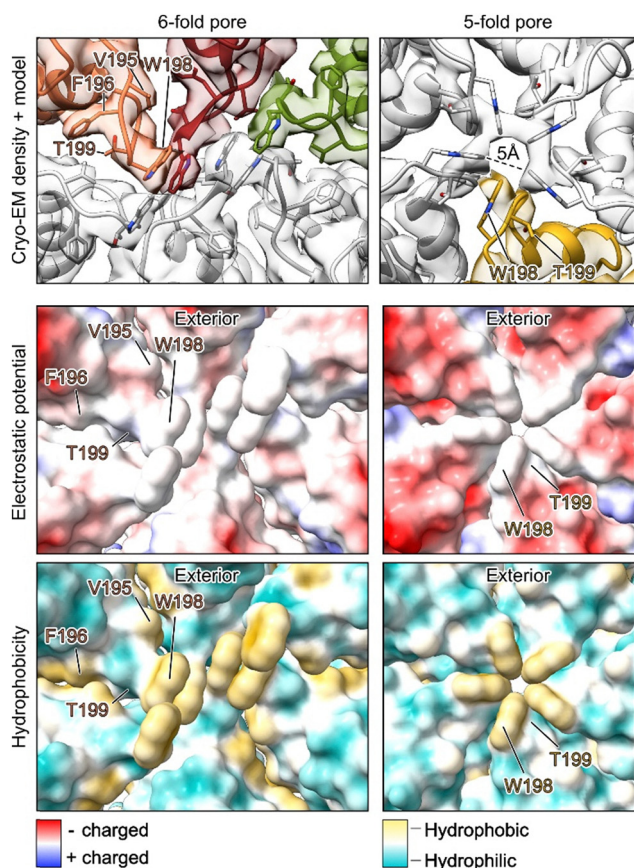


Fig. 2 Cryo-EM structure of the DendroEnc N198W CyanoPOX pores. The N198W mutation introduced a hydrophobic plug in the 6-fold pore and reduced the size of the 5-fold pore.



Fig. 3 2D class averages (above) of empty DendroEnc WT and DendroEnc N198W CyanoPOX and individual encapsulins DendroEnc N198W CyanoPOX with and without cargo (below).

Finally, to investigate the folding of CyanoPOX within DendroEnc, we compared 2D class averages of empty and cargo-loaded DendroEnc. This analysis enabled us to visualize the density corresponding to the encapsulated enzyme (Fig. 3), providing further confirmation of efficient cargo loading. However, the observed density appeared bulky and unstructured, with no indication of a defined symmetrical positioning of the cargo. Since the symmetry of the cargo might differ from that of the encapsulin shell, we performed shell subtraction to analyse the cargo alone. Further 2D classifications and 3D refinements did not reveal a clear structural organization, suggesting that the encapsulated enzymes do not adopt a fixed symmetrical arrangement within the encapsulin. These findings imply that CyanoPOX may remain in a flexible state inside DendroEnc.

Materials and methods

Chemicals and materials

The synthetic genes encoding for DendroEnc and ArthroEnc were ordered cloned in the pBAD vector (ampicillin resistance) from Twist Bioscience. The synthetic gene encoding for cargo proteins (CyanoPOX-cargo and PTDH-mFMO-cargo) were ordered at IDT (Integrated DNA Technologies). To prepare DendroEnc mutant, primers were ordered from Eurofins genomics. Superdex 200 Increase 10/300 GL column was purchased from Cytiva. Chymotrypsin from bovine pancreas and other chemicals were purchased from Sigma-Aldrich.

Cloning, protein co-expression and purification

IDT tools were used to codon optimize the sequences (Table S1, ESI†) that encode cargo enzymes (CyanoPOX-cargo and PTDH-mFMO-cargo) harboring a N-terminal $6 \times \text{His}$ -tag and the C-terminal targeting peptide to enable their expression in *E. coli*.



The targeting peptide used in this work belongs to a DyP-peroxidase¹⁰ from the same operon as the encapsulin gene. These synthetic genes were cloned in pET vector (kanamycin resistance) using the Golden Gate methodology.²⁶ 5 µg of the cargo and encapsulin PCR mixtures were added to 50 µL RbCl₂ competent *E. coli* BL21-AI cells. The cells were left to incubate on ice for 30 min followed by the heat shock for 45 seconds at 42 °C. After the heat shock, the cells were placed on ice for another 3 minutes. 500 µL of SOC media was used to recover the cells. They were then placed in the thermal shaker at 37 °C for 45 min, 700 rpm. 100 µL of the cell mixture was spread onto the LB agar plates containing 100 µg mL⁻¹ ampicillin and 50 µg mL⁻¹ kanamycin. LB agar plates were placed into an incubator at 37 °C overnight. Overnight cultures were grown in 5 mL LB medium that was supplemented with 100 µg mL⁻¹ ampicillin and 50 µg mL⁻¹ kanamycin and were incubated at 37 °C, 135 rpm. Terrific Broth medium (500 mL), supplemented with 100 µg mL⁻¹ ampicillin and 50 µg mL⁻¹ kanamycin was used for the expression. Cultures were incubated at 37 °C, 135 rpm in baffled flasks until the OD₆₀₀ reached ~0.75.

Expression of cargo enzymes and encapsulins was induced by adding IPTG (1 mM final concentration) and L-arabinose (0.02% final concentration). 5-Aminolevulinic acid (1 mM, final concentration) and iron sulfate (1 mM, final concentration) were added to the cultures containing CyanoPOX cargo in order to facilitate the biosynthesis of heme. Cultures were incubated at 24 °C at 135 rpm for 48 hours. They were then harvested by centrifugation (3700 rpm, 40 minutes, 4 °C). Cell pellets were resuspended in 35 mL lysis buffer (50 mM KPi, 150 mM NaCl, pH 7.5) and were sonicated on ice (5 s on 5 s off, 10 minutes, 70% amplitude). Cleared cell free extract was obtained by centrifugation at 12 000 rpm for 45 minutes at 4 °C. For precipitation of encapsulin-cargo constructs, equal volumes of cell free extract were mixed on ice with equal volumes of 12% PEG-8000, 50 mM KPi, pH 7.5, 2 M NaCl (used for ArthroEnc constructs) and 8% PEG-8000, 50 mM KPi pH 7.5, 2 M NaCl (used for DendroEnc constructs). The obtained solutions were incubated for 4 hours at 4 °C followed by centrifugation at 12 000 rpm for 45 minutes at 4 °C. The obtained pellets were resuspended in 50 mM KPi, 150 mM NaCl, pH 7.5. The resuspended mixture was selectively loaded onto Ni Sepharose gravity columns containing 3 mL resin in order to remove the excess unencapsulated cargo-enzyme.

Size exclusion chromatography (SEC) was used with the intention of further polishing encapsulins. A Superdex 200 increase 10/300 GL column with 20 mM KPi, 150 mM NaCl, pH 7.5 was used. Wavelengths were set at 280 nm and 420 nm corresponding to standard protein and cargo absorbance respectively. Fractions corresponding to the peak containing both wavelengths were pulled together and the SDS-PAGE analysis was then performed to assess the purity of encapsulins (Fig. S2 and S3, ESI†).

Cryo-EM, data analysis, model building

3 µL of encapsulins at concentrations 3 to 6 mg mL⁻¹, were applied onto a freshly glow-discharged copper R2/1 300 mesh grid (Quantifoil), blotted for 3 s on both sides with blotting force 0 and plunge-frozen in liquid ethane using the Vitrobot

Mark IV system (Thermo Fisher Scientific) at 13 °C and 100% humidity.

Datasets were collected using a Talos Arctica transmission electron microscope (Thermo Fisher Scientific) equipped with an XFEG at 200 kV using the automated data-collection software EPU version 2.7 (Thermo Fisher Scientific). 2 images per hole with defocus range of -0.5 to -2.5 µm were collected with K3 detector (Gatan) operated in super resolution mode. Image stacks with 50 frames were collected with the total exposure time of 3.5 s and total dose of 45 e⁻ Å⁻².

For ArthroEnc, 719 micrographs were used for data processing. After motion correction and CTF estimation performed in Relion version 3.1,²⁷ 42 006 particles were picked in crYOLO,²⁸ extracted with box size 330 and pixel size 1.04 in Relion version 3.1, and imported in CryoSPARC. 20 200 particles after 2D classification were used for *ab initio* 3D refinement with simultaneous sorting into 3 classes. One class with the largest number of particles, 15 677, was used for non-uniform 3D refinement with icosahedral (I) symmetry. The reconstruction and corresponding particle alignments were used for Bayesian polishing in Relion, followed by final non-uniform 3D refinement in CryoSPARC.

For WT DendroEnc, 4498 micrographs were used for data processing. After motion correction and CTF estimation performed in Relion version 3.1, 108 615 particles were picked in crYOLO,²⁸ extracted with box size 440 and pixel size 1.59 in Relion version 3.1, and imported in CryoSPARC. 14 526 particles after 2D classification were used for *ab initio* 3D refinement with simultaneous sorting into 3 classes. One class with the largest number of particles, 6765, was used for non-uniform 3D refinement with icosahedral (I) symmetry. These particles were subjected to one more round of 3D refinement with simultaneous sorting into 2 classes. The class with the largest number of particles, 4549, was used for non-uniform 3D refinement with icosahedral (I) symmetry. The reconstruction and corresponding particle alignments were used for Bayesian polishing in Relion, followed by final non-uniform 3D refinement in CryoSPARC.

Finally, for DendroEnc N198W, 8176 micrographs were used for data processing. After motion correction and CTF estimation performed in Relion version 3.1, 35 472 particles were picked in crYOLO, extracted with box size 440 and pixel size 1.59 in Relion, and imported in CryoSPARC. 15 958 particles after 2D classification were used for *ab initio* 3D refinement with simultaneous sorting into 3 classes. One class with the largest number of particles, 10 561, was used for non-uniform 3D refinement with icosahedral (I) symmetry. The reconstruction and corresponding particle alignments were used for Bayesian polishing in Relion, followed by one more *ab initio* 3D refinement with simultaneous sorting into 3 classes. The class with 3997 particles was used for the final non-uniform 3D refinement. To demonstrate encapsulation of DendroEnc with CyanoPOX, we performed particle subtraction in CryoSPARC using a mask covering the DendroEnc shell.

B-factor sharpened reconstructions of final refinements were used for model building. AlphaFold 2²⁹ model predictions of encapsulins were fit into the experimental density in Isolde³⁰ and refined in Phenix.³¹ The statistics presented in Table S3 (ESI†) was calculated by MolProbity.³²



Proteolytic stability of DendroEnc constructs

To assess proteolytic resistance, purified DendroEnc encapsulin obtained after SEC and non-encapsulated CyanoPOX (both at 1 mg mL⁻¹) were tested for proteolytic resistance with chymotrypsin from bovine pancreas. The protease stock solution (40 U mL⁻¹) was prepared by dissolving bovine chymotrypsin in 100 mM Tris-HCl buffer (pH 7.8) with 10 mM CaCl₂. 25 μL of 1 mg mL⁻¹ encapsulin or CyanoPOX was mixed with 475 μL of protease stock solution. The digestions were performed at 50 °C for 24 h. Samples were analyzed by SDS PAGE.

Activity of DendroEnc CyanoPOX constructs

Reactions with DendroEnc CyanoPOX (1 mg mL⁻¹) and DendroENC N198W CyanoPOX (1 mg mL⁻¹) were performed at 25 °C. Enzymatic reaction with guaiacol was tested at 10 mM concentration in a 50 mM KPi, 150 mM NaCl, pH 7.5, while ABTS was tested at 10 mM concentration in 50 mM KPi, 150 mM NaCl, pH 5.0. 0.5 mM of H₂O₂ was used in all of the reactions. Formation of oxidized products was monitored at different wavelengths on the BioTek Synergy HTX multi-mode microplate reader (ABTS $\epsilon_{420} = 36.0 \text{ mM}^{-1} \text{ cm}^{-1}$ and guaiacol $\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$).²² Reaction rates were determined from the linear regions of the reaction curves.

DendroEnc N198W mutant generation

DendroEnc mutation was performed using the QuickChange method.³³ The 25 μL PCR mix contained the following components: 12.5 μL of PfuUltra II Hotstart PCR Master Mix, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 1 μL of plasmid (100 ng μL⁻¹), 0.4 μL of DMSO, and MQ water to a final volume of 25 μL.

Conclusions

This study advances the understanding of bacterial encapsulins and their potential applications in biocatalysis through the identification and characterization of two novel systems: ArthroEnc from *Arthrobacter* sp. SLBN-53 and DendroEnc from *Dendrosporobacter quercicolus*. Structural analyses revealed that ArthroEnc assembles into a 20 nm capsid with $T = 1$ icosahedral symmetry, while DendroEnc forms a larger 40 nm structure with $T = 4$ icosahedral symmetry. Functional characterization demonstrated that DendroEnc CyanoPOX exhibits enzymatic activity with ABTS and guaiacol substrates. Notably, DendroEnc displayed resistance to proteolytic degradation and protected encapsulated enzymes during 24 hours of exposure to chymotrypsin. Furthermore, the N198W mutation in DendroEnc, designed to modulate pore size, reduced substrate accessibility, resulting in a 5-fold decrease in enzymatic activity relative to the wild type. These findings expand the known diversity of encapsulin systems and highlight their potential as robust platforms for enzyme stabilization and biocatalysis, offering promising possibilities for future applications in nanoreactor design and biotechnology.

Author contributions

M. W. F. and N. L. conceived and supervised experiments. A. B. performed the structural characterization. O. P., C. M. and R. K. carried out the identification and biochemical characterization. O. P., A. B., N. L. and M. W. F. contributed in writing the manuscript and approve the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

Coordinates for the cryo-EM structures of ArthroEnc, WT DendroEnc and DendroEnc N198W have been deposited in the Electron Microscopy Data Bank under accession numbers EMD-52714, 52715 and 52716. The corresponding molecular models have been deposited at the wwPDB with accession codes PDB 9I8D, 9I8E and 9I8F. Other relevant data supporting the findings of this study are fully included within the main article and its ESI.†

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